

Effet de la surexpression de la glycéraldéhyde 3-phosphate déshydrogénase (GAPDH) sur le développement et la fonction des lymphocytes T

Rana Mhaidly

► To cite this version:

Rana Mhaidly. Effet de la surexpression de la glycéraldéhyde 3-phosphate déshydrogénase (GAPDH) sur le développement et la fonction des lymphocytes T. Immunologie. COMUE Université Côte d'Azur (2015 - 2019), 2019. Français. NNT: 2019AZUR6021. tel-02948946

HAL Id: tel-02948946 https://theses.hal.science/tel-02948946

Submitted on 25 Sep 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.





THÈSE DE DOCTORAT

Effet de la surexpression de la glycéraldéhyde 3- phosphate déshydrogénase « GAPDH » sur le développement et la fonction des Lymphocytes T

Rana MHAIDLY

Equipe 3 : Métabolisme, Cancer et Réponses Immunes

Présentée en vue de l'obtention du grade de docteur en Sciences de la vie et de la santé de l'université Côte d'Azur, Spécialité Immunologie et Microbiologie Dirigée par : Dr Els VERHOEYEN Soutenue le : 21 Novembre 2019

Devant le jury, composé de :

Dr. Jean-Ehrland RICCI, Président du jury, Directeur de recherche DR2, C3M
Dr. Valérie DARDALHON, Rapportrice, Chargée de recherche CR1, IGMM
Dr. Fabienne MEGGETTO, Rapportrice, Directrice de recherche DR2, CRCT
Dr. Philippe GAULARD, Examinateur, professeur PU-PH, Hôpital Henri Mondor
Dr. Vahid ASNAFI, Examinateur, professeur PU-PH, Hôpital Necker- Enfants Malades
Dr. Els VERHOEYEN, Directrice de thèse, Directrice de recherche DR2, C3M



Effet de la surexpression de la glycéraldéhyde-3-phosphate déshydrogénase « GAPDH » sur le développement et la fonction des Lymphocytes T

Devant le jury, composé de : Président du jury Dr. Jean-Ehrland RICCI, Directeur de Recherche, DR1, INSERM- C3M

Rapportrices

Dr. Valérie DARDALHON, Chargée de Recherche, CR1, CNRS - IGMM Dr. Fabienne MEGGETTO, Directrice de Recherche, DR2, INSERM - CRCT

Examinateurs

Dr. Philippe GAULARD, Professeur des Universitaires, PU-PH - Hôpital Henri Mondor Dr. Vahid ASNAFI, Professeur des Universitaires, PU-PH - Hôpital Necker-Enfants Malades

Directrice de thèse

Dr. Els VERHOEYEN, Directrice de Recherche, DR2, INSERM - C3M

Résumé :

L'activation des lymphocytes T (LT) et l'acquisition des fonctions effectrices sont des phénomènes récemment décrits pour être régulés par le métabolisme cellulaire, introduisant ainsi la notion d'« immunométabolisme » dans de nombreuses études biologiques.

Dix enzymes métaboliques participent à la conversion du glucose en énergie cellulaire (glycolyse). Parmi celles-ci, des études récentes ont fait émerger la glycéraldéhyde 3-phosphate déshydrogénase (GAPDH) comme un acteur principal de la survie et de la différenciation des LT. Dans ce rôle, la balance entre ses fonctions glycolytiques (associées à la catalyse du glycéraldéhyde-3-phosphate en le 1,3-bisphosphoglycérate) et non-glycolytiques (stabilité des ARNm des cytokines et activation des voies de signalisation) est un phénomène majeur contrôlant la sélection et l'activation des LT.

Afin d'étudier les fonctions glycolytiques et non-glycolytiques de la GAPDH dans le développement lymphocytaire, nous avons généré un modèle de souris transgéniques surexprimant la GAPDH exclusivement dans les LT (plck-GAPDH).

Une première caractérisation de ces souris montre que la surexpression de la GAPDH n'a pas d'effet sur les stades précoces de développement des LT. De façon intéressante, nous avons observé dans les souris âgées (>18 mois) des changements phénotypiques évidents, notamment : une splénomégalie, des ganglions lymphatiques hypertrophiés et une infiltration lymphocytaire majeure au niveau du foie et de la moelle osseuse. Un immunophénotypage détaillé de ces tissus a montré une augmentation de la proportion des LT folliculaires helpers (Tfh) CD4⁺ Bcl-6⁺CXCR5⁺ PD-1^{high}. De plus, ces cellules, fortement prolifératives et clonales, interagissent avec les lymphocytes B CD95⁺ GL7⁺ du centre germinatif, eux-mêmes associés à des cellules dendritiques folliculaires, formant ainsi un microenvironnement tumoral dans notre modèle plck-GAPDH.

À la recherche d'un équivalent humain à notre modèle, nous avons découvert que les caractéristiques pathologiques, l'immunophénotypage et le profil génétique des souris plck-GAPDH correspondaient étroitement au lymphome T angio-immunoblastique humain (AITL). Il s'agit d'une hémopathie maligne rare pour laquelle aucune stratégie thérapeutique n'a été clairement décrite. Nous avons également mis en évidence dans les souris plck-GAPDH une activation de la voie NF-kB non canonique, également activée chez les patients AITL. Le ciblage de cette voie par un nouvel inhibiteur associé à une immunothérapie anti-PD1 a augmenté la survie des souris. Ce traitement permet une induction efficace de la réponse immunitaire anticancéreuse et représente une nouvelle perspective thérapeutique pour les patients atteints d'AITL.

En conclusion, ce nouveau modèle de souris AITL capable de mimer étroitement les caractéristiques du lymphome humain, permettra d'élucider l'origine possible de la maladie, d'identifier d'autres voies de signalisation impliquées et de tester de nouvelles lignes thérapeutiques.

Mots-clés :

GAPDH, AITL, Lymphocytes T, voie NF-kB

Abstract:

Fundamental metabolic changes are implemented by cancer cells to meet their high energy requirements, for which they show increased dependence on glycolysis (confirmed by Warburg effect). One central enzyme of glycolytic pathway is glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that is shown to be overexpressed in many cancers and emerged, few years ago, as a key player in T cell development, function and malignancies. Here we investigated the role of GAPDH in T cell development by generating a mouse model overexpressing GAPDH in the T cell lineage. From 18 months on, clear phenotypic changes were observed in these mice including: 1) splenomegaly, 2) enlarged lymph nodes, 3) lymphocytes-infiltration in the liver and bone marrow. A detailed immuno-phenotype showed an increase of strongly proliferating and clonal follicular helper Bcl6+ CXCR5+ PD1_{high} CD4+ T cells. Moreover, hyperplasic CD95+ GL7+ germinal center B cells associated with follicular dendritic cells were observed in the plck-GAPDH tumors. Additionally, tumors from plck-GAPDH mice secreted high levels of chemokines and inflammatory cytokines compared to

normal WT mice. In search for a human equivalent, we discovered that the pathological and immune-phenotypic characteristics and gene expressing profiling of the plck-GAPDH mice matched in great details human angioimmunoblastic T-cell lymphoma (AITL), a rare disease with no treatment options up to date. Even a specific mutation in *Rhoa* typical for AITL was confirmed. Further analysis demonstrated that GAPDH-induced AITL disease depends mechanistically on activation of the non-canonical NF-kB pathway also activated in human AITL. Targeting of this pathway with novel NF-kB inducing kinase (NIK) inhibitor combined with anti-PD1 immunotherapy increased mice survival to 70%. This combined treatment let to efficient induction of the anti-cancer immune response. In conclusion, this new AITL mouse model, closely mimicking the human lymphoma, will help to unravel the possible origin of the disease, novel pathways implicated and will permit us to test novel therapeutic options including immunotherapies.

Keywords:

GAPDH, AITL, T cell Lymphocyte, NF-kB pathway

À ma grand-mère... Si tu peux me voir de là-haut... J'espère que t'es fière de moi... À mes parents... Un jour j'avais promis de vous rendre fiers... Et me voilà, comme je l'avais promis...

Remerciements

Comment commencer ? Il y a tellement de personnes quí m'ont permís d'en arríver là aujourd'huí. Tellement de personnes quí m'ont accompagné et quí ont prís part de près ou de loín à cette aventure. Mercí à chacun d'entre vous !!

Je tiens d'abord à remercier tous les membres de mon jury d'avoir accepté d'être présents aujourd'hui et de juger mon travail de thèse. Un grand Merci à **Dr Valérie Dardalhon** et **Dr Fabienne Meggetto** qui ont pris le temps de lire et corriger mon manuscrit malgré leur emploi de temps chargé. Je remercie le **Pr Philippe Gaulard** qui m'a fait l'honneur d'accepter de participer à mon jury en tant qu'examinateur. Merci également à **Dr Vahid Asnafi** qui a suivi l'avancée de mes travaux durant ces trois années. Merci pour vos conseils, vos encouragements et toutes les discussions qui étaient toujours enrichissantes et surtout merci d'avoir accepté de faire partie de mon jury de thèse.

Je voudrais également exprimer toute ma reconnaissance à ma directrice de thèse, Dr **Els Verhoeyen** qui était un véritable guide scientifique pendant ces trois années. C'est difficile de trouver les mots pour te remercier. Merci pour m'avoir encadré au cours de cette thèse et m'avoir initié au monde de la recherche. Je me sentais toujours privilégiée d'avoir rencontré et travaillé avec toi. Tu m'as permis d'acquérir une autonomie scientifique dans mon travail et mes raisonnements. Je te remercie de m'avoir toujours encouragé, soutenu et donné de ton temps, tu étais toujours là quand mon moral était en baisse, lorsque les manipes ne marchaient pas. Tu étais toujours disponible pour me débloquer à tout moment. Nous avons formé ensemble un super binôme. Ce travail n'était jamais possible sans ta volonté et ta patience. Si je suis là aujourd'hui c'est en grande partie grâce à toi. Je garderai un super souvenir des bons moments passés ensemble au labo (à 7h du matin ou même 21h du soir), mais aussi durant nos voyages ensemble. Merci encore une fois pour tout !

Je voudrais également remercier tout particulièrement mon chef d'équipe, Dr **Jean Ehrland Ricci**. Merci Jean Ehr de m'avoir accueilli au sein de ton équipe et de m'avoir donné tous les moyens pour effectuer ma thèse. Mes premiers pas dans la recherche ont commencé au sein de ton équipe depuis mon M1. Je suis arrivée dans le labo une étudiante toute timide, et je pars aujourd'hui avec mon diplôme de docteur. Tu as toujours cru en moi, peut-être plus que je croyais en moi-même. Tu m'as soutenu tout au long de ces années depuis la décision de faire une thèse jusqu'à aujourd'hui lors de la recherche de mon post-doctorat. Je te remercie pour ta confiance, tes conseils et pour tes encouragements incessants qui m'ont permis de surmonter de nombreux obstacles. Sans ton aide, je n'aurais pas pu y arriver.

<u>Je remercie du fond de mon cœur tous mes collègues de la TEAM 3 qui sont devenus plus que ça...</u>

Aux membres présents :

Jojo, tu étais comme une grande sœur pour moi, avec toi j'ai toujours pu parler de tout. On partageait les mêmes pensées et on se comprenait sans rien dire. Merci pour m'avoir appris à jamais baisser les bras, à faire, et refaire et encore refaire pleins de fois pour réussir. Merci pour le savoir-faire que tu m'as transmis. β -Gal est fini maintenant !! je repars pour une nouvelle aventure ! je n'oublierai jamais les manipes de curiosité, le bloc opératoire de Mme Chiche et ta passion pour le Taboulé Libanais !! je te souhaite beaucoup de bonheur à côté de ta petite famille, Denis, Ève et le petit bébé !!

Sandrine, toujours prête à nous aider quand tu pouvais. J'admire sincèrement ta passion et ton enthousiasme pour la recherche. Tu fais tout dans la bonne humeur !! T'es un vrai cadeau pour l'équipe 3. Je te remercie de m'avoir toujours encouragé et motivé et surtout appris à réfléchir à tous les contrôles d'une expérience.

Romain, voilà c'est toi le prochain, tu n'as plus le choix !!! je ne serai pas là mais j'ai confiance que tu finiras avec une super thèse !! T'as fait ton mieux pour arriver là !! Tu mérites un grand succès. Je te remercie surtout d'avoir pris soin de ma santé, même plus que moi. Merci d'être toujours présent avec ta bonne humeur et tes blagues « nulles » (mais on a beaucoup rigolé quand même). C'est rare de rencontrer des personnes comme toi qui, malgré tout leur travail et leur responsabilité, gardent toujours leur sourire et leur enthousiasme. Merci pour tout ça !!

Adriana, my crime partner. I never though I will have such an amazing friend in the lab. You were always here for me. Thank you for your advices, your support and your friendship during these two years. We survived through good and bad times, mainly with your mice!! We shared together PHD depression and few hours in the gym. I will miss you a lot, but I will wait for you in Paris for new adventures (with the Chicas). Stay the person you are, with the same motivation and energy and you will reach your goals.

Thank you Manuel for your kindness and the happy moments we spent in the office with captain

Poo. I wish you a lot of success in your professional as well as your private life. I know the big challenge that you have in front of you but I am sure you can manage. I will be always present if you need any support or help.

Ashaina, je suis très contente de partager les derniers mois de ma thèse à côté de toi, on s'est bien amusé !! Malgré ton emploi de temps chargé cet été, on avait trouvé du temps pour papoter et partager nos avis sur des robes, des tatouages et... Merci pour ton écoute quand je n'arrêtais pas de râler (évidemment quand Blandine est partie, il fallait que quelqu'un prenait sa place). C'était un vrai plaisir de travailler à tes côtés et j'espère qu'on restera en contact, peu importe où la vie nous amènera !!

Rachel, la Reine des rangements, merci pour rendre notre labo tellement bien organisé, j'espère juste que ça dure le plus longtemps possible ! Merci d'être toujours prête à nous aider !!

Blandine, Merci pour tout ce qu'on a partagé quand tu étais au labo, pour nos fous-rires, nos joies, nos moments de craquage et de fatigue. Merci d'avoir toujours été là, merci de m'avoir soutenu, conseillé et surtout réconforté. Les journées ne sont plus pareilles depuis ton départ, surtout personne ne partage le goûter avec moi !! Je te souhaite beaucoup de succès et de bonheur aussi bien dans ta vie professionnelle que personnelle.

Aux anciens membres :

Florian, je préfère ma nouvelle voisine de bureau maintenant !! elle est plus organisée et elle bavarde moins !!! Flo Flo !! t'as laissé un grand vide dans le bureau après ton départ !! Personne n'a redit « Vivement le weekend » et personne n'est arrivé le matin en râlant parce qu'il y a du monde dans le parking !! Et surtout je peux éteindre la clim quand je veux !!! Les trois ans sont passés vite !! Merci pour ta gentillesse, ton soutien et ton aide précieux à l'animalerie. Bon courage pour la suite !!!

Jozef, Thank you for always being nice, friendly and for your supporting messages. I missed you a lot since you left. You learn me to keep my energy and positive attitude in all circumstances. I hope that your projects will go as you expect and that you keep enjoying your stay in New York.

Elo, j'ai commencé ma thèse pendant que toi tu finissais la tienne. Quelques mois ensemble au labo étaient suffisants pour te connaître et admirer ta gentillesse ; c'est rare de rencontrer des

personnes comme toi qui donnent autant d'elles-mêmes pour aider les autres. Merci de m'avoir transmis ton esprit d'équipe. Merci pour m'avoir toujours soutenu dès mon premier jour dans le labo. Un grand merci pour tout !!

Camilla, un grand merci pour ton soutien et ta gentillesse. Et bon courage pour la suite de tes aventures !

Emma, je te remercie de m'avoir pris par la main la première semaine au labo et de m'avoir initié au monde des manipes. Un grand merci pour ta patience et ta bonne humeur !!

Adrien, le petit bébé de notre équipe, je n'ai toujours pas cru que t'as que 20 ans. Merci pour ta bonne humeur et ton optimisme durant les 5 mois de stage. J'espère que tu atterriras dans une super équipe pour ton stage et tu feras surtout la bonne décision pour la thèse. Je serais toujours là quand t'as besoin (pas seulement dans le pire des cas).

Pauline, brillante, motivée, folle, aimable. Merci pour tout !! et surtout un énorme merci pour tous les burgers Kings que tu m'avais ramenés pendant que je rédigeais ma thèse, tu savais que c'était le seul moyen de me motiver. J'espère que tu trouveras ta joie dans ce que t'as choisi !!

Sonia, since your first day in the lab, you were very smart and helpful. It was a great pleasure working with you and sharing happy memories together. I am so happy to see you again and thank you so much for coming.

Un grand merci à toutes les membres de l'équipe 2 et l'équipe 4.

Marwa, j'ai la chance d'avoir rencontré quelqu'un comme toi, tellement brillante et passionnée de son travail. Merci pour ton soutien permanent même aux moments les plus durs, tu as su toujours me réconforter. Ne lâches pas !!! J'ai trop confiance en toi ! Ton travail se payera un jour !!

Je remercie également notre gestionnaire Virginie, pour sa patience et sa disponibilité.

Un énorme merci aux personnels de l'animalerie, Véro, Alex, Sandrine, Yannick et Vincent pour votre aide et votre suivi quotidien de nos souris.

Merci à nos collaborateurs, **Dr. Thierry Passeron**, **Dr Rachid Benhida** et tous les chimistes en particulier **Pascal Dao** et **Anthony Martin**.

Je remercie tout particulièrement **Marie Tosolini** pour toutes les analyses bio-informatiques qui m'ont permis d'avancer dans mon projet. Merci pour ta présence discrète tout au long de ces années.

Merci à tous les membres du C3M avec qui j'ai collaboré de près ou de loin au cours de ma thèse.

Je tiens après à adresser ma profonde gratitude à mon entourage, qui m'a permis d'avancer tous les jours. Parce qu'on a une vie à côté de la thèse…

Maya, voilà pour moi aujourd'hui c'est la fin de mon chemin, pour toi, c'est bientôt : comme on s'est promis, on continue jusqu'à la fin malgré toutes les contraintes, tu seras la meilleure, je n'ai aucun doute. Merci d'être toujours présente dans les bons comme dans les mauvais moments. Tu ne m'as jamais laissé tomber, je savais toujours que je pourrais compter sur toi. Reste comme ça, ne change pas. Dans les moments les plus difficiles, penses que ça ne va pas durer éternellement, tu arriveras toujours avec ta bonne volonté et ta patience (Surtout ne râles pas !!! ça ne servira à rien !!!!), Dieu ne nous prépare que du bien !

Hawraa, même si on n'était pas toujours d'accord, on a réussi à traverser les mauvaises périodes. Je n'oublierai pas que t'étais la première personne qui m'a aidé sur Nice et depuis là on est resté des amis. On a partagé ensemble les bons et les mauvais souvenirs, t'étais toujours là pour les grosses joies comme pour les moments de détresse. C'est parti maintenant pour une nouvelle aventure !! je serais toujours là pour t'écouter et t'aider même si je pars. Notre amitié ne s'arrêtera pas !! comme je t'ai toujours dit, ait confiance en toi-même, sois gentil qu'avec les gens qui méritent et fais tes choix !!!! Prends soin de toi et de ta fille !! T'es une super maman, Mimi sera fière de toi !! J'espère que tu trouveras ton chemin de bonheur avec une personne qui te mérites.

Mimi, le petit ange qui a rayonné mon chemin à Nice. Merci de me faire revivre les joies de l'enfance. Je t'embrasse fort !!

Hassan, Essayes dans nos prochaines rencontres d'être à l'heure !! Toujours en retard, toujours surchargé, toujours sur ton téléphone, tout ce que je n'aimais pas tu faisais !!! mais je t'aime quand même. Merci d'être tout simplement toi !! avec cette gentillesse et cette humeur. Tu fais partie de ces personnes que je ne voudrais pas perdre peu importe où je partirais !! *Mhamad,* merci pour ton inestimable gentillesse et ta bonne volonté. Tu étais toujours à l'écoute même quand je parlais pendant deux heures des sujets si éloignés des tiennes. T'es une personne exceptionnelle. Merci d'avoir rapporté un peu d'humour et de fraîcheur « Baalbakienne » à notre groupe.

Yara, toujours en paix, toujours prête à tout faire sans des conflits, sans chercher les problèmes ; j'ai toujours admiré ta joie pour vivre, ta capacité à cacher ta fatigue et ta tristesse. Malgré que t'es la plus petite parmi nous, tu gardais ton sourire, et ton enthousiasme et rien ne pouvait te démotiver. Je me sens toujours responsable de toi. Tu sais très bien que si un jour t'as besoin de moi tu me trouveras. J'espère que tu garderas ta bonne volonté pour toujours. je t'aime fort fort !!!

La famille Haidar, merci pour votre présence et votre joie de vivre que vous avez toujours répandue entre les libanais de Nice.

Janwa, Janah, Georges, Merci pour vos conseils, vos encouragements et votre confiance qui ne me permettaient pas de baisser les bras. Merci de m'avoir suivie dans cette aventure et d'être toujours présents pour me réconforter.

À tous les gens que j'ai rencontré au cours de ces trois années, merci pour votre doux passage, pour vos conseils, votre support et votre amitié.

Amani et Mhamad, un seul Merci suffira jamais, Merci à vous deux:

Amani, quoi dire, ma meilleure amie, ma confidente, ma sœur jumelle. On a parcouru un long chemin ensemble, depuis la maternité, depuis plus que vingt ans. Qui dirait qu'on allait se revoir à Nice !! Rien que t'avoir si proche était un réconfort pour moi. Merci pour toutes ses années d'amitié, merci pour les bons moments qu'on a pu partager, merci d'avoir été toujours à mes côtés dans le bon comme dans le pire. Merci pour tes encouragements, ton soutien et surtout pour ta motivation et tes ondes POSITIVES que tu m'as toujours transmises. Même si maintenant, chacun d'entre nous a choisi son chemin, nous resterons toujours proches, tu resteras toujours ma meilleure amie. Merci à toi **Mhamad**, merci pour tout... tu n'as jamais cessé de croire en moi et de m'encourager. T'as fait preuve d'énormément de patience et de véritable amitié durant ces 5 ans. Tu sais que si je suis arrivée là aujourd'hui c'est en grande partie grâce à toi, tu m'as toujours écoutée et conseillée, tu as supporté mon sale caractère, mes crises de pleures et de fatigue. Tout ce que je pourrais écrire ne sera jamais assez pour te dire à quel point ta présence est importante dans ma vie !!

À mes parents, frères et sœurs sans qui ce travail n'aurait pas vu le jour.

Enfin, un grand merci à ma famille qui m'a soutenue de manière inconditionnelle durant ce long parcours. Je savais que m'éloigner de vous pendant toutes ces années, afin d'aller au bout de mes rêves n'a pas été une décision facile à prendre. Sans votre aide et vos encouragements, je n'aurais pas pu arriver. Je voulais m'excuser de ne pas être présente avec vous quand vous aviez besoin de moi, mais je pensais toujours à vous

Zeinab, ma grande sœur, je ne pourrais jamais résumer tout l'aide que tu m'avais apporté tout au long de ces années d'étude. Tu m'as toujours poussé vers le haut, même dans les moments les plus difficiles. Je savais que tu vivais mon rêve avec moi, tu comprenais que ce n'était pas facile et malgré ça tu me soutenais, tu calmais ma colère, et tu essayais toujours de me redonner le sourire. Tout ce que je pourrais dire ne pourra jamais suffire à exprimer tout ce que je te dois et combien ta présence était importante pour faire de moi ce que je suis aujourd'hui.

Samar, ma petite sœur, qui n'est plus si petite que ça aujourd'hui. Avec toi, je ne voyais pas les années passées, tu savais toujours ce que tu voulais de la vie. Je suis très fière de ce que t'es devenue aujourd'hui. Tu m'as toujours rappelé que la vie est belle et qu'il faut bien profiter de chaque instant. Merci simplement d'être celle que tu es, brillante, forte et courageuse. Je pensais souvent à toi quand je voyais le ciel bleu. Gardes ton sourire et ton plaisir de vivre

Abbas, comment te remercier ? le seul qui pourra en un mot transformer une crise de fatigue en un moment de fous rires. Merci pour tous les moments que tu as pu me réconforter avec ton sourire et ta bonne humeur... Cette année, on a décidé de ne pas baisser les bras pour y arriver, on voulait être tous les deux docteurs et voilà on y est. Je te vois aujourd'hui marcher ton premier pas vers un avenir plein de succès et de bonheur. Je suis tellement fière de toi. J'ai confiance que tu feras toujours de bons choix. J'ai toujours demandé à nos parents pourquoi je n'ai pas un grand frère pour compter sur lui, je ne savais pas que le petit grandira si vite. Malgré les 7 ans d'écart, je sais que tu es toujours là pour moi. Je t'aime très fort.

Adnan, mon petit frère. La première fois que j'ai quitté la maison, tu pleurais et tu ne comprenais pas pourquoi je suis partie. Ça m'a pris du temps pour te dire que je ne reviendrais pas toute de suite et qu'un jour tu comprendras pourquoi. Maintenant, tu es plus grand et t'as sûrement compris que pour aller au bout de notre rêve il faut parfois faire des sacrifices. Merci pour tous les bonbons et chocolats que je trouvais dans mes valises. Je savais sans doute que c'était ta façon de dire « Au revoir ». Je t'adore mon petit et même si je resterais loin, je prendrais toujours soin de toi.

À toute ma famille, mes oncles, mes tantes, mes cousines, et particulièrement Alissar, Rima, Nano, et Rola, je vous aime tous !! Merci pour votre soutien et vos encouragements.

Les meilleurs pour la fin toujours :

بدي إختم بشخصين لو قد ما شكرتن رح ضل مقصرة بحقهن.. شخصين وصلوني لهون... وبعرف قديش شايفين حالن فيي اليوم.. **بابا و ماما، مثالي الأعلى وسندي..** و عدتكن من خمس سنين، ونشالله كون اليوم وفيت بو عدي.. ولو قد ما رح إحكي، ما بوفيكن تعبكن عليي..

__*ĻĻ*

وقفت حدِّي من أول نهار قررت إمشي بهالطريق.. وبوجودك كنت أكيدة إني ما رح إحتاج حدا.. بدي إشكرك عكل دقيقة تعبت فيها لتشوفني هون اليوم.. بدي إشكرك إنك وثقت فيي وآمنت بحلمي.. بدي إشكرك إنو طول هالسنين كنت حدي وما حرمتني من شي.. بتمنى اليوم كون رفعتلك راسك وردَّيتلك لو شي بسيط من جميلك عليي..

ماما.. بتكفيني دعواتك اللي رافقتني من أول نهار فتحت عيوني عالدَّني.. بعرف قدَّيش غيابي عن البيت بي وجعك، بس رغم هيك، كنتي دايما قوية وعم تتحملي.. ما رح لاقي حدا بهالدَّني يحبني ويخاف عليي قدَّك...

كنت ورح ضل قول لما الله وزَّع النعم عالأرض، عطاني أحن إم وبي... الله يخليلي ياكن وتضلو دايما فوق راسنا..

بحبكن كتير....

TABLE DES MATIÈRES

REMERCIEMENTS	9 -
TABLE DES MATIÈRES	18 -
LISTE DES ABRÉVIATIONS	21 -
LISTE DES FIGURES	25 -
LISTE DES TABLEAUX	26 -
INTRODUCTION	28 -
I. LES LYMPHOCYTES T	30 -
A. Développement et sélection thymique des LT	30 -
1. Développement précoce et expression du pré-TCR	30 -
	31 -
2. La sélection positive	- 31 -
5. La selection negative	
$C_{\rm Les}$ lymphocytes T CD ⁴	
C. Les lymphocytes T CD4	32 -
a Les lymphocytes Th	- 33 -
b. Les lymphocytes Th2	
c. Les lymphocytes Th17	34 -
d. Les lymphocytes T régulateurs	35 -
e. Les lymphocytes T folliculaires Tfh	36 -
► Bcl-6 :	37 -
CD40L/CD40	37 -
	- 38 -
 CXCR5/CVC113 	- 38 -
 Les cvtokines stimulatrices IL-21 et IL-4. 	39 -
II. METABOLISME ENERGETIQUE DES LT	41 -
A. Les différentes voies métaboliques impliquées dans le développement des LT	- 41 -
1. Glycolyse	41 -
2. Le cycle de Krebs	42 -
3. La phosphorylation oxydative	42 -
4. La β-oxydation des acides gras	43 -
B. La reprogrammation métabolique des LT	44 -
C. Métabolisme énergétique des LT folliculaires (Tfh)	47 -
III. GLYCERALDEHYDE-3-PHOSPHATE DESHYDROGENASE (GAPDH)	51 -
A. Fonction glycolytique	51 -
B. Fonctions non-alvcolvtiques	52 -
1. Rôle dans la composition cellulaire	52 -
2. Rôle de GAPDH dans la régulation des voies de signalisation	53 -
3. Rôle de la GAPDH dans la mort cellulaire	55 -
4. Rôle de la GAPDH dans le développement tumoral	57 -
5. Rôle de la GAPDH dans le développement et la fonction des cellules T	59 -
IV. NF-κB	63 -
A. Voie de signalisation canonique (classique)	63 -
B. Voie de signalisation non-canonique (alternative)	64 -
C. Interaction entre les deux voies	65 -
D. Gènes cibles de la voie NF-κB	66 -
E. Rôle de NF-κB dans le développement et la différenciation des LT	67 -
1. NF-κB et développement thymocytaire	68 -
2. NF-κB et développement des LT CD4+ conventionnels	69 -

	3.	NF-κB et développement des Tregs thymiques	70 -
	4.	NF-κB et développement des Tfh	71 -
F.		Rôle de NF-κB dans le développement tumoral	74 -
	1.	Les leucémies myéloïdes	74 -
	2.	Les leucémies lymphoïdes	75 -
	3.	Les lymphomes B	76 -
	4.	Les lymphomes T	77 -
G		Inhibiteurs de la voie NF-κB	78 -
	1.	Les inhibiteurs du protéasome	78 -
	2.	Les anti-inflammatoires non stéroïdiens	79 -
	3.	Les glucocorticoïdes	80 -
	4.	Les inhibiteurs de ΙΚΚβ	80 -
	5.	Les inhibiteurs d'Ezh2	81 -
	6.	Les innibiteurs de Nik	83 -
۷.	LY	MPHOME I ANGIO-IMMUNOBLASTIQUE	86-
A.		Epidémiologie et facteurs de risque	87 -
В.		Aspects cliniques de l'AITL	87 -
С.		Pathogenèse de l'AITL	89 -
D		Immuno-phénotypage des cellules tumorales de l'AITL	91 -
F.		Caractéristiques moléculaires et aénétiques de l'AITI	93 -
	1.	Clonalité	93 -
	2.	Mutations génétiques observées dans les AITL	93 -
		a. Mutation Rhoa G17V	93 -
		b. Mutation de Tet2	94 -
		c. Mutation d'IDH2	95 -
		d. Mutation de DNMT3a	95 -
		e. Mutations dans les gènes du TCR	95 -
_	3.	Anomalies cytogénétiques	96 -
<i>F.</i>		Options thérapeutiques	97 -
G		Modèles murins de l'AITL	99 -
	1.	Modèle Roquin ^{san/+}	100 -
	2.	Modèle d'invalidation de Tet2 « Tet2 ^{gt/gt} »	101 -
	3.	Modèle Swiss Jim Lambert SJL	101 -
	4.	RhoA G1/V TET2 19/11	102 -
	5.	Modele de Xenotransplantation (PDX)	104 -
VI.	Ľ		- 105 -
A.		Anti-CTLA-4	- 106 -
В.		Anti-PD-1/PD-L1	- 107 -
С.		L'immunothérapie dans les lymphomes T	- 109 -
OBJEC			- 111 -
RESULT	ΓΑΊ		- 115 -
		1	- 117 -
		י ז	121
	CLE	2	- 121 -
DISCUS	SSI	ONS ET PERSPECTIVES	- 127 -
	1.	Rôle de la GAPDH dans la pathogenèse de l'AITL	129 -
	2.	Activation et ciblage de la voie de signalisation NF-κB dans les tumeurs AITL	132 -
	3.	Révéler de nouvelles options thérapeutiques pour le traitement de l'AITL	135 -
	4.	Métabolisme énergétique des cellules tumorales Tfh et des cellules du microenvironnement	137 -
	5.	Analyse des modifications épigénétiques dans les tumeurs mAITL	141 -
CONCL	US	ION	- 144 -
	GR	<u>арніғ</u>	- 148 -
	ыл. /Ес	· · · · · · · · · · · · · · · · · ·	174
ANNEX	E2	~	- 1/4 -
ARTI	CLE	3	-1/6-
Arti	CLE	4	- 179 -

Article 5	182 -
Article 6	- 184 -
Article 7	187 -
Article 8	189 -
Article 9	191 -
CURRICULUM VITAE	195 -
LISTE DE PUBLICATIONS	199 -
RESUME	203 -

LISTE DES ABRÉVIATIONS

- ADN : Acide désoxyribonucléique
- ADCC : Antibody-Dependent Cell-Mediated cytotoxicity
- AINS : Anti-inflammatoires non-stéroidiens
- AITL : Angioimmunoblastic T cell lymphoma

Aldoc : Aldolase c

- AMP : Adénosine monophosphate
- AMPK : AMP-activated protein kinase
- ANK : Répétitions Ankyrines
- ATP : Adénosine triphosphate
- ATG5 : Autophagy related 5
- αKG : alpha-kéto-glutarate
- BACH1 : BTB Domain and CNC Homolog 1
- BCR-ABL : Breakpoint Cluster Region-Abelson
- Bcl-2 : B-cell lymphoma 2
- Bcl-6 : B-cell lymphoma 6
- Bcl-xl : B cell lymphoma-extra large
- Blimp1 : B lymphocyte-induced maturation protein 1
- CARD11 : Caspase Recruitment Domain Family Member 11
- CCND1 : Cyclin D1
- CD : cellule dendritique
- CG : Centre germinatif
- CICD : caspase independent cell death
- c-IAP1/2 : Cellular inhibitor of apoptosis 1/2
- CMH : Complexe majeur d'histocomaptibilité
- COX : Cyclooxygénase
- CPA : Cellule présentatrice d'antigène
- CSH : Cellule souche hématopoïétique
- CTLA-4 : cytotoxic T-lymphocyte-associated protein 4
- CTNNB1 : Catenin Beta 1
- CXCR5 : C-X-C chemokine receptor type 5
- CXCL-13 : C-X-C chemokine ligand type 13

DLBCL : Diffuse large B cell lymphoma

DN : Double négatif

DNMT3a : DNA méthyl-transférase

DP : Double positif

1,3-DPG: 1,3-diphosphoglycérate

DZNep : Déaazaneplanocine

EBV : Epstein Bar Virus

Ezh2 : Enhancer of zeste homolog 2

FAD : Flavine adénine dinucléotide

FLT3 : Fms-like tyrosine kinase 3

Foxp3 : Forkhead box P3

FYN : Tyrosine Protein Kinase

GAPDH : glycéraldéhyde 3-phosphate déshydrogénase

GC : Glucocorticoïdes

Glut1 : Transporteur de glucose 1

GSTP1 : Glutathione S-transferase P

GTP : Guanosine triphosphate

G3P : glycéraldéhyde 3-phosphate

GVH : Réaction du greffon contre l'hôte

 $HIF1\alpha$: Hypoxia-inducible factor 1-alpha

HK2 : Héxokinase 2

ICOS : Inducible T-cell costimulator

IDH2 : Isocitrate Déshydrogénase

IFNγ: Interféron gamma

IgM : Immunglobuline M

IgG : Immunoglobuline G

 $I\kappa B$: Inhibitor of κB

IKK : Inhibitor of nuclear factor kappa-B kinase

Klf2 : Krueppel-like factor 2

LAM : Leucémie Myéloïde Aigue

LB : Lymphocyte B

LED : Lupus Érythémateux Disséminé

LDH : Lactate Déshydrogénase

- LLC : Leucémie Lymphoïde Chronique
- LMC : Leucémie Myéloide chronique
- LPS : Lipopolysaccharide
- LT : Lymphocyte T
- LTC : Lymphocyte T cytotoxique
- LTh : Lymphocyte T helper
- LTβR : Lymphotoxin Bêta Receptor
- M2 : Macrophage de type M2
- Mdh : Malate Dehydrogenase
- mTOR : mechanistic target of rapamycin
- MYD88 : Myeloid differenciation primary response
- MM : Myélome multiple
- $TNF\alpha$: Tumor necrosis factor alpha
- NAD : Nicotinamide adénine dinucléotide
- NIK : NF-κB inducing kinase
- NBD : NEMO Binding Domain
- NF-κB : Nuclear factor-kappa B
- NK : Cellule Natural Killer
- NOD : Non-Obese Diabetic
- NSG : NOD-SCID IL2γnull
- OXPHOS : phosphorylation oxidative
- **OPN** : Ostéopontine
- PD-1 : Programmed Cell Death 1
- PDL-1/2 : Programmed Cell Death Ligand 1/2
- PTCL : Peripheral T cell lymphoma
- PLCG1 : Phospholipase C Gamma 1
- PI3K : Phosphoinositide 3-Kinase
- PDX : Patient derived xenograft
- Rc3h1 : Ring Finger And CCCH-Type Domains 1
- RhoA : Ras homolog gene family, member A
- RE : Réticulum Endoplasmique
- Rab2 : RAS-related protein 2

R-CHOP : Rituximab - Cyclophosphamide - Hydroxy Doxorubicine - Vincristine -

Prednisone

RHD : Rel homology domain

RORyT : RAR-related orphan receptor gamma

Rpb4 : RNA Polymerase II subunit

Sdhc : Succinate Dehydrogenase complex subunit C

SH2 : SRC Homology 2

Siah1 : Seven In Absentia Homolog 1

Sirt1 : Sirtuine 1

SJL : Swiss Jim Lambert

Slc2a1 : Solute Carrier Family 2 Member 1

Slc2a3 : Solute Carrier Family 2 Member 3

Slc25a1 : Solute Carrier Family 25 Member 1

SOCS1 : Suppressor of cytokine signaling protein 1

SP : Simple positif

T-bet : T-box transcription factor 2

TCA : Tricarboxylic acid cycle

TCR : T-cell receptor

Tet2 : Ten-eleven-translocation 2

Tfh : Lymphocyte T folliculaire helper

TME : Tumor Microenvironment

TNFAIP3 : TNF alpha induced protein 3

TRAF5 : TNF receptor-associated factor 5

TRAF2 : TNF receptor-associated factor 2

Tregs : Lymphocyte T régulateur

UTR : Untranslated Transcribed Protein

VEGF : Vascular Endothelial Growth Factor

VHL : Von Hippel-Lindau

LISTE DES FIGURES

FIGURE 1 : REPRESENTATION DU DEVELOPPEMENT INTRA-THYMIQUE DES LYMPHOCYTES T : 31	-
FIGURE 2 : PRINCIPALES POPULATIONS DES LYMPHOCYTES T CD4+ EFFECTEURS :	-
Figure 3 : Differenciation des TFH et generation des plasmocytes 40	-
FIGURE 4 : LES DIFFERENTES VOIES METABOLIQUES DE DEGRADATION DU GLUCOSE ET DE L'ACIDE	
GRAS 44	-
Figure 5 : Reprogrammation metabolique des LT au cours d'une reponse immunitaire - 47	-
FIGURE 6 : DEUX HYPOTHESES EXPLIQUANT LA REPROGRAMMATION METABOLIQUE DES TFH 50	-
FIGURE 7 : FONCTION GLYCOLYTIQUE DE LA GAPDH 52	-
FIGURE 8 : MECANISME DE REGULATION DE LA VOIE NF-KB PAR LA GAPDH 54	-
FIGURE 9 : MECANISME DE REGULATION DE LA MORT CELLULAIRE INDEPENDANTE DES CASPASES PAR	
LA GAPDH 57	-
FIGURE 10 : ROLE DE LA GAPDH DANS LA REGULATION DES FONCTIONS EFFECTRICES DES CELLULES	
Т60	-
FIGURE 11 : LES DEUX VOIES D'ACTIVATION CANONIQUE ET NON-CANONIQUE DE NF-KB 65	-
FIGURE 12: MECANISME D'ACTIVATION DE LA VOIE NF-KB PAR LA SIGNALISATION TCR 69	-
FIGURE 13 : REPRESENTATION DES DIFFERENTS INHIBITEURS PROPOSES POUR CIBLER L'ACTIVATION	
CONSTITUTIVE DE LA VOIE NF-KB 85	-
FIGURE 14 : SIGNES CLINIQUES DE L'AITL 89	-
FIGURE 15 : PATHOGENESE DE L'AITL	-
FIGURE 16 : MODELE DE DEVELOPPEMENT DE L'AITL 104	-
FIGURE 17 DIFFERENTES METHODES D'IMMUNOTHERAPIE PASSIVE ET ACTIVE 106	-
FIGURE 18 : MECANISME D'ACTION DE L'ANTI PD-1 ET DE L'ANTI PD-L1 109	-
FIGURE 19:MODELE DE DEVELOPPEMENT DE L'AITL BASE SUR L'EXPRESSION DE LA GAPDH ET	
L'ACTIVATION DE LA VOIE NF-KB 130	-
Figure 20:Correlation entre les niveaux d'expression de la GAPDH et de l'IFN γ 132	-
FIGURE 21:SCHEMA RECAPITULATIF DE L'IMPLICATON DE GAPDH ET DE LA VOIE NF- κB dans le	
DEVELOPPEMENT DE L'AITL 133	-
FIGURE 22:SCHEMA RECAPITULATIF DES DEUX OPTIONS THERAPEUTIQUES PROPOSEES DANS NOTRE	
ETUDE POUR LE TRAITEMENT DE L'AITL 134	-
FIGURE 23:CORRELATION ENTRE LE NIVEAU D'EXPRESSION DE BCL-6 ET LE METABOLISME	
ENERGETIQUE DES CELLULES TUMORALES DES SOURIS PLCK-GAPDH (A) OU DANS LES BIOPSIES	
DE PATIENTS AITL (B) 138	-
FIGURE 24: MESURE DE LA PRODUCTION DE L'ATP GLYCOLYTIQUE SUR LES CELLULES B CD19+ (A)	
ET LES CELLULES T CD8+ (B) ISOLEES A PARTIR DES TUMEURS SPLENIQUES DES SOURIS PLCK-	
GAPDH EN COMPARAISON A DES CELLULES NORMALES ISOLEES DE LA RATE DES SOURIS	
CONTROLES 140	-
FIGURE 25 : POURCENTAGE DE L'ATP GLYCOLYTIQUE PRODUITE PAR LES CELLULES T CD4+PD-	
1 HIGH ISOLEES A PARTIR DE LA TUMEUR PLCK-GAPDH D'ORIGINE OU LA TUMEUR TRAITEE PAR	
LA METFORMINE 141	-
FIGURE 26 : LISTE DES GENES CODANT POUR LES REGULATEURS EPIGENETIQUES QUI SONT MODIFIES	
DANS SIX TUMEURS PLCK-GAPDH DIFFERENTS, DETECTES PAR UN SEQUENÇAGE DU GENOME	
ENTIER 143	-

LISTE DES TABLEAUX

TABLEAU 1: FONCTIONS NON-GLYCOLYTIQUES DE LA GAPDH	61 -
TABLEAU 2:ROLE DE LA VOIE NF-κB DANS LE DEVELOPPEMENT DES LT	72 -
TABLEAU 3: AGENTS ANTI-TUMORAUX UTILISES DANS LE TRAITEMENT DE L'AITL ET LEUR MOD	E
D'ACTION	98 -

Introduction

I. Les lymphocytes T

A. Développement et sélection thymique des LT

Le système immunitaire est constitué d'une diversité de cellules, toutes impliquées dans la protection de l'organisme contre les éléments reconnus comme étrangers.

Parmi ces cellules, les lymphocytes T (LT) représentent les acteurs principaux qui contrôlent la réponse immunitaire. L'efficacité de cette réponse dépend alors de la génération de ces cellules et de l'acquisition de leurs fonctions qui leur permettent de reconnaître de manière spécifique un pathogène et de déclencher une attaque adaptée.

Le développement des LT nécessite l'arrivée des cellules souches hématopoïétiques (CSH) provenant de la moelle osseuse dans le thymus où elles se différencient en progéniteurs lymphoïdes communs (Figure 1). La différenciation de ces progéniteurs en LT se divisent en trois étapes se basant sur l'expression ou pas des marqueurs de surface CD4 et CD8 (Figure 1).

1. Développement précoce et expression du pré-TCR

Cette étape est menée par les cellules doubles négatives (DN) CD4-CD8-. Ces cellules représentent 1 à 5% des thymocytes et correspondent aux thymocytes les plus immatures. Elles sont divisées en 4 stades distingués sur la base de l'expression de la molécule d'adhésion CD44 et du récepteur de l'interleukine 2, CD25, ainsi nous distinguons : DN1 (CD44⁺CD25⁻), DN2 (CD44⁺CD25⁺), DN3 (CD44⁻CD25⁺) et DN4 (CD44⁻CD25⁻) (Figure 1). Durant cette étape, les cellules réarrangent leur chaîne β du TCR grâce à des combinaisons diverses des segments V (Variable), J (Joining) et D (Diversity). Ce réarrangement est contrôlé par deux enzymes recombinases RAG1 et RAG2 et s'effectue en deux étapes initiées par un réarrangement D→J et suivi par V \rightarrow DJ. Les premières chaînes TCR β matures apparaissent au stade DN3 à la surface des thymocytes en association avec une chaîne pré-TCR α pour former les pré-TCR- $\alpha\beta$. Si le réarrangement de la chaîne β est complet, la cellule reçoit un signal de survie lui permettant de se proliférer et de passer au stade DN4. Ce processus correspond à la « β sélection ». Les cellules déclenchent ensuite le réarrangement de la chaîne α et passent en stade double positif (DP) CD4⁺CD8⁺ exprimant un TCR $\alpha\beta$ mature (Figure 1). Ces thymocytes subissent après la sélection positive et négative qui permettent la survie des cellules capables de reconnaitre le complexe « CMH-Antigène » à une affinité appropriée.



Figure 1 : Représentation du développement intra-thymique des lymphocytes T :

Les LT sont issus des cellules souches hématopoïétiques qui quittent la moelle osseuse et colonisent le thymus et ainsi débute la différenciation des LT. Les phases précoces de développement dépendent des réarrangements du locus β qui ont lieu dans les thymocytes doubles négatifs. L'expression du pré-TCR déclenche un signal anti-apoptotique et permet le passage des cellules au stade de la β sélection. Ensuite, la transition au stade CD4⁺CD8⁺ s'accompagne des réarrangements du locus α aboutissant à la génération d'un récepteur mature TCR $\alpha\beta$. Dès lors, le développement des thymocytes est dépendant des interactions du TCR avec les complexes peptides-CMH exprimés à la surface des cellules stromales thymiques. Ces étapes correspondent aux sélections thymiques positives et négatives. Les thymocytes sélectionnés achèvent leur maturation et deviennent des LT CD4⁻CD8⁺ ou CD4⁺CD8⁻ et gagnent les organes lymphoïdes secondaires. CSH: cellule souche hématopoïétique; DN: Double Négatif; ISP: Immature Single positive; DP: Double Positif; SP: Simple Positif

2. La sélection positive

Durant cette étape, des antigènes du soi sont présentés par des cellules épithéliales corticales aux thymocytes DP. Les thymocytes dont le TCR ne reconnaît pas ou reconnaît avec une faible affinité le complexe « CMH-peptide » ne reçoivent pas un signal de survie et meurent. 90% des thymocytes sont éliminés à cette étape.

3. La sélection négative

Contrairement à l'étape précédente, cette étape permet d'éliminer les thymocytes auto réactifs ayant une forte affinité pour les antigènes du soi. En effet, le passage de ces thymocytes dans la circulation peut induire des réactions d'auto-immunité d'où la nécessité de les éliminer. À la suite de ces étapes de différenciation et de sélection, les thymocytes survivants vont se différencier en lymphocytes T simples positifs SP CD4⁺ ou SP CD8⁺ capables de quitter le thymus pour rejoindre la circulation sanguine. À ce stade, les LT sont appelés naïfs. Afin d'être activés, leur TCR doit être en contact d'un antigène du non-soi associé aux molécules CMH présentés à la surface des cellules présentatrices d'antigènes (CPA), majoritairement les cellules dendritiques.

B. Les lymphocytes T CD8⁺

Les lymphocytes T CD8⁺ représentent en majorité les lymphocytes T cytotoxiques (LTC) et jouent un rôle majeur dans les réponses immunitaires anti-tumorales mais également dans les réactions de défense contre les pathogènes intracellulaires. La réponse immunitaire des LT CD8⁺ est initiée lorsque ces derniers reconnaissent les peptides antigéniques associés à des molécules CMH de classe I présentées à la surface des cellules dendritiques. Cette interaction permet l'activation et la prolifération des cellules CD8⁺ naïves et leur différenciation en cellules effectrices. Les LTC utilisent plusieurs mécanismes pour éliminer les cellules infectées. Ils peuvent sécréter des cytokines pro-inflammatoires comme le TNF- α ou l'IFN- γ qui causent la mort des cellules cibles. Ils peuvent également libérer dans la zone de contact entre la cellule effectrice et la cellule cible, le contenu des granules cytotoxiques présentes dans leur cytoplasme. Ces granules contiennent deux molécules : La perforine qui, comme son nom l'indique, permet de créer des pores dans la membrane plasmique des cellules cibles via lesquels le granzyme B y rentre. Une fois libéré dans le cytoplasme, le granzyme B qui est une protéase à cystéine active les voies d'apoptose et provoque la mort de la cellule cible.

Par ailleurs, plusieurs études ont également mis en évidence des populations immunosuppressives au sein des LT CD8⁺ appelées les LT CD8⁺ régulateurs. Ces cellules, par la sécrétion de plusieurs cytokines inhibitrices (IL-10, IL-16, TGF- β), jouent un rôle efficace dans le maintien de l'homéostasie immunitaire, en réponse à des signaux inflammatoires excessives ou à une transplantation allogénique (Robb et al., 2012; Yu et al., 2018).

C. Les lymphocytes T CD4⁺

Les lymphocytes T CD4⁺ sont en majorité reconnus en tant que cellules auxiliaires (helper) pour leur capacité à activer d'autres cellules du système immunitaire. Ils reconnaissent quant à eux les peptides antigéniques présentés par des molécules CMH de classe II exprimées à la surface des cellules présentatrices de l'antigène (CPA). À la rencontre de leurs antigènes spécifiques, les LT naïfs vont s'activer, proliférer de manière clonale et se différencier en lymphocytes T effecteurs. En fonction de la nature du pathogène, les LT CD4⁺ peuvent se différencier en plusieurs sous populations de LT effecteurs dépendant du panel de cytokines produites par les cellules présentatrices d'antigènes après leur interaction avec les LT. Ces différentes sous populations peuvent être distinguées en fonction de leurs marqueurs de surface et des cytokines sécrétées (Figure 2).



Figure 2 : Principales populations des Lymphocytes T CD4+ effecteurs : Selon l'environnement cytokinique assuré par les cellules présentatrices de l'antigène, le LT CD4⁺ naïf se différencie en un LT effecteur. Chaque population de LT est associée à l'expression d'un facteur de transcription particulier qui lui-même régule la production des cytokines nécessaires à leurs fonctions effectrices.

1. La diversité des lymphocytes T CD4⁺

a. Les lymphocytes Th1

Les Th1 correspondent à une population de cellules T caractérisées par la sécrétion de forte quantité d'IFN- γ et d'IL-2. Leur différenciation à partir des cellules T naïves est due à

l'expression du facteur de transcription *Tbet* (Figure 2). Les Th1 orchestrent les réponses immunitaires à médiation cellulaire dirigées contre les pathogènes intracellulaires comme les virus et les antigènes tumoraux. L'IFN- γ , sécrété par les Th1, permet d'activer les cellules Naturel Killer (NK) et stimule les fonctions phagocytaires des macrophages. Les Th1 sécrètent également de l'IL-2 qui favorise la prolifération et l'activation des lymphocytes T cytotoxiques CD8⁺ permettant l'élimination des pathogènes infectieux. Par ailleurs, plusieurs travaux récents soulignent une participation des cellules Th1 à la réponse anti-tumorale. En effet chez les animaux présentant des réponses Th1 réduites, à savoir déficientes en IFN- γ , l'incidence tumorale est augmentée (Shankaran et al., 2001). Il a été également montré que la restauration de l'expression de *T-bet* chez des souris invalidées pour ce gène (T-bet^{-/-}) inhibe le développement des métastases pulmonaires (Lee et al., 2010).

b. Les lymphocytes Th2

Les lymphocytes Th2 sont les médiateurs de la réponse immunitaire adaptative contre les pathogènes extracellulaires (toxines, parasites). À l'inverse des Th1, la différenciation en Th2 est stimulée par le facteur de transcription *GATA3*. Les Th2 ne produisent pas de l'IFN-γ mais sécrètent de l'IL-4, l'IL-5 et l'IL-13 (Figure 2), ainsi que de faibles quantités d'IL-9 et d'IL-10. Ces cytokines favorisent la réponse immunitaire humorale en induisant la sécrétion des immunoglobulines par les lymphocytes B et permettent le recrutement des éosinophiles suite à des réactions allergiques. D'autre part, les cellules Th2 présentent une action pro-tumorale en stimulant la survie et la prolifération des cellules cancéreuses par l'intermédiaire des cytokines qu'elles sécrètent. La sécrétion d'IL-4 par les Th2 favorise notamment la polarisation des macrophages pro-tumoraux de type M2 (DeNardo et al., 2009; Mantovani et al., 2002). En outre, l'IL-4 agit d'une façon délétère sur les CD8⁺ cytotoxiques en diminuant leur activité anti-tumorale (Olver et al., 2013).

c. Les lymphocytes Th17

Les lymphocytes Th17 sont des cellules productrices de cytokines pro-inflammatoires telles que l'IL-17a, IL-17f et l'IL-22 (Figure 2). Leur génération est contrôlée par le facteur de transcription ROR γ t. Ils sont activés en réponse à des bactéries extracellulaires et des champignons. En réponse à l'IL-17a et l'IL-17f, les cellules endothéliales et les fibroblastes sécrètent des cytokines et des chimiokines (TNF- α , GM-CSF, CXCL1 et CXCL8) dont le but principal est l'activation et le recrutement des cellules de l'immunité innée comme les

neutrophiles. Le rôle des cellules Th17 dans l'immunité anti-tumorale reste un sujet de débat vu les résultats contradictoires observés, probablement liés au type de tumeur étudié et à son environnement (Najafi and Mirshafiey, 2019). D'une part, ces cellules présentent un effet protumorale. Il a été démontré que l'IL-17 stimule la production des facteurs angiogéniques comme le VEGF par les cellules stromales et les fibroblastes, ce qui favorise la construction d'un réseau vasculaire alimentant les cellules tumorales et permettant leur migration vers d'autres sites (Du et al., 2012; Numasaki et al., 2003). En outre, les Th17 exercent des fonctions immunosuppressives par l'expression des ectonucléotidases CD39 et CD73. Cette expression altère la conversion de l'ATP en AMP et inhibe par la suite la prolifération et la survie des cellules T effectrices (Dwyer et al., 2007). D'autres part, de nombreuses études menées chez la souris suggèrent un rôle anti-tumoral des cellules Th17 et alimentent la controverse. Les cellules Th17, de la même façon que les Th1, permettent d'une manière indirecte le recrutement et l'activation des cellules CD8⁺ cytotoxiques. En effet, elles favorisent la production de CCL20 qui à son tour induit le recrutement et la migration des cellules dendritiques et par la suite l'activation des cellules CD8⁺ (Martin-Orozco et al., 2009). En effet, chez les souris IL-17^{-/-}, la croissance tumorale est plus importante et est souvent associée à la formation des métastases. Ce mécanisme est corrélé à une diminution de l'infiltration des cellules NK et des cellules t sécrétrices d'IFN- γ , proposant ainsi un rôle protecteur des cellules Th17. Les cellules Th17 présentent alors deux phénotypes mixtes, selon l'environnement cytokinique.

d. Les lymphocytes T régulateurs

Les lymphocytes T régulateurs (Tregs) représentent une sous-population des lymphocytes T CD4⁺ qui a pour rôle de contrôler en périphérie le comportement lymphocytaire contre les antigènes du soi et de maintenir un état de tolérance immunitaire. En effet, suite à la clairance des pathogènes, les Tregs rabaissent les réponses immunitaires et préviennent ainsi l'apparition des maladies auto-immunes (Grant et al., 2015; Tao et al., 2017). Plusieurs populations de Treg ont été identifiées : les Th3, Tr1, iT35 et iTreg. Dans le cadre de cette introduction, seulement les iTreg (Treg inductibles) seront décrites. Ce sont les cellules T régulatrices immunosuppressives induites en périphérie. Les iTregs sont caractérisés par l'expression du facteur de transcription *Foxp3* et par la présence de la chaîne α du récepteur de l'IL-2 (CD25 ou IL2ra). Ils produisent des cytokines immunosuppressives telles que le TGF- β et l'IL-10 (Figure 2). Des pertes de fonction des gènes *IL2ra* et *FOXP3* sont à l'origine de développement des maladies auto-immunes qui peuvent être corrigées par un transfert adoptif des Tregs
(Sakaguchi et al., 1995; Wing and Sakaguchi, 2010). Cependant les Tregs n'ont pas toujours un rôle bénéfique dans la réponse immunitaire. De nombreux travaux semblent montrer que l'obstacle majeur face à l'immunosurveillance anti-tumorale et à la mise en place des stratégies thérapeutiques efficaces est dû à l'action suppressive des Tregs (Takeuchi and Nishikawa, 2016; Tanaka and Sakaguchi, 2017). En effet, il a été démontré que l'élimination sélective des Tregs par des anticorps déplétants anti-CD25 induit une diminution de la croissance tumorale et favorise un rejet de la tumeur (Shimizu et al., 1999). Supportant ces travaux réalisés chez la souris, l'étude de l'impact des Tregs sur l'immunité anti-tumorale chez les patients atteints de cancer colorectal a montré qu'une augmentation de la fréquence des Tregs au sein de la tumeur est souvent associé à un mauvais pronostic (Shang et al., 2015; Saito et al., 2016).

e. Les lymphocytes T folliculaires Tfh

Les lymphocytes Tfh ont été découverts dans les années 2000 et sont devenus depuis un sujet d'intenses recherches pour comprendre leur origine et leur fonction. Contrairement aux cellules non Tfh qui après activation migrent vers les sites d'infection ou d'inflammation, les cellules Tfh restent présentes dans les ganglions lymphatiques (LN) et la rate parce qu'elles ont pour but principal d'aider les lymphocytes B et d'induire leur différenciation en cellules productrices d'anticorps. La différentiation des Tfh est un processus complexe qui se déroule en plusieurs étapes successives (Martin-Orozco et al., 2009) et dépend majoritairement de la présence des cytokines (IL-6 et IL-21) (Figure 2). Elle consiste en une migration des LT stimulés du compartiment T (appelé également zone T) vers les zones de prolifération et de maturation des LB (appelées zone B) pour former ainsi les centres germinatifs.

De la même façon que les autres sous-populations des cellules T, l'initiation de la différenciation des LT naïfs en Tfh dépend de l'interaction avec les cellules dendritiques présentatrices de l'antigène. Une forte affinité de liaison du TCR au complexe CMH-II/peptide combinée à l'interaction des molécules de co-stimulation entre les cellules T et les cellules dendritiques induisent une signalisation permettant la génération du lymphocyte T précurseur du Tfh (pré-Tfh). Cette signalisation est associée à une expression précoce du répresseur transcriptionnel *Bcl-6 (B-cell lymphoma)* qui est le facteur de transcription clé dans ce processus. Bcl-6 favorise l'expression, à la surface du pré-Tfh, du récepteur CXCR5 qui lie la chimiokine CXCL13 produite essentiellement par les cellules dendritiques folliculaires localisées dans la zone B. CXCL13 peut-être également produite par les pré-Tfh induisant une action autocrine. L'ensemble de ces interactions favorise la colocalisation des lymphocytes T

et B qui migrent au sein des follicules déterminant la zone B. La différentiation terminale des Tfh conduit à la formation du centre germinatif (CG) où s'opère la maturation des lymphocytes B, représentée par l'hypermutation somatique et la commutation de classe isotypique du BCR (B cell receptor). Au sein du CG, les Tfh effecteurs vont permettre le développement des plasmocytes à longue durée de vie et de haute affinité, et la génération des LB mémoires. La coopération qui s'opère entre les Tfh et les LB implique la présence des facteurs solubles (IL-21, IL-4, CXCL13) et des protéines de surface (CD40L, PD1, ICOS, CXCR5) (Figure 3).

➢ <u>Bcl-6 :</u>

Bcl-6 est souvent désigné comme le facteur de transcription clé dans la différenciation des Tfh (Crotty, 2019; Hatzi et al., 2015; Nurieva et al., 2009). Une étude a montré que la différenciation en Tfh est interrompue dans des modèles murins où le gène *Bcl-6* est inactivé (Bcl-6^{-/-}) (Yu et al., 2009). En effet, Bcl-6 oriente la différenciation des LT naïfs en LT folliculaires helper en inhibant la transcription des gènes impliqués dans la génération des autres populations des LT notamment *T-bet* (Th1) et *ROR* γ *T* (Th17). En plus, Bcl-6 active l'expression de toutes les molécules fonctionnelles des Tfh (PD-1, CXCR5, ICOS, IL-21). Ce mécanisme est dû à l'inhibition par Bcl-6 des micro-ARN dont le rôle est l'invalidation de l'expression de ces molécules (Jogdand et al., 2016).

➢ CD40L/CD40

CD40 est exprimé par le lymphocyte B et interagit avec son ligand spécifique, CD40L, fortement exprimé par le lymphocyte T CD4⁺ après activation. Des études ont montré que la perturbation des interactions CD40L/CD40 par l'administration d'un anticorps monoclonal anti-CD40L se traduit par l'absence des centres germinatifs, ceci démontre son implication dans le dialogue Tfh/LB (Foy et al., 1994). En plus, l'engagement CD40L/CD40 est critique pour le maintien de la survie des LB du CG. Cette liaison induit une augmentation de l'expression de la molécule anti-apoptotique *Bcl-xl*, rendant ainsi les LB résistants à l'apoptose (Zhang et al., 1996). En retour, le LT, via le signal CD40L, va libérer certaines cytokines jouant un rôle important dans le maintien du CG (Zhang et al., 1996).

➢ <u>PD-1/PDL-1</u>

PD-1 (Programmed cell death-1) est un récepteur inhibiteur fortement exprimé par les Tfh. Son expression, en association avec CXCR5, reste le moyen le plus simple pour l'identification des Tfh par cytométrie en flux. Il interagit avec PD-L1 et PD-L2 exprimés à la surface des cellules

B du CG. Des modèles de souris déficientes en PD-1 révèlent son importance dans la survie et la différenciation des cellules B en plasmocytes (Good-Jacobson et al., 2010). PD-1 est considéré comme un marqueur de « fatigue cellulaire » exprimé après une stimulation prolongée du TCR et semble être entretenu par les interactions T-B dans le CG. L'expression de PD-1 permet de distinguer deux catégories de cellules Tfh : les Tfh CG (CXCR5^{high} PD-1^{high}) et les Tfh périphériques (CXCR5^{high} PD-1^{low}). Ces deux catégories sont induites après infection ou vaccination. Les Tfh CG (CXCR5^{high} PD-1^{high}) ont été identifiés comme ayant une fonction effectrice de courte durée et alors perdent leur phénotype Tfh après élimination des pathogènes. Alors que les Tfh PD-1^{low} semblent pouvoir générer des cellules de type mémoire capables de retourner aux ganglions et ainsi persister pendant une longue durée (Tangye et al., 2013).

► <u>ICOS/ICOS-L</u>

ICOS (Inducible T cell costimulatory), comme son nom l'indique, est une molécule stimulatrice des cellules T, appartenant à la même famille que CD28 et CTLA-4. Son expression est indispensable à la génération des Tfh (Xu et al., 2013). En effet, chez les souris déficientes en ICOS, le développement lymphocytaire des populations non-Tfh reste normal, mais les réponses de type Tfh sont sévèrement impactées (Dong et al., 2001). L'expression d'ICOS est également requise pour le maintien du phénotype Tfh, en stabilisant une faible expression du facteur de transcription *Klf2*, un régulateur négatif des Tfh. En plus, le blocage de la liaison ICOS-ICOSL induit le départ des Tfh de la zone B et la perte de la réponse immunitaire présente dans les centres germinatifs.

<u>CXCR5/CXCL13</u>

CXCR5 (C-X-C chemokine receptor type 5) est le récepteur de la chimiokine CXCL13, sécrétée par les cellules stromales et dendritiques folliculaires (Breitfeld et al., 2000). CXCL13 a pour rôle de guider les Tfh dans les follicules, et par suite d'induire leur interaction avec les lymphocytes B. CXCR5 est exprimé précocement par les cellules T activées, suite au signal délivré par ICOS et s'accompagne par une perte de l'expression de CCR7, ce qui permet aux cellules de sortir de la zone T pour se rendre dans la zone B (organes lymphoïdes secondaires) (Haynes et al., 2007).

Les cytokines stimulatrices IL-21 et IL-4

Ces deux cytokines, caractéristiques des Tfh, agissent de façon autocrine sur leur différenciation et la maintenance de leur phénotype, mais sont également essentielles à la génération du centre germinatif et à la maturation des cellules B. *Weinstein et al.* propose dans leur étude un modèle de différenciation séquentielle des Tfh se basant sur la sécrétion de ces deux cytokines : les Tfh du CG expriment d'abord l'IL-21 seule (Tfh₂₁), puis en combinaison avec l'IL-4 (Tfh₂₁₊₄), et enfin l'IL-4 seule (Tfh₄) (Weinstein et al., 2016). D'un point de vue mécanistique, il explique que l'IL-21 induit directement l'expression de Bcl-6, qui permet une sélection des cellules B matures à haute affinité (Linterman et al., 2010; Ozaki et al., 2004; Zotos et al., 2010). L'IL-4 intervient plus tardivement pour réguler les processus d'hypermutation et de commutation isotypique des immunoglobulines (IgM vers IgG1) et ainsi la différenciation des LB en plasmocytes.

Enfin, la différenciation des Tfh et leur interaction avec les LB au sein du CG forme une boucle de régulation, dans laquelle un signal alimente l'autre, aboutissant à affiner la réponse immunitaire.



Figure 3 : Différenciation des Tfh et génération des plasmocytes

La différenciation des Tfh est un processus multi-étapes nécessitant des interactions avec les cellules du microenvironnement. Une première interaction avec les cellules dendritiques présentatrices de l'antigène permet d'initier la différenciation dans la zone T. les LT activés migrent plus tard vers la bordure de la zone B formant le complexe pré-Tfh/LB activés. Cette interaction est assurée grâce à la présence de plusieurs complexes ligand-récepteur qui induisent la migration des deux types cellulaires vers la zone B pour former un centre germinatif. Des contacts répétés entre les LB et les Tfh permettent la différenciation des LB en plasmocytes et la production des anticorps à haute affinité grâce à l'hypermutation somatique et la commutation de classe isotypique.

DC : *Dendritic cell; TCR: T cell receptor; CMH: complexe majeur d'histovompatibilité; Tfh: lymphocyte T folliculaire helper; IL: interleukine; LB: lymphocyte B; CG: centre germinatif; GCB: Lymphocyte B du centre germinatif*

II. <u>Métabolisme énergétique des LT</u>

Le métabolisme définit l'ensemble des réactions chimiques intracellulaires permettant de convertir les nutriments en énergie et en métabolites nécessaires à la survie de l'organisme. La cellule animale saine est capable de générer de l'énergie chimique sous forme d'ATP (Adénosine Tri Phosphate). La quantité d'énergie produite ou consommée par une cellule dépend de sa fonction et de son niveau de prolifération et de différenciation. Dans cette partie, je m'intéresserais spécifiquement aux différentes voies métaboliques intervenant au cours du développement et de la maturation des LT et je détaillerais la reprogrammation métabolique ayant lieu suite à leur stimulation ou leur différenciation en Tfh.

A. Les différentes voies métaboliques impliquées dans le développement des LT

1. Glycolyse

La glycolyse ou la voie d'Embden-Meyerhof est l'une des premières voies métaboliques à avoir été décrite. Elle consiste en une succession de dix réactions enzymatiques conduisant à la dégradation du glucose en 2 molécules de pyruvate, associée à la production de 2 molécules d'ATP (Lunt and Vander Heiden, 2011). Elle peut être divisée en deux phases, la première est une phase de préparation qui consomme de l'énergie (utilisation de 2 molécules d'ATP) et la deuxième phase correspond à la récupération de l'énergie avec un gain de 4 molécules d'ATP. L'ensemble de ces réactions a lieu exclusivement dans le cytosol chez tous les organismes eucaryotes. Elles peuvent se dérouler soit en présence d'oxygène, on parle alors de « glycolyse aérobie », soit en absence d'oxygène ou en hypoxie ($< 2\% O_2$), et on parle alors de « glycolyse anaérobie ». Ces deux phases ne diffèrent que par l'utilisation du produit final de la glycolyse : le pyruvate. En aérobie, celui-ci entre dans la mitochondrie pour être transformé en acétyl-CoA, intermédiaire du cycle tricarboxylique (TCA ou cycle de Krebs), permettant l'initiation de la phosphorylation oxydative qui produit une quantité importante d'ATP (Figure 4). Par contre, la glycolyse anaérobie a lieu chez les organismes qui ne possèdent pas de chaîne respiratoire (e.g. les érythrocytes) ou dans les tissus lorsqu'une diminution de la concentration d'oxygène a lieu (e.g. les cellules musculaires lors d'un effort physiques). Elle consiste à la fermentation du pyruvate en lactate ou en alcool (Donnelly and Finlay, 2015). De façon intéressante, la

glycolyse anaérobie peut aussi avoir lieu dans les cellules cancéreuses y compris en présence d'oxygène. C'est ce que l'on appelle l'effet Warburg.

2. Le cycle de Krebs

Le cycle de Krebs ou également appelé cycle des acides tricarboxyliques est le carrefour métabolique intermédiaire où se rejoignent les produits dérivés du catabolisme du glucose, des acides gras et de nombreux acides aminés (Akram, 2014). Cette voie métabolique a lieu dans la matrice mitochondriale et se fait exclusivement en condition aérobie. Elle comprend une succession de huit réactions d'oxydo-réduction qui utilisent un substrat unique, l'acétyl-CoA, pour produire de l'énergie. Ainsi, comme décrit précédemment, en conditions d'aérobie, le pyruvate se transforme en acétyl-CoA qui représente le substrat de départ du cycle de Krebs. La première étape consiste à transférer un groupement acétyl provenant de l'acétyl-CoA sur une molécule d'oxaloacétate pour former une molécule de citrate. Celle-ci se transforme ensuite en isocitrate qui subit une décarboxylation oxydative permettant la formation de l' α -kétoglutarate. Un enchaînement de réactions enzymatiques a lieu ainsi permettant une production successive de métabolites à base de succinate, fumarate et malate. Au cours de ces étapes, le cycle de krebs va permettre la production d'énergie, soit directement sous forme d'ATP, soit indirectement en fournissant du pouvoir réducteur (sous forme de NADH et FADH₂) nécessaire au bon fonctionnement de la chaîne respiratoire (Figure 4).

3. La phosphorylation oxydative

La phosphorylation oxydative (OXPHOS), appelée aussi chaîne respiratoire, a lieu dans la mitochondrie au niveau de la membrane interne. Elle consiste en un transfert d'électrons, depuis les substrats énergétiques comme le NADH et le FADH₂ vers l'accepteur final : l'oxygène. Ce transfert d'électrons se fait à travers 5 groupes de transporteurs : les complexes I à V et nécessite une série de réactions d'oxydo-réduction, catalysée par les enzymes de la chaîne respiratoire (Figure 4). Chaque complexe dans la chaîne respiratoire présente une affinité plus grande pour les électrons que son prédécesseur, et ainsi les électrons passent d'un complexe à l'autre jusqu'à être finalement transférés à l'oxygène, qui a l'affinité la plus grande pour les électrons. L'OXPHOS débute avec l'entrée des électrons à travers le complexe I et II lors de l'oxydation du NADH ou du FADH₂. Ces électrons sont ensuite transportés par l'ubiquinone jusqu'au complexe III qui les transfère après au cytochrome c. Le complexe IV complète la séquence en transférant les électrons du cytochrome c à l'oxygène. Ce transport des électrons est couplé à

une translocation de protons à travers la membrane mitochondriale interne, vers l'espace intermembranaire. Ce gradient de protons nommé potentiel mitochondrial constitue une réserve d'énergie qui est finalement utilisée par le complexe V pour produire de l'ATP. Ainsi, le système OXPHOS génère la majorité de l'ATP cellulaire (36 molécules d'ATP/molécule de glucose), et est composé de plus de quatre-vingts protéines différentes, treize d'entre elles étant codées par l'ADN mitochondrial et les autres par le génome nucléaire (Donnelly and Finlay, 2015).

4. La β-oxydation des acides gras

La β-oxydation des acides gras est la voie métabolique permettant la dégradation des acides gras et la libération de leur potentiel énergétique. Les acides gras, issus de la circulation sanguine, pénètrent dans les cellules pour être oxydés principalement au niveau de la mitochondrie. Par contre, les acides gras, reconnus par les cellules sous forme de gros complexes de triglycérides, ne peuvent y rentrer que s'ils sont sous forme libre. Cette étape d'hydrolyse est assurée par la lipoprotéine lipase qui facilite l'accès des acides gras au sein des cellules. Dans le cytosol, les acides gras sont couplés au coenzyme A par l'acyl-CoA synthétase formant les acyl-CoA. Une fois formés, les acyl-CoA sont transportés dans la mitochondrie où une série de transformations se déroule progressivement et de façon répétée. Cette série de réactions enzymatiques aboutissent à la génération de plusieurs molécules d'acétyl-CoA qui seront métabolisées par le cycle de Krebs (Figure 4). L'oxydation des acides gras est un processus hautement énergétique. Par exemple, le bilan énergétique finale de l'oxydation d'une molécule de palmitate (un acide gras à 16 carbones) correspond à la production de 129 molécules d'ATP (Carracedo et al., 2013)



Figure 4 : Les différentes voies métaboliques de dégradation du glucose et de l'acide gras

En conditions physiologiques normales, le glucose pénètre dans la cellule et se transforme en pyruvate via une succession de dix réactions enzymatiques (la glycolyse). Le pyruvate peut après, soit être métabolisé par une fermentation anaérobie pour produire du lactate, soit, en présence d'oxygène, pénétrer à l'intérieur de la mitochondrie pour être transformé en acétyl-CoA et rejoindre le cycle de Krebs. Celui-ci permet l'obtention des cofacteurs fortement énergétiques comme le NADH qui sera utilisé par les complexes de la chaîne respiratoire pour produire de l'ATP.

De manière similaire, la β -oxydation des acides gras se fait en quatre étapes successives permettant de générer de l'acétyl-CoA qui rejoint après le cycle de Krebs et la phosphorylation oxydative.

B. La reprogrammation métabolique des LT

L'activation des lymphocytes T (LT), leur expansion clonale et l'acquisition des fonctions effectrices sont des phénomènes récemment décrits pour être régulés par le métabolisme cellulaire, introduisant ainsi la notion d'« immunométabolisme » dans de nombreuses études biologiques (Pearce and Pearce, 2013). En effet, le passage des cellules T de l'état quiescent à l'état activé s'accompagne d'un « switch » métabolique principalement dû aux besoins énergétiques accrues nécessaires à leurs fonctions effectrices (Almeida et al., 2016; Pearce and

Pearce, 2013; Slack et al., 2015). Une des questions qui s'est alors posé est : « Comment ce switch métabolique se déroule-t-il et par quels mécanismes est-il contrôlé ? »

En réalité, après avoir quitté le thymus, les LT naïfs (non stimulés) demandent un niveau basal de nutriments pour achever leur migration dans les organes lymphoïdes secondaires et garantir une surveillance immunitaire adéquate. Bien que ce processus nécessite de faibles besoins métaboliques il demande un apport énergétique important. Pour répondre à ces besoins, les LT naïfs utilisent principalement les deux voies métaboliques connues pour fournir un rendement énergétique important qui sont : la β -oxydation des acides gras et la phosphorylation oxydative . Cependant, après activation antigénique, les LT subissent une reprogrammation métabolique, caractérisée par une augmentation de la glycolyse (Figure 5). Cette réorientation permet aux LT activés de diriger rapidement les intermédiaires métaboliques vers la synthèse de macromolécules requises pour leur prolifération et leur fonction effectrice. Par ailleurs, le passage vers un métabolisme glycolytique dans les cellules T matures dépend de la présence du transporteur de glucose Glut1. Il a été démontré que l'activation des LT via une costimulation des deux récepteurs TCR et CD28 induit une surexpression de Glut1 qui favorise l'apport en glucose. L'importance du métabolisme glycolytique dans les mécanismes d'activation des LT a été également observé dans les modèles de souris Glut1^{-/-} qui présentait un déficit d'apport en glucose. Ces souris présentent une perte de l'activation des LT, une inhibition de la sécrétion des cytokines et une susceptibilité à induire des maladies inflammatoires (Frauwirth et al., 2002; Macintyre et al., 2014). De manière intéressante il a été démontré que l'expression de Glut1 a un rôle primordial dans la sécrétion d'IFN-γ aussi bien en normoxie qu'en hypoxie (Cretenet et al., 2016).

Cependant, en plus de l'importance de la glycolyse dans l'activation des LT, la phosphorylation oxydative joue également un rôle dominant. En effet, la production des espèces réactives d'oxygène, régule l'expression de l'IL-2 jouant un rôle primordial dans les fonctions effectrices des LT (Sena et al., 2013). Il a été également démontré que l'inhibition de l'OXPHOS par l'oligomycine, un inhibiteur de l'ATP synthase (complexe V) de la chaîne mitochondriale, bloque les stades précoces d'activation des LT en inhibant l'expression de leurs marqueurs d'activation (Chang et al., 2013). En plus, une étude récente a montré, grâce à l'utilisation de plusieurs approches pharmacologiques et génétiques, la présence de différents changements métaboliques qui interviennent suite à la stimulation des LT naïfs. En effet, il semble que le processus d'activation des LT se fait en deux étapes successives métaboliquement contrôlées par deux complexes différents de la chaîne mitochondriale. Plus précisément, les auteurs ont prouvé que l'inhibition du complexe I de la chaîne de transport des électrons par un inhibiteur

pharmacologique, la roténone, a bloqué les stades précoces de différenciation des Th1 y compris l'entrée dans le cycle cellulaire et les modifications épigénétiques nécessaires à leurs fonctions effectrices. Cette fonction est principalement dépendante des transporteurs de malate et d'aspartate parce que l'invalidation génétique de ces transporteurs ($Slc25a1^{-/-}/Mdh^{--/}$) a également inhibé l'activation des LT. Alors que, le blocage du complexe II de la chaîne mitochondriale par le diméthylmalonate a révélé son rôle dans la finalisation de la différenciation des LT y compris la sécrétion des cytokines et l'élimination des agents pathogènes. Cette observation a été confirmé dans un modèle murin présentant une invalidation de l'enzyme responsable de l'activité du complexe II ($sdhc^{-/-}$) et qui a montré également une déficience des fonctions effectrices des LT (Bailis et al., 2019).

Enfin, l'ensemble de ces résultats confirme la complexité et le dynamisme de l'activation des LT, et montre une communication entre les différentes voies métaboliques pour assurer une fonction normale du système immunitaire.

Une fois le pathogène éliminé, 90% des LT meurent par apoptose. Un compartiment mémoire persiste dans l'organisme et présente la capacité de répondre de façon plus rapide et plus efficace contre une réinfection. Afin de persister pour une longue durée, les LT mémoires gardent leurs mitochondries intactes et utilisent la synthèse et l'oxydation des acides gras pour leur survie et leur persistance (Figure 5) (Bailis et al., 2019; O'Sullivan et al., 2014; van der Windt and Pearce, 2012).

De même, la différenciation des LT naïfs en Th17 s'accompagne d'une reprogrammation métabolique d'un état quiescent à dominance de phosphorylation oxydative à un état activé orchestré par le métabolisme glycolytique et la synthèse de novo des acides gras.

D'autre part, les Tregs présentent un métabolisme énergétique indépendant des autres populations des LT. Il semble que la glycolyse joue un rôle dans la génération et l'expansion des Tregs mais cette voie doit être complètement supprimée pour que ces cellules soient fonctionnelles. Plusieurs études ont montré l'implication du métabolisme oxydative dans l'expression de *Foxp3* et au cours des fonctions suppressives des Tregs (Beier et al., 2015; Galgani et al., 2016; Zeng and Chi, 2015).



Etapes d'activation des lymphocytes T

Figure 5 : Reprogrammation métabolique des LT au cours d'une réponse immunitaire

Les LT naïfs sont à l'état quiescent et produisent leur ATP via la phosphorylation oxydative. Cette voie métabolique est suffisante pour avoir un niveau basal de consommation des nutriments. En réponse à une stimulation, les LT prolifèrent et deviennent fonctionnels. Cette transition phénotypique requiert une augmentation de la glycolyse aérobie qui est indispensable aux fonctions effectrices des LT y compris la production des cytokines. Suite à l'élimination du pathogène, les LT qui survivent se différencient en LT mémoires et reviennent à un état métabolique oxydatif principalement basé sur l'oxydation des acides gras.

 T_N : LT naïfs ; T_{eff} : LT effecteurs ; T_m : LT mémoires

C. Métabolisme énergétique des LT folliculaires (Tfh)

Les lymphocytes T folliculaires helper diffèrent des autres populations de LT effecteurs par leur dialogue avec les lymphocytes B du centre germinatif afin de contrôler leur différenciation en plasmocytes et la production des anticorps. Comme il a été bien établi que la génération des Tfh à partir des CD4⁺ naïfs se fait en plusieurs étapes (initiation, expansion et maintenance) les changements métaboliques semblent être un des mécanismes régulant le passage des Tfh d'une étape à une autre. Plusieurs études ont révélé une reprogrammation métabolique différente entre les Th1 et les Tfh et même au sein des différents stades de maturation des Tfh. Le rôle de la

glycolyse au cours de la génération des Tfh a été longtemps étudié aboutissant à deux conclusions contradictoires qui restent à être clarifiées. Tout d'abord, un rôle favorable de la glycolyse dans la génération des Tfh a été démontré aussi bien in vitro qu'in vivo. En effet, Zeng et ses collègues ont montré que la stimulation des Tfh par le biais de leur récepteur ICOS active la voie de signalisation mTOR qui a un rôle majeur dans l'activation du métabolisme glycolytique et dans l'augmentation de la consommation du glucose (Figure 6). Ainsi, l'invalidation de cette voie dans des modèles murins (mTORC^{-/-}) bloque la signalisation ICOS et par suite inhibe la différenciation des Tfh et le développement des centres germinatifs (Zeng et al., 2016). En plus, un traitement des Tfh en culture avec un inhibiteur de la glycolyse (le 2-Déoxyglucose) inhibe la sécrétion des cytokines IL-21 et IL-4 responsables de la réponse humorale induite par les Tfh (Zeng et al., 2016). De manière similaire, une deuxième étude renforce cette hypothèse justifiant l'implication de la glycolyse dans le développement Tfh en proposant d'utiliser un inhibiteur du métabolisme glycolytique « le 2-déoxyglucose » dans le traitement des maladies auto-immunes. De manière intéressante, dans cette étude, il a été démontré une implication des cellules Tfh dans la mise en place du lupus érythémateux disséminé en raison d'une activation accrue de la réponse humorale et une production excessive d'auto-anticorps. Une inhibition de la glycolyse dans ces Tfh entraîne une élimination des LT auto-réactifs en laissant intactes les autres composants de la réponse immunitaire, montrant un effet majeur de la glycolyse sur la survie des Tfh (Choi et al., 2018). De plus, une étude récente a montré également l'implication de la glycolyse et plus précisément de la signalisation E3 ligase (VHL)/HIF-1 α au cours de l'initiation de développement des Tfh (Zhu et al., 2019). Il semble qu'une invalidation de cette voie de signalisation ralentit la différenciation des Tfh, en raison des modifications épigénétiques atténuant l'expression des gènes clés (principalement la méthylation m⁶A au niveau de l'ARN messager d'ICOS).

D'autre part, comme décrit précédemment l'une des caractéristiques des Tfh est l'expression du facteur de transcription Bcl-6 qui en plus de son rôle dans la génération et la maturation des Tfh, émerge récemment comme ayant un rôle majeur dans leur reprogrammation métabolique, et plus précisément dans l'inhibition du métabolisme glycolytique. Il a été démontré qu'en absence d'IL-2, Bcl-6 induit une répression des gènes impliqués dans la glycolyse y compris les gènes codant pour les transporteurs de glucose GLUT1 et GLUT3 (*Slc2a1 et Slc2a3*) ou pour certains enzymes glycolytiques (*HK2 et Aldoc*). Cela est dû dans les Tfh à l'absence de l'expression de *T-bet* qui exerce un contrôle négatif sur l'expression de Bcl-6 dans les autres populations de cellules T (Oestreich et al., 2014). De manière identique, Ray et ses collègues ont décrit dans leur étude que les besoins énergétiques des Tfh ainsi que les voies de

signalisation activées au cours de leur différenciation sont différents de ceux des cellules Th1. Ainsi ils ont montré que la production de l'IL-2 par les Th1 active la voie de signalisation mTOR/AKT qui favorise le métabolisme glycolytique et augmente l'expression du facteur de transcription *Blimp1* qui s'oppose à la différenciation des Tfh. Cette signalisation est complètement inversée suite à l'expression de Bcl-6 qui le facteur clé de la différenciation des Tfh. Les auteurs ont proposé ainsi que les Tfh sont moins prolifératives et métaboliquement moins actives que les autres populations des LT activés (Ray et al., 2015). Par ailleurs, le récepteur inhibiteur PD-1 a été identifié comme un élément clé dans la reprogrammation métabolique des Tfh. En effet, PD-1 inhibe l'apport et l'utilisation du glucose au sein des cellules et réduit le taux de lactate sécrété dans le surnageant des cellules in vitro (Patsoukis et al., 2015). Cette inhibition de la glycolyse s'accompagne d'une augmentation de la synthèse et de l'oxydation des acides gras. Il a été également démontré que la différenciation des LT CD4+ in vitro en absence de glucose induit une augmentation de l'expression de PD-1 à la surface des cellules, un mécanisme accompagné par un métaolisme oxydatif (Figure 6) (Chang et al., 2013). Ainsi la régulation métabolique des Tfh semble être un mécanisme dynamique jouant un rôle majeur au cours de leur fonction et reste à être clairement définie.



Figure 6 : Deux hypothèses expliquant la reprogrammation métabolique des Tfh

Deux hypothèses contradictoires se sont émergés récemment pour mettre en évidence la voie métabolique contrôlant la différenciation des Tfh.

D'une part (en bleu), la stimulation par ICOS active la voie de signalisation mTOR qui elle, active la transcription du gène codant pour Glut1, le transporteur de glucose. L'augmentation de la consommation du glucose active ainsi la glycolyse et favorise la différenciation des Tfh.

D'autre part (en rouge), PD-1, marqueur essentiel des Tfh, inhibe la voie glycolytique et réoriente le métabolisme cellulaire vers la dégradation et la β -oxydation des acides gras. De même, l'expression de Bcl-6, facteur clé de la différenciation des Tfh, inhibe l'expression des enzymes de la glycolyse.

III. <u>Glycéraldéhyde-3-Phosphate Déshydrogénase</u> (GAPDH)

Parmi les enzymes qui régulent la production d'énergie par la glycolyse (Figure 4), la glycéraldéhyde-3-phosphate déshydrogénase semble avoir une importance particulière. Elle était initialement identifiée comme étant une enzyme métabolique. Mais, son niveau d'expression au sein des cellules a fait qu'elle est souvent considérée comme un gène de contrôle. Cependant, de nombreuses études décrivent la GAPDH comme ayant de nombreuses fonctions cellulaires non-glycolytiques. Dans cette partie, je détaillerai l'ensemble de ces fonctions en me concentrant sur le potentiel rôle de cette enzyme dans le développement et l'activation des LT.

A. Fonction glycolytique

La glycéraldéhyde-3-phosphate déshydrogénase (GAPDH) est l'une des enzymes clés de la glycolyse. Elle catalyse, en présence du phosphate inorganique, la conversion du glycéraldéhyde-3-phosphate (G3P) en 1,3-biphosphoglycérate (1,3-DPG) (Figure 7). Cette réaction est l'unique, parmi les dix réactions de la glycolyse, permettant la réduction du NAD⁺ en NADH,H⁺, fournissant ainsi pour les cellules une première source d'énergie. La GAPDH se présente sous la forme d'un homotétramère dont les sous-unités ont une masse moléculaire variante de 34 à 38 kDa selon les espèces. Chaque sous-unité est composée d'environ 330 acides aminés qui se replient pour former deux domaines, un premier domaine qui se lie au cofacteur NAD⁺ et un second domaine impliqué dans la fixation et la catalyse du substrat. Les fonctions glycolytiques de la GAPDH dépendent princiapalement de deux acides aminés, la cystéine 152 et l'histidine 179, essentiels aux mécanismes catalytiques (Colell et al., 2009).



Figure 7 : Fonction glycolytique de la GAPDH

La GAPDH catalyse la conversion du glycéraldéhyde 3-phosphate en 1,3biphosphoglycérate. Cette réaction est couplée à la réduction du NAD+ en NADH,H⁺.

B. Fonctions non-glycolytiques

En plus de son rôle en tant qu'une enzyme métabolique, de nombreuses études ont identifié la participation de la GAPDH dans de nombreuses fonctions cellulaires non-glycolytiques (Tableau 1).

1. Rôle dans la composition cellulaire

Un grand nombre de fonctions non-glycolytiques de la GAPDH provient de sa capacité à se lier à différentes macromolécules dans la cellule. En effet, plusieurs études ont mis en évidence l'implication de la GAPDH dans l'endocytose (Robbins et al., 1995), dans l'assemblage de la membrane nucléaire (Nakagawa et al., 2003) ainsi que dans le transport membranaire entre le réticulum endoplasmique et l'appareil de golgi grâce à sa liaison à la GTPase Rab2 (Tisdale et al., 2004). La GAPDH intervient également dans l'assemblage des microtubules jouant ainsi un rôle dans la formation du cytosquelette (Huitorel and Pantaloni, 1985; Tisdale et al., 2009). Elle interagit également avec les acides nucléiques. Les premières études ont montré une grande affinité de la GAPDH à discriminer les formes sauvages et mutants des ARN de transfert afin de réguler leur translocation nucléaire (Singh and Green, 1993). Des études ultérieures ont caractérisé la GAPDH comme étant capable de se lier aux ARN par l'intermédiaire du domaine de fixation du NAD⁺ (Nagy et al., 2000). Ces résultats ont été confirmé tardivement en montrant que la GAPDH se lie à l'ARNm préférentiellement au niveau des éléments riches en résidus AU dans la région 3'UTR, assurant ainsi la stabilité de cet ARN et inhibant sa traduction en protéines (Rodríguez-Pascual et al., 2008; Zhou et al., 2008).

En plus de ces rôles cytosolique et membranaire, la GAPDH a la capacité de se transloquer dans le noyau présentant ainsi de nombreux fonctions nucléaires. En effet, il a été démontré que la GAPDH permet un recrutement du complexe co-activateur de la transcription (OCA-S) au niveau du promoteur de l'histone 2B (Zheng et al., 2003). De la même façon, une étude effectuée sur des levures a montré que la GAPDH interagit avec la sous-unité Rpb4 de l'ARN polymérase II permettant l'initiation de la transcription (Mitsuzawa et al., 2005). D'autre part le rôle de la GAPDH dans la réparation de l'ADN a été mis en évidence dans des cellules de leucémie *in vitro* suite à un traitement avec un agent altérant la séquence d'ADN. Cette étude a montré que la GAPDH s'associe à un système de réparation des mésappariements (mismatch repair MMR) qui permet de détecter les dommages de l'ADN et de les réparer (Krynetski et al., 2001). De façon intéressante il a été aussi démontré que la GAPDH joue un rôle dans l'échappement des cellules cancéreuses aux agents chimiothérapiques. En effet, dans une lignée cellulaire d'adénocarcinome, suite à une exposition aux céramides, la GAPDH est capable de se lier aux télomères permettant de les protéger d'une dégradation et induisant ainsi une progression tumorale (Sundararaj et al., 2004).

2. Rôle de GAPDH dans la régulation des voies de signalisation

En plus de ses fonctions dans la régulation des processus cytoplasmiques et nucléaires, la GAPDH a été identifié comme un facteur activant plusieurs voies de signalisation. En effet, il a été démontré que la GAPDH interagit avec la protéine TRAF2 (TNF receptor-associated factor 2), et régule sa polyubiquitination, une étape indispensable à la transmission du signal et l'activation de la voie NF- κ B (Figure 8). Cette interaction a été identifiée pour la première fois dans un modèle d'infection par E.coli. En effet, il a été démontré que la protéine bactérienne NleB, empêche la liaison GAPDH-TRAF2 par glycosylation de la GAPDH et par suite inhibe

l'activation de la voie NF- κ B. Ce mécanisme est considéré comme un moyen d'échappement des bactéries aux réponses immunitaires innées (Gao et al., 2013). De manière similaire, l'interaction GAPDH-TRAF2 a été observée dans les modèles de lymphomes B diffus à grandes cellules. Ainsi, mon équipe a montré qu'en condition d'hypoxie, une expression élevée de la GAPDH, mais non des autres enzymes de la glycolyse, favorise l'activation de la voie NF- κ B par l'intermédiaire de la liaison à TRAF2. Cette activation se traduit par une augmentation de l'expression des gènes *HIF1* α et *VEGFa* qui jouent un rôle clé dans la vascularisation et l'agressivité du lymphome (Chiche et al., 2015).

Par ailleurs, la GAPDH semble jouer un rôle dans la réponse immunitaire innée par l'intermédiaire de la signalisation p38/MAPK. Suite à une infection, GAPDH interagit avec LL-37, un peptide anti-microbien, pour augmenter la signalisation p38/MAPK. Cette interaction induit la transcription des gènes anti-inflammatoires et des chimiokines qui favorisent le recrutement des monocytes nécessaires à l'élimination des pathogènes (Mookherjee et al., 2009).



Figure 8 : Mécanisme de régulation de la voie NF-KB par la GAPDH

La GAPDH interagit avec la protéine TRAF2 et permet sa stabilisation, une étape indispensable à la transmission du signal et l'activation de la voie NF- κB .

De manière intéressante, il a été également démontré que la GAPDH joue un rôle majeur dans la régulation de la voie de signalisation mTOR, impliqué dans l'activation du métabolisme glycolytique. En effet, suite à une faible consommation du glucose, la GAPDH se lie à Rheb, un composant majeur de la voie mTOR, et inhibe ainsi cette signalisation. Par ccontre, la présence du glycéraldéhyde 3-phosphate, l'intermédiaire glycolytique qui se lie à la GAPDH, déstabilise l'interaction GAPDH-Rheb et réactive ainsi la voie mTOR (Chiche et al., 2019; Lee et al., 2009).

3. Rôle de la GAPDH dans la mort cellulaire

Le rôle de la GAPDH dans la régulation de la mort cellulaire semble être un processus complexe montrant des résultats contradictoires dépendants principalement de son niveau d'expression. Bien que la GAPDH a été décrite initialement comme ayant un rôle pro-apoptotique, plusieurs études ont démontré plus tard sa capacité à protéger les cellules dans les conditions où les caspases sont inhibées comme c'est le cas de la mort cellulaire indépendante des caspases ou « CICD ». Les premières preuves du rôle pro-apoptotique de la GAPDH ont été abordées sur des neurones cérébraux en culture, dans lesquels il a été démontré qu'une augmentation de l'expression de la GAPDH est associée à la mort cellulaire accrue suite à un traitement avec des agents apoptotiques. De manière intéressante, l'utilisation des oligonucléotides anti-sens dirigés contre la GAPDH empêche la translocation nucléaire de l'enzyme et assure un mécanisme de protection cellulaire contre l'apoptose mettant en place ainsi un rôle proapoptotique de la GAPDH (Ishitani et al., 1998). Ces observations ont été étendues plus tard sur plusieurs modèles cellulaires en expliquant plus en détails les mécanismes de cette régulation. En effet, il a été décrit que l'accumulation nucléaire de la GAPDH favorise l'induction de la mort cellulaire par une inhibition de la protéine anti-apoptotique Bcl-2 (Dastoor and Dreyer, 2001; Maruyama et al., 2001). Il semble également que le passage de la GAPDH dans le noyau est accompagné par l'apparition de plusieurs formes alkalines qui renforcent sa fonction pro-apoptotique (Saunders et al., 1999).

Un deuxième mécanisme de mort cellulaire induit par la GAPDH a été expliqué ultérieurement. En effet, suite à un stress cellulaire ou un signal de mort, l'enzyme oxyde nitrique synthase est activée et induit la S-nitrosylation de la GAPDH qui favorise sa liaison à Siah1. Cette protéine contient dans sa séquence un domaine de translocation nucléaire qui favorise le passage de la GAPDH dans le noyau. Siah1 est une E3 ubiquitine ligase qui permet une poly-ubiquitination des protéines nucléaires et favorise la mort cellulaire (Hara et al., 2005). L'implication de la S- nitrosylation dans la fonction pro-apoptotique de la GAPDH a été confirmé dans les cellules de cancer thyroïdien (Du et al., 2007).

D'une manière intéressante, Ricci & Colell et leurs collègues ont montré, par l'utilisation d'une banque rétrovirale, que la GAPDH est impliquée dans la protection des cellules contre la mort cellulaire indépendante des caspases (CICD). En effet, suite à un signal de mort, la surexpression de la GAPDH protège les cellules et favorise leur prolifération dans les conditions où les caspases sont inhibées. De manière intéressante, les auteurs ont mis en place deux mutants de la GAPDH, un premier incapable de transloquer au noyau et un deuxième qui ne peut pas remplir sa fonction glycolytique. Ainsi, chacun de ces mutants n'a pas été capable de protéger les cellules de la CICD. Ces résultats ont indiqué qu'aussi bien les fonctions glycolytiques que nucléaires sont nécessaires pour la protection contre la CICD. De plus il a été montré que ce mécanisme se fait par l'intermédiaire de l'induction de l'autophagie. En effet, en condition où l'autophagie est inhibée par une invalidation du gène Atg5, la GAPDH n'est plus capable de protéger les cellules. L'ensemble des résultats de ces études, ont montré que la translocation nucléaire de la GAPDH induit une transcription des gènes régulateurs de l'autophagie, assurant ainsi une survie des cellules en condition où l'apoptose est inhibée (Colell et al., 2007) (Figure 9). Dans le même contexte, mon équipe a montré plus tard que la GAPDH, lorsqu'elle est surexprimée, se lie à la forme active d'Akt bloquant sa déphosphorylation. Une activation constitutive d'Akt empêche la localisation nucléaire de FoxO ce qui diminue l'expression de Bcl-6 et conduit à la surexpression de Bcl-xl. Cette surexpression est capable de protéger les mitochondries de la perméabilisation. Cette étude a mis en évidence un mécanisme d'échappement des cellules tumorales aux chimiothérapies (Jacquin et al., 2013). D'autre part, une étude effectuée en condition de déprivation en glucose, a confirmé l'implication de la GAPDH dans l'induction de l'autophagie. Suite à un manque en glucose, la GAPDH cytoplasmique est phosphorylée par l'AMPK activée, ce qui induit sa translocation nucléaire. À l'intérieur du noyau, la GAPDH active Sirt1, une histone déacétylase qui cible les gènes de l'autophagie (Atg5 et Atg7) induisant la formation des autophagosomes (Chang et al., 2015).



Figure 9 : Mécanisme de régulation de la mort cellulaire indépendante des caspases par la GAPDH

Suite à un stress cellulaire, dans les conditions où les caspases sont inhibées, la GAPDH favorise la survie cellulaire grâce à ses fonctions cytosoliques et nucléaires. Dans le cytosol, la GAPDH participe à la voie de la glycolyse. Dans le noyau, la GAPDH participe à la transcription du gène régulant l'autophagie, Atg12. Une fois dans le cytoplasme, Atg12 favorise l'élimination des mitochondries endommagées suite à la perméabilisation de la membrane mitochondriale, permettant la protection et la survie cellulaire.

4. Rôle de la GAPDH dans le développement tumoral

Vu son rôle pro-apoptotique décrit dans certains lignées cellulaires, il était inattendu de détecter une surexpression de la GAPDH au cours du développement tumoral. Néanmoins, des taux élevés de la plupart des enzymes glycolytiques, y compris la GAPDH ont été observés dans la plupart des cancers humains (Altenberg and Greulich, 2004), et ont été souvent associés à un mauvais pronostic. Par exemple, une surexpression de la GAPDH est observée dans les cancers du sein et de la prostate à un stade avancé de la progression tumorale et au cours de la formation des métastases (Epner and Coffey, 1996; Lu et al., 2002). En effet, ces mécanismes de surexpression étaient partiellement expliqués par les travaux de Warburg en 1929 montrant que les cellules cancéreuses utilisent fréquemment le métabolisme glycolytique pour leur invasion, ce qui nécessite une expression et une activité abondantes des enzymes régulant cette voie

énergétique. Par la suite et comme décrit précédemment, la GAPDH a été identifiée comme ayant un rôle dans la protection de la mort cellulaire dans les conditions où les caspases sont inhibées. Ce mécanisme de protection confère aux cellules tumorales une capacité de résister aux traitements chimiothérapiques et met en évidence une nouvelle fonction pro-tumorale de la GAPDH en plus de sa fonction glycolytique. Cette observation a été confirmée dans un modèle cellulaire de leucémie myéloïde chronique (LMC) résistant au traitement de référence « l'imatinib ». En effet, les cellules LMC résistantes ont été protégées de la mort cellulaire par une surexpression de la GAPDH et par suite une invalidation, même partielle de cette enzyme a resensibilisé les cellules au traitement (Lavallard et al., 2009). Les mêmes résultats ont été observées dans le cas de leucémie lymphoïde aigue, montrant que le ciblage de la GAPDH peut être une nouvelle stratégie thérapeutique qui pourrait module l'échappement des cellules tumorales au traitement de référence (Hulleman et al., 2009). D'autre part, mon équipe a montré qu'une expression élevée de la GAPDH, mais pas des autres enzymes glycolytiques testées, augmente l'agressivité et la vascularisation des lymphomes B non-hodgkiniens. Ce mécanisme est dû à l'activation par la GAPDH de la voie de signalisation NF-κB qui ciblent la transcription des gènes oncogènes (Chiche et al., 2015).

De plus, une étude récente a mis en évidence une implication de la GAPDH dans la formation des métastases pulmonaires par l'intermédiaire du facteur de transcription BACH1. En effet, les antioxydants utilisés en clinique pour s'opposer au stress oxydatif des cellules tumorales, stabilisent l'expression de BACH1. Celui-ci à son tour active la transcription de *GAPDH* et de l'*Hexokinase2* qui stimulent la consommation du glucose et le métabolisme glycolytique et par suite favorisent la croissance et la progression tumorale (Wiel et al., 2019).

Récemment, une nouvelle étude de mon équipe a identifié la GAPDH comme étant un biomarqueur qui pourrait prédire la réponse des patients atteints de lymphome B diffus à grandes cellules DLBCL (Diffuse Large B Cell Lymphoma) au traitement R-CHOP (Chimiothérapie associée à la Rituximab). En effet, dans une cohorte de patients, une faible expression de la *GAPDH* (GAPDH^{low}) est associée à un échappement des cellules tumorales aux traitements. Ces cellules, contrairement aux cellules GAPDH^{high}, présentent un métabolisme basé sur la respiration mitochondriale et la glutaminolyse, et par suite sont sensibles à des inhibiteurs spécifiques de ces deux voies (Phenformine et L-Asparaginase). Ce modèle a été validé aussi bien sur des modèles murins que sur des patients en rechute au R-CHOP (Chiche et al., 2019).

5. Rôle de la GAPDH dans le développement et la fonction des cellules T

À ce jour, nous connaissons très peu de choses sur le rôle de la GAPDH au cours du développement et de la différenciation des LT. Néanmoins, certaines études ont mis en évidence une implication de la GAPDH dans la régulation des fonctions effectrices des LT et la génération des LT mémoires. Comme nous l'avons vu précédemment, l'activation des cellules T s'accompagne d'une augmentation rapide de la voie glycolytique. En 2013, Chang et ses collègues ont identifié que la GAPDH, suite à des changements métaboliques cellulaires et à des fluctuations de son taux d'expression, est capable de contrôler les fonctions effectrices des LT ainsi que la sécrétion des cytokines. En effet, ils expliquent qu'après stimulation des LT, la GAPDH est impliquée dans le métabolisme glycolytique pour assurer l'énergie nécessaire à leur activation. Par contre, dans les conditions où la glycolyse est inhibée, la GAPDH est libérée de ses fonctions glycolytiques et est liée aux éléments riches en AU au niveau de l'extrémité 3'UTR de l'ARNm de l'IFN- γ , induisant sa stabilisation et une inhibition de sa traduction (Chang et al., 2013). Cette régulation de l'IFN- γ est accompagnée d'une augmentation de l'expression de PD-1 qui envoie des signaux inhibitrices à l'activation cellulaire. De cette façon, la GAPDH inhibe l'activation et la sécrétion des cytokines dans les LT naïfs en absence de la glycolyse (Figure 10). De la même manière, un rôle de la GAPDH dans la régulation de l'expression de $HIF1\alpha$ dans les LT naïfs et mémoires a été également identifié. En effet, il semble que l'expansion des LT mémoires effectrices dans des conditions d'hypoxie est liée à une forte expression du facteur HIF1 α et une activité accrue du métabolisme glycolytique. Ce mécanisme est absent dans les LT naïfs et cela est due à l'activité de la GAPDH qui, en absence de la glycolyse, régule négativement l'expression d' $HIF1\alpha$ en se liant à son ARNm (Xu et al., 2016). Ces travaux montrent que la GAPDH joue un rôle fondamental dans la régulation des fonctions effectrices des LT par l'intermédiaire de sa capacité à stabiliser les ARNm.

De manière intéressante, il a été décrit que, suite à un stress cellulaire, comme dans le cas des infections bactériennes, plusieurs tissus libèrent de l'acétate. Au sein des cellules, une augmentation du taux d'acétate provoque sa conversion en acétyl-CoA qui favorise l'acétylation de la GAPDH. Cette modification post-traductionnelle augmente l'activité glycolytique de l'enzyme et entraîne une reprogrammation métabolique au sein du système immunitaire à l'origine de l'activation des cellules T CD8⁺ mémoires (Balmer et al., 2016).



Figure 10 : Rôle de la GAPDH dans la régulation des fonctions effectrices des cellules T

Suite à la stimulation des LT, la GAPDH est impliquée dans le métabolisme glycolytique pour assurer l'énergie nécessaire à leur activation et la production des cytokines (IFN γ). Dans les conditions où la glycolyse est inhibée comme par les signaux de co-inhibition émis par PD-1, la GAPDH est libérée de ces fonctions glycolytiques et est capable de se lier à l'ARNm de l'IFN γ , induisant sa stabilisation et une inhibition de sa traduction

Fonction	Mécanisme	Référence
Formation du cytosquelette	Par assemblage des microtubules polymérisées	(Huitorel and Pantaloni, 1985)
Augmentation de l'endocytose	Par liaison aux microtubules du cytosquelette membranaire	(Robbins et al., 1995)
Assemblage de la membrane nucléaire	Par induction de la fusion des vésicules phospholipidiques	(Nakagawa et al., 2003)
Transport membranaire entre le réticulum endoplasmique et l'appareil de golgi	Par interaction avec la GTPase Rab2 qui joue un rôle majeur dans le transport vésiculaire entre le RE et l'appareil de Golgi	(Tisdale et al., 2004)
Stabilisation de l'ARNm et inhibition de la traduction	Par liaison aux éléments riches en résidus AU dans la région 3'UTR de l'ARNm	(Rodríguez-Pascual et al., 2008; Zhou et al., 2008)
Activation du cycle cellulaire	Par association au complexe OCA-S qui est recruté au niveau du promoteur de l'histone 2B durant la phase S	(Zheng et al., 2003)
Réparation de l'ADN	Par liaison à un complexe ADN- protéine qui constitue un système de réparation des mésappariements	(Krynetski et al., 2001)
Initiation de la transcription	Par liaison à la sous-unité Rpb7 de l'ARN polymérase II	(Mitsuzawa et al., 2005)
Échappement des cellules cancéreuses aux agents chimiothérapiques	Par liaison aux télomères permettant de les protéger de la dégradation	(Sundararaj et al., 2004)
Activation de la voie de signalisation NF-κB	Par interaction avec la sous-unité TRAF2 qui permet la transmission du signal d'activation	(Chiche et al., 2015, 2015; Mondragón et al., 2019)
Activation de la réponse immunitaire innée et recrutement des monocytes	Par interaction avec le peptide antimicrobien LL-37 pour activer la signalisation MAPK p38 et activer la transcription des gènes codant pour les protéines anti-inflammatoires	(Mookherjee et al., 2009)
Inhibition de la voie de signalisation mTOR	Par lisaion GAPDH-Rheb	(Chiche et al., 2019; Lee et al., 2009)
Rôle pro-apoptotique	Par inhibition de la protéine anti- apoptotique Bcl-2	(Dastoor and Dreyer, 2001)
de la GAPDH favorise l'induction de l'apoptose)	Suite à sa S-nitrosylation, la GAPDH se lie à Siah1 qui induit une polyubiquitination des protéines	(Hara et al., 2005)

Tableau 1: Fonctions non-glycolytiques de la GAPDH

	nucléaires et favorise la mort cellulaire	
Protection des cellules contre la mort cellulaire indépendante des caspases	Par induction de l'autophagie qui permet une élimination des mitochondries endommagées	(Colell et al., 2007)
	Par une induction constitutive de la voie de signalisation AKT qui induit une surexpression de Bcl-xl et protège les mitochondries intactes de la perméabilisation	(Jacquin et al., 2013)
Rôle dans la progression tumorale	Résistance des cellules cancéreuses aux traitements chimiothérapiques à cause de son rôle de protection contre la mort cellulaire non apoptotique	(Lavallard et al., 2009)
	Par activation de la voie NF-κB qui induit une augmentation de l'agressivité et de la vascularisation des lymphomes B	(Chiche et al., 2015)
	Biomarqueur qui pourrait prédire la réponse des patients atteints de lymphome B aux traitements de référence	(Chiche et al., 2019)

IV.<u>NF-κB</u>

La régulation de l'expression des gènes est un élément central de la physiologie cellulaire et représente la clé de l'adaptation des cellules aux différents stress environnementaux. Depuis sa découverte, la voie NF- κ B a servi de modèle dans l'étude des facteurs de transcription inductibles. Beaucoup de nos connaissances actuelles découlent des efforts visant la compréhension de cette voie de signalisation dans le fonctionnement, la régulation des réponses immunes mais également dans des processus cellulaires fondamentaux tels que la prolifération, la différenciation et la survie cellulaire. Chez les mammifères, la famille des facteurs de transcription NF- κ B est constituée de cinq membres : p65 (RelA), RelB, c-Rel, NF- κ B1 (p105/p50) et NF- κ B2 (p100/p52) (Gilmore, 2006; Scheidereit, 2006).

Ces protéines partagent en N-terminal un domaine conservé de 300 acides aminés appelé « Rel homology domain (RHD) ». Ce domaine contient des séquences nécessaires à la dimérisation, la fixation à l'ADN, l'interaction avec les protéines inhibitrices (IκB) ainsi qu'à la translocation nucléaire des protéines NF-κB.

Les membres de la famille NF-κB ne sont transcriptionnellement actifs que sous forme d'homo ou d'hétéro-dimères. Ils influent sur la biologie de la cellule à travers deux voies principales de signalisation qui se distinguent, non seulement par les intervenants moléculaires, mais également par les voies d'activation ainsi que les réponses cellulaires induites. Il s'agit des voies classiques et alternatives appelées également canoniques et non-canoniques (Figure 11). Pour l'une comme pour l'autre, en absence de stimulation, les dimères NF-κB sont maintenus inactifs dans le cytoplasme par leur interaction avec des protéines inhibitrices de la famille IκB. Suite à un stimulus extracellulaire, ces protéines sont phosphorylées, ubiquitinées et puis dégradées par le protéasome. Les dimères NF-κB sont ainsi redistribués vers le compartiment nucléaire où ils exerceront leur activité transcriptionnelle au niveau des gènes cibles.

A. Voie de signalisation canonique (classique)

La voie de signalisation classique est la plus connue et par suite la mieux étudiée des deux. Sa principale unité fonctionnelle est l'hétérodimère p65(RelA)/p50 qui est maintenu inactif grâce à son interaction avec I κ B α . L'activation de cette voie est déclenchée par de nombreux stimuli extracellulaires tels que les stress cellulaires (les UV ou les irradiations gamma), les produits viraux ou bactériens (LPS) ou également certains cytokines (IL-1 et TNF α). Lors d'une

stimulation, la fixation du ligand sur son récepteur spécifique induit un recrutement des protéines adaptatrices (TRAF2/TRAF5) qui à leur tour recrutent un complexe de protéines kinases IKK (IKK α /IKK β /NEMO). Ces kinases induisent une phosphorylation des protéines inhibitrices I κ B α , qui seront ensuite ubiquitinées et dégradées permettant la libération de l'hétérodimère RelA/p50 et sa translocation nucléaire. Une fois dans le noyau, ce complexe peut alors se fixer sur les séquences promotrices et activer ou réprimer les gènes cibles. Parmi les premiers gènes à transcrire, cette voie induit l'expression du gène codant pour I κ B α (NF- κ Bia) établissant ainsi une boucle de rétrocontrôle négatif. Ce mécanisme permet aux protéines inhibitrices de rentrer dans le noyau et récupérer le complexe NF- κ B1 pour arrêter la transcription (Figure 11) (Hayden and Ghosh, 2012).

B. Voie de signalisation non-canonique (alternative)

Les principaux acteurs moléculaires de la voie alternative ou non canonique NF- κ B sont les sous-unités RelB et p100. En absence de stimulation, les dimères RelB/p100 sont maintenus inactifs dans le cytoplasme par l'action inhibitrice du domaine ANK de la sous-unité p100 montrant que celle-ci a un rôle d'auto inhibition de la voie de signalisation. L'activation de cette voie est induite par les cytokines de la superfamille des TNF tels que RANK, lymphotoxine- β , BAFF, CD40-L et leurs récepteurs respectifs. Une protéine clé de cette voie est la kinase NIK (NF- κ B inducible kinase) qui est associée dans le cytoplasme à un complexe de protéines inhibitrices (TRAF3/TRAF2/c-IAP1/2) (Sun, 2011). En absence de stimulation, c-IAP1/2 induit la dégradation de NIK par des cycles continus de phosphorylation et d'ubiquitination. La fixation d'un ligand à son récepteur permet la dégradation des protéines inhibitrices et la stabilisation de NIK. NIK, activée ainsi par autophosphorylation, phosphoryle IKK α , qui à son tour induit la dégradation de la sous-unité p100 par le protéasome en réponse à son ubiquitinylation. Ceci permet la conversion de RelB/p100 en RelB/p52 qui, libéré de l'action inhibitrice de p100, peut-être transloqué au noyau où il régulera l'expression de gènes cibles (Figure 11).



Figure 11 : les deux voies d'activation canonique et non-canonique de NF-kB

La voie canonique est activée par la fixation du TNF α , de LPS, d'IL-1 sur leur récepteurs spécifiques induisant l'activation de complexe IKK composé de NEMO, IKK α et IKK β . Ce complexe phosphoryle I κ B α provoquant son ubiquitination puis sa dégradation protéasomale libérant les complexe p50 et RelA qui migrent dans le noyau.

La voie non canonique est activée par la stimulation du CD40L, BAFF activant la kinase NIK qui vient se fixer sur le complexe IKK α /IKK β associé à RelB et p100. p100 est alors dégradé et les sous-unités RelB/p52 sont libérées.

L'activation des deux voies induisent l'expression des gènes cibles. (NF- κ Bia, Ezh2...). Plusieurs liens existaient entre ces deux voies permettant notamment la régulation d'une voie par l'autre

C. Interaction entre les deux voies

Ces deux voies ont souvent été considérées comme deux voies bien distinctes et indépendantes l'une de l'autre. Mais des études récentes ont prouvé des liens qui existaient permettant notamment la régulation d'une voie par l'autre (Figure 11) (Sun, 2012). Un mécanisme bien reconnu de l'interaction entre les voies canonique et non canonique est l'induction de

l'expression du gène NF- κ B2 (p100) par la voie canonique (Dejardin et al., 2002). Ce processus est principalement observé suite à une stimulation de la voie LT β R, normalement stimulatrice de la voie non canonique. Ainsi la liaison ligand-récepteur induit la rupture du complexe inhibiteur I κ B δ libérant l'hétérodimère RelA/p50 : les effecteurs de la voie canonique. Ceux-ci migrent alors dans le noyau et induisent la transcription du gène codant pour la sous-unité p100, qui en présence d'une stimulation continue par LT β R, se clive et génère la sous-unité active p52 induisant une activation de la voie non canonique.

Comme décrit précédemment, la voie NF- κ B est activée par une dimérisation de ces différents sous-unités. Plusieurs études ont montré que l'interaction entre les deux voies canoniques et non canoniques peut avoir lieu également au niveau de cette dimérisation mais en induisant un rétrocontrôle négatif. Par exemple, lors d'une stimulation des cellules par TNF- α , la sous-unité RelA fonctionne comme un répresseur de RelB (Jacque et al., 2005). Ainsi, la formation de l'hétérodimère RelA/RelB empêche la liaison de RelB à l'ADN de son gène cible et par suite le maintient sous sa forme inactive dans le noyau. Cette suppression a également été détectée dans des cellules dendritiques humaines stimulées par le ligand d'une lectine de type C, la dectine, bloquant par la suite la génération des lymphocytes T folliculaires helper (Gringhuis et al., 2009). L'interdépendance des deux voies peut-être également contrôlée par la kinase NIK (NF- κ B inducing Kinase). La fonction physiologique de NIK est principalement l'activation de la voie non canonique NF- κ B. Toutefois, sous certaines conditions, NIK peut également contribuer à l'activation de la forme canonique. C'est principalement observé quand NIK est accumulée de façon aberrante dans les cellules comme dans le cas de beaucoup de cancers tel que le multiple myélome (Demchenko et al., 2010).

D. Gènes cibles de la voie NF-ĸB

La voie NF-κB régule l'expression d'une grande variété de gènes cibles intervenant notamment dans la survie cellulaire, la prolifération et l'apoptose. Ces gènes présentent dans leur régions promotrices la même séquence consensus 5'-GGGRNWYYCC-3' (Où R = A ou G, W=A ou T, Y= C ou T et N=A, T, C ou G) permettant la fixation des dimères de NF-κB.

Parmi les gènes cibles de NF- κ B, nous citons principalement les gènes régulant la réponse immunitaire. En effet, NF- κ B intervient dans l'inflammation en régulant l'expression des gènes majeurs de la réponse inflammatoire comme le TNF α , les interleukines (IL-6, IL-8, IL-1), les chimiokines (MIP-1, MCP1 RANTES and eotaxin) et les molécules d'adhésion permettant la migration des lymphocytes (Ghosh and Karin, 2002; Oeckinghaus and Ghosh, 2009). Elle régule également les gènes impliqués dans l'activation et la maturation des lymphocytes T et B comme par exemple les molécules du complexe majeur d'histocompatibilité (CMH-I et CMH-II) ainsi que les gènes codant pour les chaînes d'immunoglobulines (IgG et IgE) (Pahl, 1999). À part son rôle dans la réponse immunitaire, NF-κB est connue pour contrôler l'expression des gènes régulateurs du cycle cellulaire comme les cyclines (cyclin D1 et D3), les gènes codant pour les protéines anti-apoptotiques comme Bcl-2 ainsi que les gènes impliqués dans l'angiogenèse comme le VEGF (Vascular Endothélial Growth Factor). Ces gènes sont régulés aussi bien par la voie canonique que par la voie non canonique.

Par ailleurs, la voie NF- κ B non-canonique régule également l'expression d'Ezh2 (Enhancer of zeste homolog 2). C'est une méthyltransférase qui cible de nombreux gènes suppresseurs de tumeurs et réprime leur expression en catalysant la triméthylation de H3K27. Il a été démontré récemment que les sous-unités RelB/p52 de la voie non-canonique se lient à la région promotrice d'Ezh2 et active sa transcription (De Donatis et al., 2016). Plusieurs études ont montré une corrélation entre le niveau d'expression d'Ezh2 et un mauvais pronostic chez les patients atteints de cancer de poumon (Sato et al., 2013b). D'une façon identique, Ezh2 intervient dans les mécanismes d'échappement des cellules tumorales aux réponses immunitaires en induisant une répression des gènes du complexe CMH (Robbins et al., 2012). Enfin, NF- κ B, par l'intermédiaire de ces gènes cibles, joue un rôle important au cours du développement de certains types cellulaires ainsi que dans les processus de migration et d'invasion des cellules tumorales.

E. Rôle de NF-kB dans le développement et la différenciation des LT

La voie de signalisation NF- κ B est reconnue comme étant un régulateur majeur des réponses immunitaires innées et adaptatives, mais son rôle dans le développement des cellules immunitaires et en particulier les cellules de la lignée lymphocytaire T est de plus en plus étudié. Comme détaillé précédemment, le développement des LT débute au niveau du thymus à partir d'un progéniteur lymphocytaire immature qui, par le biais d'une progression ordonnée de plusieurs étapes, aboutit à la génération des LT capables de reconnaître de manière spécifique un pathogène donné et de déclencher une attaque adaptée. Parmi les nombreux réseaux de signalisation intracellulaire impliqués dans la maturation des LT, la voie NF- κ B semble intervenir au cours des différentes étapes y compris, l'engagement des progéniteurs lymphoïdes envers la lignée T, la sélection et la maturation des thymocytes et plus tard l'acquisition des fonctions effectrices (Tableau 2) (Gerondakis and Siebenlist, 2010; Gerondakis et al., 2014).

1. NF-kB et développement thymocytaire

Les premières observations du rôle de la voie NF-KB dans le développement thymique se sont principalement focalisées sur sa fonction d'induction des molécules anti-apoptotique vu l'importance des mécanismes de la mort cellulaire au cours de la sélection des LT. Alors qu'un niveau basale d'activation de la voie NF- κ B a été observé au niveau des stades DN1 et DN2, une augmentation significative de cette voie représente une étape cruciale au cours du passage au stade pré-TCR (Sen et al., 1995). Il a été démontré que la signalisation pré-TCR associée aux réarrangements des gènes codant pour la chaîne β induit l'activation du facteur de transcription NF-κB qui protège les thymocytes DN3 de la mort cellulaire. Par ailleurs, une inhibition de la voie NF- κ B dans des modèles de culture des thymocytes in *vitro* ou dans les modèles murins génétiquement invalidés pour la voie NF-κB provoque l'apoptose des cellules en stade pré-TCR. De façon intéressante, ce processus de mort cellulaire est restauré par une activation constitutive de la voie NF-KB (Voll et al., 2000). D'autre part, il semble que ce mécanisme de protection par le biais de la voie NF-kB se fait par l'intermédiaire de l'expression du gène de la molécule anti-apoptotique Bcl-2 qui contrôle la maturation des thymocytes par une inhibition de l'activité de la caspase 3 (Mandal et al., 2005). Cependant, il a été démontré qu'une activation accrue de la voie NF-kB au cours de la maturation des thymocytes affecte également leur survie, suggérant ainsi la nécessité d'une régulation fine de cette voie de signalisation pour assurer un développement normal des LT (Goudeau et al., 2003).

D'autre part, la voie NF- κ B régule également les deux étapes de sélection thymique qui aboutissent à la génération des LT matures. Par contre, cette régulation semble être dépendante du type des LT vu que différents niveaux d'activation de la voie NF- κ B sont observés dans les thymocytes engagés soit dans la lignée cellulaire CD4⁺ soit dans la lignée CD8⁺. En effet, il a été démontré qu'une activation modérée de la voie NF- κ B est indispensable à la sélection des LT CD8⁺ spécifiques capables de reconnaitre les antigènes pathogènes associés aux molécules CMH de classe I. D'une façon remarquable, cette voie n'est pas activée dans les thymocytes CD4⁺ au cours de leur sélection positive, au contraire une activation constitutive de cette voie aboutit à une suppression presque complète des thymocytes SP CD4⁺ (Jimi et al., 2008). En outre, NF- κ B, par la régulation des gènes anti-apoptotiques, est nécessaire pour l'élimination des thymocytes DP après une double stimulation TCR-CD3 (Hettmann et al., 1999). L'ensemble de ces études révèle une différence surprenante dans l'activation de la voie NF- κ B dans les sous-populations de thymocytes et suggère que la voie NF- κ B établit un seuil de signalisation qui détermine l'acheminement de la maturation.

2. NF-kB et développement des LT CD4+ conventionnels

Un certain nombre d'études a mis en évidence un rôle majeur de la voie NF- κ B dans le contrôle de l'activation et la prolifération des LT CD4⁺. En premier lieu, l'activation des LT et leur expansion clonale suite à la reconnaissance d'un antigène spécifique induisent des cascades de signalisation dont la voie NF- κ B. En effet, il a été démontré que la stimulation du complexe CD3/TCR permet l'activation de NF- κ B, et une co-activation de CD28 par ses ligands potentialise cette activation. Cette cascade de signalisation est médiée par des protéines à activité kinase de la famille Src, LCK et FYN qui phosphorylent des protéines activatrices de la voie NF- κ B (CARMA1, BCL-10, MALT1) (Figure 12).



Figure 12: Mécanisme d'activation de la voie NF-kB par la signalisation TCR

A la suite de la stimulation du complexe CD3/TCR, un grand nombre de protéines kinases (Lck, FYN, Src) est recrutée au niveau de la synapse immune. Ces kinases phosphorylent la protéine CARM1 qui interagit avec Bcl-10 et MALT1 formant le complexe CBM. Ce complexe est à l'origine de l'activation de la voie NF- κ B

En plus, il a été montré que la délétion de la sous-unité IKK/NEMO, se traduisant par une perte de l'activation de la voie canonique, conduit à une perte de la capacité des cellules CD4⁺ à proliférer et survivre en périphérie (Chen et al., 2015; Schmidt-Supprian et al., 2003). D'autre part, il a été démontré un rôle de la voie alternative dans la réaction du greffon contre l'hôte (GVH). En effet, les LT déficientes pour la kinase NIK sont incapables d'induire un rejet d'un greffon incompatible à l'organisme du receveur. Par contre, une surexpression de cette kinase est à l'origine du développement des maladies auto-immunes caractérisées par des LT CD4⁺ effectrices hyperactives. D'où la nécessité d'une auto-régulation de la voie NF-κB pour veiller une activation normale du système immunitaire (Murray et al., 2011).

NF-κB joue un rôle important dans la régulation des fonctions effectrices des différentes populations de cellules CD4⁺ T helper. En effet, il a été démontré que l'invalidation spécifique de la voie NF-κB/Rel dans les CD4⁺ conduit à un défaut de production de l'IFN- γ par les Th1 (Aronica et al., 1999). Plus tard, le mécanisme expliquant ce dysfonctionnement a été clarifié, en montrant le recrutement de la sous-unité RelA par le facteur de transcription T-bet, et sa fixation sur la région promotrice du gène IFN- γ favorisant sa transcription (Balasubramani et al., 2010). De même, les LT CD4⁺ invalidés pour le gène RelB sont incapables de se différencier en cellules Th1, indiquant un rôle de RelB dans l'induction de l'expression du facteur de transcription T-bet et de la phosphorylation de Stat4 (Corn et al., 2005). De façon similaire, RelA active la transcription du gène de l'IL-4 en se fixant sur un site actif de son promoteur montrant ainsi un rôle de la voie NF- κ B dans la différenciation des Th2 (Li-Weber et al., 2004).

3. NF-kB et développement des Tregs thymiques

L'implication de NF- κ B dans la génération des Tregs a été étudiée par plusieurs équipes de recherche mettant en évidence un rôle essentiel des différentes sous-unités de NF- κ B dans le développement thymique et la fonction immunosuppressive des Tregs (Oh et al., 2017). Au niveau des précurseurs de Tregs, c-Rel se lie directement au niveau de la région codante CNS3 de *Foxp3*. Il a été proposé que cette fixation initierait la déméthylation du promoteur de *Foxp3*, permettant à d'autres facteurs de transcription de se fixer dessus et d'initier son expression. En effet, le développement des Tregs est altéré dans les modèles de souris dépourvus de c-Rel en comparaison à des souris normaux (Isomura et al., 2009; Ruan et al., 2009). Il a été également démontré que les délétions spécifiques de IKK α et IKK β dans les cellules T conduisent à une forte diminution du nombre des Tregs dans le thymus, la rate et les ganglions (Chen et al., 2015). Par ailleurs, une population de précurseurs pré-Tregs, CD4⁺CD122⁺CD25⁻Foxp3⁻, a été

mise en évidence. Ces cellules sont capables de se différencier en Tregs CD25⁺Foxp3⁺ suite à la fixation de c-Rel sur le locus du gène de CD25 (Schuster et al., 2017).

De point de vue fonctionnel, Chang et al ont montré que les cellules Tregs déficientes pour IKK β présentent un défaut de production d'IL-10, cytokine clé dans les fonctions suppressives de Tregs, et de SOCS1, facteur impliqué dans la stabilité des Tregs (Chang et al., 2012). D'autre part, *Messina et al* ont montré que l'absence de RelA dans les Tregs conduit au développement d'une maladie auto-immune. Par l'utilisation de différents modèles de souris, ils concluent que le développement de la pathologie est dû à un défaut de la capacité des Tregs à acquérir leur fonction effectrice (Messina et al., 2016). D'autre part, un rôle majeur de la voie NF- κ B alternative dans la génération des Tregs fonctionnels a été également mis en évidence. En effet, une activation forcée de cette voie par délétion de la protéine inhibitrice TRAF3 (TRAF3^{-/-}) qui induit une stabilisation de NIK, ou une surexpression directe de NIK induit une augmentation de la différenciation en Tregs (Murray et al., 2011; Muto et al., 2013). En plus, une délétion du gène codant pour la sous-unité p100 induit des maladies inflammatoires dues principalement à un dysfonctionnement des cellules Tregs (Grinberg-Bleyer et al., 2018a).

4. NF-KB et développement des Tfh

Comme décrit précédemment, le développement des Tfh se déroule en deux étapes successives, les LT doivent d'abord s'activer dans la zone T par une interaction avec les cellules dendritiques présentatrices de l'antigène formant ainsi les pré-Tfh. Ces précurseurs interagissent ensuite avec les LB en présence des signaux de co-stimulation y compris la signalisation ICOS pour aboutir à la formation des Tfh. Ainsi il a été observé chez les souris invalidées pour la voie noncanonique (NIK-'-), un développement défectueux des cellules Tfh. Ainsi, les auteurs de cette étude ont expliqué que ce déficit de génération des cellules Tfh n'était pas dû à un effet intrinsèque dépendant des cellules T mais plutôt à une absence de la population des cellules B du centre germinatif. En effet, une expression modérée de NIK est essentielle pour maintenir des niveaux d'expression élevés d'ICOSL à la surface des cellules B. En absence de NIK, les sous-unités RelB et p52 de la voie non-canonique ne s'activent plus, et par suite sont incapables de se lier sur le promoteur d'ICOSL pour activer sa transcription. L'absence de la signalisation ICOS-ICOSL se traduit alors par une population immature de cellules B incapable d'interagir avec les pré-Tfh pour induire leur maturation (Hu et al., 2011). D'autre part, il a été également démontré chez les souris déficientes en NF- κ B₁, un déficit de développement des cellules Tfh, dû à une diminution du niveau d'expression de CXCR5. Bien que l'expression des autres
marqueurs des Tfh ne soit pas affectée, l'absence de CXCR5 inhibe la différenciation des Tfh et par suite la formation des centres germinatifs (Serre et al., 2011). De façon similaire, la production d'IL-21 a été altérée chez les souris déficientes en c-Rel, induisant par la suite une altération du développement des Tfh et des cellules B du centre germinatif. En effet, c-Rel se lie au promoteur d'IL-21 et régule directement sa transcription (Chen et al., 2010).

L'ensemble de ces résultats met en évidence le rôle des membres de la famille NF- κ B dans la régulation des différentes étapes de développement et du fonctionnement des Tfh, non seulement en agissant sur les LT mais aussi par leur effet sur les cellules B du centre germinatif.

Population de LT	Stade de différenciation et de fonction	Observations	Références
Thymocytes	Réarrangement de la chaîne β	L'inhibition de la voie NF-kB dans les thymocytes provoque leur apoptose en stade pré- TCR	(Voll et al., 2000)
	Sélection positive des cellules CD8 ⁺	Une activation modérée de la voie NF-κB est nécessaire à la sélection des LT CD8 ⁺	(Jimi et al., 2008)
	Sélection positive des cellules CD4 ⁺	Une activation constitutive de NF-κB aboutit à une suppression complète des LT CD4 ⁺	(Jimi et al., 2008)
LT CD4 ⁺	Prolifération des cellules CD4 ⁺	Une délétion de la sous- unité IKK induit une perte de la capacité des CD4 ⁺ à proliférer et survivre en périphérie	(Schmidt-Supprian et al., 2003)
	Contrôle de la réaction immunitaire dans les cas de greffe	Les LT déficientes pour la kinase NIK sont incapables d'induire le rejet d'un greffon incompatible à l'organisme du receveur	(Murray et al., 2011)
	Régulation de la fonction des Th1	RelA se fixe sur la région promotrice de l'IFN-γ	(Balasubramani et al., 2010)

Tableau 2:Rôle de la voie NF-ĸB dans le développement des LT

		pour activer sa transcription	
		RelB induit l'expression de T-bet, facteur clé dans la différenciation des Th1	(Corn et al., 2005)
	Régulation de la génération des Th2	RelA se fixe sur la région promotrice d'IL-4 pour induire sa transcription	(Li-Weber et al., 2004)
	Expression de <i>Foxp3</i>	c-Rel se lie sur la région codante de Foxp3 pour activer sa transcription	(Isomura et al., 2009)
	Génération des Tregs	L'invalidation du gène codant pour IKK induit une forte diminution du nombre des Tregs	(Chen et al., 2015)
		Les Tregs déficientes en IKKβ présentent un défaud de production de l'IL-10	(Chang et al., 2012)
Tregs		L'invalidation de RelA aboutit à un défaut de la capacité des Tregs à acquérir leur fonction effectrice	(Messina et al., 2016)
	Fonction des Tregs	Une stabilisation de NIK ou une délétion de la protéine inhibitrice TRAF3 induit une augmentation de la différenciation en Tregs	(Murray et al., 2011)
		Une délétion de p100 induit un dysfonctionnement des Tregs	(Grinberg-Bleyer et al., 2018b)
Tfh	Interaction Tfh-GCB	Chez les souris déficientes en NIK, les cellules B du centre germinatif sont absentes bloquant ainsi la maturation des pré-Tfh	(Hu et al., 2011)
	Activation de l'expression de CXCR5 par les Tfh	L'invalidation de NF-ĸB1 inhibe l'expression de CXCR5 et induit un	(Serre et al., 2011)

	déficit de développement des Tfh	
Sécrétion d'IL-21	L'invalidation de c-Rel inhibe la production de l'IL-21 et altère à la fois le développement des Tfh et des LB du centre germinatif	(Chen et al., 2010)

F. Rôle de NF-kB dans le développement tumoral

De nombreuses études révèlent un rôle majeur de la voie NF- κ B dans le développement et la progression tumorale. Un tel rôle est attendu principalement vu l'ensemble de gènes dont l'expression est contrôlée par NF- κ B y compris les gènes impliqués dans la prolifération, l'échappement à la mort cellulaire ou également la résistance aux traitements. NF- κ B est retrouvée constitutivement activée dans un grand nombre de cancer, y compris le mélanome, le cancer de la tête, du sein ou les leucémies myéloïdes. Cette activation non contrôlée peut-être due aux molécules inflammatoires sécrétées dans le microenvironnement tumoral (chimiokines et cytokines) ou à des mutations induisant une activation aberrante d'une ou de plusieurs sous-unités de la voie NF- κ B.

Le développement et la progression tumorale des hémopathies malignes dépendent d'une dérégulation des voies de signalisation impliquées dans la différenciation et l'activation des cellules du système immunitaire y compris la voie NF-KB. Comme cité précédemment, il est devenu évident au cours de ces dernières années que l'activation déréglementée de cette voie est à l'origine d'une progression tumorale rapide.

1. Les leucémies myéloïdes

La leucémie aiguë myéloïde (LAM) est liée à la multiplication incontrôlée des blastes qui envahissent la moelle osseuse et perdent leur capacité à produire des cellules sanguines normales. Dans les LAM, l'activation de la voie NF- κ B semble avoir un rôle important. Elle a été observée pour la première fois en 2001 quand Guzman et ses collègues ont détecté une activation aberrante de la voie NF- κ B dans les cellules progénitrices des LAM (Guzman et al., 2001). Un an plus tard, une étude a montré que cette activation peut-être due à la dérégulation

de la signalisation IKK entrainant une dégradation des protéines inhibitrices (Baumgartner et al., 2002). Cette activation est associée à la survie des cellules et à leur résistance aux mécanismes de mort cellulaire. Un autre mécanisme d'activation de la voie NF-κB est la présence dans les LAM du facteur FLT3 (Fms-like tyrosine kinase 3) qui pourrait interagir avec IKK puis activer NF-κB (Takahashi et al., 2005). Ainsi, l'inhibition de FLT3 réduit la phosphorylation d'IKK et entraine l'apoptose des cellules cancéreuses (Grosjean-Raillard et al., 2008).

En ce qui concerne la leucémie myéloïde chronique (LMC), elle apparaît à la suite d'une modification de l'ADN d'une cellule souche hématopoïétique en croissance. En effet, les patients atteints de LMC présentent un chromosome anormal appelé « chromosome Philadelphie » qui résulte d'une translocation entre les chromosomes 22 et 9 et qui entraine le développement d'un oncogène appelé BCR-ABL. Dans la LMC, la protéine de fusion BCR-ABL entraine une activation constitutive de la voie NF- κ B suite à une interaction spontanée avec les sous-unités p65/RelA dans les blastes du sang périphérique (Carrà et al., 2016). D'autre part, dans la LMC, le microenvironnement joue un rôle majeur dans la régulation de la voie NF- κ B. Par exemple, le TNF α , la première cytokine capable de moduler la voie NF- κ B, est contrôlée par le gène du BCR-ABL directement. Les cellules leucémiques augmentent la production de TNF α induisant l'activation de NF- κ B et favorisant la survie des cellules tumorales progénitrices.

2. Les leucémies lymphoïdes

La leucémie lymphoïde chronique (LLC) se caractérise par l'accumulation progressive des lymphocytes B monoclonaux matures dans le sang périphérique, ou aussi dans les organes lymphoïdes secondaires tels que les ganglions lymphatiques. Dans la LLC, la voie NF- κ B est activée pratiquement dans tous les cas. Mais, contrairement à de nombreux lymphomes des cellules B matures, seuls certains gènes semblent être impliqués dans l'activation aberrante de la voie NF- κ B dans la LLC (Mansouri et al., 2016). Parmi les modifications génétiques dérégulant la voie NF- κ B dans la LLC, une mutation du gène I κ B ϵ induit une perte de sa fonction d'inhibition favorisant ainsi une augmentation de la phosphorylation de la sous-unité RelA et de sa translocation nucléaire (Mansouri et al., 2015).

De façon identique à ce qui a été observée dans d'autres lymphomes B, une mutation récurrente du gène MYD88 (L-255P) est identifié dans 2-4% des cas de LLC. Cette protéine mutée coimmunoprécipite avec IRAK1, une molécule effectrice importante pour l'activation de la voie classique de NF- κ B (Puente et al., 2011), induisant ainsi une expression continue de ses gènes cibles, à l'origine de la progression tumorale.

3. Les lymphomes B

Les lymphomes B se définissent comme étant des proliférations malignes des cellules B intervenant au cours des différents stades de leur maturation. Il a été souvent démontré que ces hémopathies sont caractérisées par une activation constitutive de la voie NF-kB qui semble être associée à un mauvais pronostic chez les patients (Davis et al., 2001; Pavan et al., 2008). Cette activation est principalement due à des modifications génétiques affectant les régulateurs de cette voie de signalisation. Par exemple, des mutations inactivatrices (délétion) ou des modifications épigénétiques (méthylation du promoteur) du gène TNFAIP3, qui code pour le régulateur négatif majeur de la voie NF-κB, sont observées dans 24.3% des cas de lymphome B diffus à grandes cellules (DLBCL) et peuvent être à l'origine de la dérégulation de la voie NF- κ B. En plus la ré-introduction de ce gène dans les lignées de DLBCL sensibilise les cellules cancéreuses à la mort cellulaire induite par les agents chimiothérapiques (Honma et al., 2009). Nous retrouvons également dans les DLBCL une contribution de la voie non-canonique de NFκB dans la progression tumorale. En effet, une délétion totale de l'extrémité 3' du gène NF-kB2 (p100) a été identifié dans les lignées de DLBCL. Cette délétion est à l'origine d'une augmentation du taux d'ARNm de NF-kB2 induisant une activation constitutive de cette voie de signalisation (Neri et al., 1991). Des mutations de TRAF3 ont été aussi retrouvés dans environ 15% des échantillons de DLBCL, expliquant l'origine de l'activité constitutive de la kinase NIK (Zhang et al., 2015).

Par ailleurs, l'activation de la voie NF-κB dans les lymphomes B est principalement contrôlée par trois molécules: CARD11, Bcl-10 et MALT1 qui forment le complexe CBM à l'origine de l'activation du complexe IKK. Des mutations au niveau de ces molécules induisent une signalisation chronique du BCR qui peut être à l'origine d'une activation oncogénique de la voie NF-κB. En effet, des translocations chromosomiques affectant les gènes de ce complexe sont fortement décrites dans les lymphomes B de la zone marginale et interviennent dans sa pathogenèse (Akagi et al., 1999; Du, 2016; Morgan et al., 1999; Streubel et al., 2003; Willis et al., 1999; Zhang et al., 1999)

Concernant les lymphomes de la zone marginale spléniques, la voie NF- κ B a été retrouvée dérégulée dans 30-40% des cas, ce qui suggère que cette voie intervient dans la pathogénicité de cette maladie. Comme détaillé précédemment, cette dérégulation est principalement due à

des mutations affectant les gènes codant pour les régulateurs positifs ou négatifs de cette voie (Spina and Rossi, 2016).

Des altérations génétiques sont également identifiés dans plus que 30% des lymphomes hodgkiniens, y compris les mutations de type perte de fonction qui affectent les gènes de contrôle négatif de la voie NF- κ B (TNFAIP3, NFKbia) (Liu et al., 2010; Nomoto et al., 2012) ou des mutations entrainant une amplification de certaines kinases activatrices de la voie NF- κ B (NIK, TRAF3) (Otto et al., 2012).

4. Les lymphomes T

Les lymphomes T représentent un sous-groupe d'hémopathies malignes rares appartenant à la catégorie des lymphoproliférations matures des lymphocytes T. Parmi ce groupe, les lymphomes T périphériques (PTCL) représentent 5 à 10% des lymphomes non-hodgkiniens et montrent à ce jour un pronostic défavorable avec une absence d'une identification claire des mécanismes impliqués dans la mise en place de cette pathologie. Malgré le rôle de la voie NFκB dans le développement des cellules T, l'implication de cette voie dans la pathogénicité des lymphomes T est encore très peu caractérisée. En 2005, une analyse transcriptomique a montré que 64% des PTCL présentent une augmentation de l'expression du gène codant pour la sousunité NF- κ B₁ leur permettant de distinguer deux groupes de patients : « NF- κ B⁺ et NF- κ B⁻ » D'une façon inattendue, et contrairement à d'autres types de cancer cette étude a montré que l'activation de la voie NF-κB est corrélée à une meilleure survie des patients (Martínez-Delgado et al., 2005). Une étude contradictoire réalisé par Odqvist et ses collègues en 2013 a mis en place un rôle de la voie NF- κ B, et plus précisément de la kinase activatrice NIK, dans la progression et la survie des cellules tumorales. En effet, l'invalidation de cette kinase sensibilise les cellules tumorales à l'apoptose et induit une réduction importante de l'expression des gènes du cycle cellulaire et les gènes anti-apoptotiques (Odqvist et al., 2013).

Enfin, une délétion monoallélique du gène codant pour TNFAIP3 (impliqué dans la régulation négative de la voie NF- κ B) a été mise en place dans 18% des cas de lymphome T mais l'association de ce résultat à une signification clinique chez les patients reste à clarifier (Ahn et al., 2018).

Un deuxième sous-groupe de lymphome T est le lymphome T cutané (CTCL) qui se développe à partir des lymphocytes T se multipliant dans les couches superficielles de la peau. Le CTCL est caractérisé par une activation constitutive de la voie NF-κB qui joue un rôle majeur dans la survie des cellules tumorales et la résistance à l'apoptose (Izban et al., 2000; Sors et al., 2008). D'autre part, l'implication de la voie non-canonique de NF- κ B a été clairement étudié, montrant une translocation chromosomique du gène *NF-\kappaB2* dans 10-15% des cas de CTCL. Cette modification génétique induit la génération d'une protéine tronquée constitutivement localisée dans le noyau favorisant l'expression de ces gènes cibles (Döbbeling, 2007). De même, une analyse immuno-histochimique sur des coupes de biopsies cutanées a confirmé la présence des sous-unités p50 et RelB dans les noyaux des cellules malignes.

Les éléments présentés ci-dessus indiquent qu'une activation constitutive du facteur de transcription NF- κ B semble favoriser le développement et la progression tumorale aussi bien dans les tumeurs solides que dans les hémopathies malignes. Cela conduit à suggérer que les agents inhibiteurs de cette voie de signalisation pourraient faire objet des traitements anticancéreux.

G. Inhibiteurs de la voie NF-ĸB

1. Les inhibiteurs du protéasome

L'étape cytoplasmique finale de l'activation de la voie NF- κ B implique la phosphorylation, l'ubiquitination et la dégradation de la protéine inhibitrice IkB. Comme décrit précédemment, un des mécanismes d'activation de la voie NF-kB observé au cours de la progression tumorale est une dégradation spontanée d'IkB par le protéasome, ce qui rend les inhibiteurs du protéasome attractifs en tant qu'agents thérapeutiques (Figure 13). Le protéasome est un complexe formé de multiples sous-unités catalytiques dont les protéases qui constituent les premières cibles thérapeutiques. Il existe huit familles d'inhibiteurs protéasomiques, qui peuvent être classés selon plusieurs critères, y compris leur structure, leur origine (naturel ou artificiel), leur site de fixation et notamment s'ils forment une liaison covalente ou non covalente avec leur site actif (Gandolfi et al., 2017). Les inhibiteurs protéasomiques, connus auparavant comme étant des anti-inflammatoires, représentent un domaine prometteur dans le traitement des cancers. Parmi ces inhibiteurs, le bortézomib (PS-341) est devenu le traitement de référence dans le myélome multiple (MM) utilisé chez les patients en rechute au traitement standard par la dexaméthasone. En effet, les données d'études in vitro (sur des lignées de MM), ou *in vivo* (sur des modèles précliniques de tumeurs) utilisant le traitement avec le bortezomib ont montré la capacité de cet inhibiteur à bloquer le cycle cellulaire, à induire l'apoptose et par suite à réduire la croissance tumorale. Les effets cytotoxiques du bortezomib ont été également observé dans les modèles de cancer colorectal, les cancers du pancréas, de la prostate ou également les cancers du sein. De façon intéressante, il a été montré dans un modèle murin d'adénocarcinome rénal, une implication du bortezomib dans l'activation des cellules CD8⁺ et l'induction de ses fonctions cytotoxiques (Pellom et al., 2017). Par contre, les essais cliniques montrent des effets secondaires majeurs causés par le bortezomib, y compris une faiblesse généralisée, une neuropathie périphérique, des nausées, des vomissements et une diminution du taux des plaquettes et des globules rouges (Chen et al., 2011).

2. Les anti-inflammatoires non stéroïdiens

Les anti-inflammtoires non stéroidiens (AINS) constituent un groupe hétérogène de substances chimiques, indiqués dans le traitement des cancers à origine inflammatoire. Ils agissent en bloquant la formation des prostaglandines, substances responsables de l'inflammation et présentées comme ayant un rôle oncogénique. Les AINS exercent leur effet anti-inflammatoire en inhibant la synthèse des prostaglandines via un blocage de l'activité enzymatique de la cyclooxygénase (COX). L'inhibition de la voie NF-κB par les AINS a un effet indirect lié à une diminution des signaux pro-inflammatoires. En plus de leurs activités anti-inflammatoires, les AINS exercent également un fort potentiel anti-tumoral. Par exemple les effets antitumoraux du sulindac et de l'aspirine ont été confirmés dans des modèles murins d'adénocarcinome du colon. Ces deux molécules présentent des modes d'action bien différents. Bien que le sulindac était capable de supprimer la tumorigénèse dans ce modèle (Boolbol et al., 1996), l'aspirine quant à elle diminue l'incidence, le nombre et la taille des nodules formés (Reddy et al., 1993). Les AINS exercent aussi leur potentiel anti-tumoral via des voies COX-2 indépendantes. En effet, il a été démontré que certains agents inflammatoires tels que l'aspirine ou le salicylate de sodium inhibent spécifiquement l'activité IKKβ aussi bien in vitro qu'in vivo. Le mécanisme de cette inhibition est dû à la liaison compétitive de ces molécules anti-inflammatoires sur les sites de fixation de l'ATP, empêchant ainsi l'activation de la voie NF-κB (Yin et al., 1998). En outre, l'ibuprofène semble inhiber l'activation constitutive de la voie NF-KB dans des modèles cellulaires de cancer de prostate, suite à une dégradation des kinases activatrices de la famille IKK (Palayoor et al., 1999).

3. Les glucocorticoïdes

Les glucocorticoïdes (GC) représentent une deuxième classe d'anti-inflammatoires largement prescrits dans le traitement des leucémies, des lymphomes et du myélome multiple. Les GC exercent un effet anti-inflammatoire majeur, en inhibant la sécrétion des cytokines proinflammatoires et en agissant directement sur la voie de signalisation NF-KB. Il a été montré que la dexaméthasone induit une augmentation de la transcription du gène codant pour la protéine inhibitrice IkB entraînant une augmentation de sa synthèse protéique. Dans les conditions de croissance tumorale, la stimulation par le facteur de nécrose tumorale (TNF α) provoque une dégradation de la protéine ΙκΒ et une activation accrue de la voie NF-κB. Cependant, en présence de dexaméthasone, le facteur de transcription libéré après stimulation se réassocie rapidement à la protéine IkB nouvellement synthétisée, permettant de la stabiliser et réduisant par la suite la translocation nucléaire de NF-κB (Scheinman et al., 1995). Par ailleurs, les GC permettent la re-sensibilisation des cellules tumorales aux traitements chimiothérapiques. Il a été démontré dans des modèles cellulaires du carcinome cervical que les GC stimulent les effets cytotoxiques de la cisplatine via une suppression de l'activation de la voie NF- κ B (Lu et al., 2006). En plus, la déxamethasone augmente l'activité anti-tumorale de l'adriamycine dans un modèle syngénique de cancer de sein (Wang et al., 2007). Cette étude a montré qu'un prétraitement avec la dexaméthasone renforce l'effet thérapeutique de l'adriamycine induisant une inhibition presque complète de la croissance tumorale. En plus le co-traitement inhibe l'activation de la voie NF- κ B, par une régulation positive d'I κ B ce qui entraîne une diminution de la sécrétion des cytokines IL-1 β et VEGF et une inhibition de la prolifération cellulaire accompagnée d'une sensibilisation des cellules tumorales à l'apoptose (Wang et al., 2007).

4. Les inhibiteurs de IKKβ

IKK, une kinase clé dans l'activation de la voie NF-κB, a été un centre d'intérêt pour la plupart des sociétés pharmaceutiques qui avaient mis au point un certain nombre de composés synthétiques inhibant son activité (Figure 13). La plupart des composés sont soit des inhibiteurs compétitifs bloquant le site de liaison à l'ATP, soit des inhibiteurs allostériques qui modifient la conformation d'IKK et la rende inactive. Parmi ces inhibiteurs, PS-1145 est un composé dont l'effet anti-prolifératif a été initialement démontré dans le myélome (Hideshima et al., 2002). Cependant, un effet pro-apoptotique de PS-1145 a été mis en place dans les cellules cancéreuses

de prostate. Cet inhibiteur favorise l'apoptose de ces cellules par activation des caspases 3 et 7 et diminution de l'expression des gènes du cycle cellulaire (cyclines D1 et D2) ainsi que les molécules pro-inflammatoires telles que l'IL-6 (Yemelyanov et al., 2006). Une étude similaire a montré un effet favorable de PS-1145 sur l'inhibition de la prolifération des lignées cellules de CML ainsi que les progéniteurs de la moelle osseuse. De manière intéressante, cet inhibiteur resensibilise les cellules CML résistantes au traitement par l'imatinib (Cilloni et al., 2006). Un second inhibiteur compétitif d'IKK « ML 120B » a montré une efficacité à inhiber les signaux pro-inflammatoires dans les cas d'arthrite rhumatoïde (Wen et al., 2006) et à resensibiliser les cellules cancéreuses aux traitements chimiothérapiques dans les cas de lymphomes folliculaires ou les lymphomes B diffus à grandes cellules (Al-Katib et al., 2010). Une nouvelle approche pour l'inhibition d'IKK consiste à cibler les interactions entre les différentes sous-unités du complexe IKK et à rompre les liaisons protéine-protéine. Un exemple de ces inhibiteurs est le peptide ciblant le domaine de liaison de la sous-unité NEMO (NBD : NEMO-Binding domain). Ce peptide a été validé pour sa capacité à inhiber la translocation nucléaire de p65 et par suite l'activation de la voie NF- κ B. Son application *in vitro* et *in vivo* sur des lignées cellulaires de cancer de sein ou du cancer épidermoïde confirme une efficacité dans l'inhibition de la prolifération et l'induction de la mort des cellules cancéreuses (Biswas et al., 2004; Furuta et al., 2012). En outre, les résultats de la phase I d'un essai clinique utilisant ce peptide sur des modèles de chiens atteints de lymphome B (DLBCL) ont montré qu'une administration systémique de ce peptide inhibe la voie NF-kB et réduit la croissance tumorale (Habineza Ndikuyeze et al., 2014).

5. Les inhibiteurs d'Ezh2

Bien qu'Ezh2 soit un gène cible de la voie NF-κB non-canonique, sa fonction de régulateur épigénétique a été largement analysé dans le cadre du développement tumoral. En effet, des anomalies d'expression d'un ou de plusieurs régulateurs épigénétiques sont de plus en plus courantes dans les cancers et sont à l'origine des processus d'hypo-méthylation entraînant une surexpression des gènes oncogènes ou d'hyper-méthylation induisant une perte d'expression des gènes suppresseurs de tumeurs. Ezh2 est une histone méthyltransférase impliquée dans les processus de régulation du cycle cellulaire ou de réparation de l'ADN (Genta et al., 2019; Stazi et al., 2017). Dans le système hématopoïétique, Ezh2 régule la prolifération cellulaire, la différenciation des LT et les recombinaisons des chaînes lourdes des immunoglobulines dans les cellules B. En plus, Ezh2 est fortement exprimée dans les cellules B du centre germinatif vu

son rôle dans les processus d'hypermutation somatique (Su et al., 2003). Des altérations d'expression d'Ezh2 ont été décrites dans de multiples cancers y compris le cancer du sein, le cancer de la prostate, les lymphomes non-hodgkiniens ou aussi les lymphomes des cellules T. Dans ces tumeurs, la surexpression d'Ezh2 est associée à l'agressivité tumorale et à la formation des métastases. En plus, un rôle majeur d'Ezh2 dans la progression tumorale des mélanomes cutanées a été démontré récemment en mettant en évidence une nouvelle fonction d'Ezh2 dans l'inhibition des gènes régulant la sénescence dans les cellules cancéreuses (De Donatis et al., 2016). Ainsi, au cours des dernières années, plusieurs inhibiteurs d'Ezh2 ont été mis au point et testés cliniquement (Figure 13). Le premier composé mis en place pour inhiber Ezh2 est le 3-déazaneplanocine (DZNep) qui agit indirectement par une inhibition compétitive du substrat de l'enzyme inhibant ainsi son activité. Par contre, des données ont montré que le DZNep a un vaste effet inhibiteur sur d'autres méthyltransférases et n'est donc pas très spécifique (Miranda et al., 2009). Ce qui a encouragé le développement de petites molécules plus puissante et plus sélectives, telles que les inhibiteurs catalytiques d'Ezh2 comme EPZ0005687, EI1 et GSK126. Bien que la plupart des composés soient encore en développement préclinique, le DS-3201b, le tazemetostat et le CPI-1205 sont récemment passés en phase I/II des essais cliniques.

En effet, l'efficacité du tazémetostat a été validée dans des modèles précliniques de tumeurs rhabdoïdes malignes. Ce sont des tumeurs pédiatriques rares et agressives qui prennent naissance dans l'encéphale et la moelle épinière et apparaissent surtout chez les jeunes adultes. Suite à ces observations, le tazémétostat a été évalué dans une première étude clinique de phase I chez des patients en rechute ou réfractaires atteints de tumeurs rhabdoïdes mais également chez des patients atteints de lymphomes B non hodgkiniens, et les premiers résultats ont montré une activité clinique efficace avec des résultats favorables (Italiano et al., 2018).

Par ailleurs, les DS-3201b est un inhibiteur puissant fortement spécifique d'Ezh2 introduit récemment dans la phase I d'un essai clinique chez des patients atteints de lymphome non hodgkinien réfractaire, notamment les lymphomes T. Les analyses préliminaires de cette étude ont montré des résultats satisfaisants portant sur la pharmacocinétique, la pharmacodynamique et l'activité anti-tumorale de cet inhibiteur (Maruyama et al., 2017). D'autres molécules inhibitrices d'Ezh2 sont également introduites dans les essais cliniques surtout chez les patients atteints de lymphomes B en rechute aux traitements de bases (Harb et al., 2018; Yap et al., 2016).

6. Les inhibiteurs de NIK

La dérégulation de la signalisation non-canonique de NF-κB peut avoir de graves conséquences sur le développement cellulaire aboutissant finalement à l'oncogenèse. La stabilisation de la kinase NIK est l'évènement en amont menant à l'activation de cette voie de signalisation. Plusieurs études récentes ont montré que des altérations de NIK sont fréquentes au cours du développement tumoral ce qui a mis en évidence NIK comme une possible cible thérapeutique (Figure 13) (Maubach et al., 2019).

Pour cela, deux inhibiteurs catalytiques de NIK (AM-0216, AM0561) ont été testés sur des modèles cellulaires de myélome multiple présentant une surexpression de NIK associée à une activation constitutive de la voie NF- κ B alternative. Ces deux inhibiteurs présentent un effet cytotoxique sur les cellules tumorales et les sensibilisent à la mort cellulaire par apoptose. Leur mécanisme d'action est basé sur une inhibition de la translocation nucléaire des sous-unités p52 et RelB. De façon intéressante, la même étude a montré qu'un traitement combiné ciblant les deux kinases NIK et IKK β (comme étant la kinase activatrice de la voie classique de NF- κ B) présente des effets anti-tumoraux prometteurs (Demchenko et al., 2014). D'autre part, un deuxième inhibiteur a été mis en place, le B022. C'est une molécule chimique puissante et sélective de NIK. Il a été montré que l'utilisation de B022 dans une lignée cellulaire d'hépatocytes inhibe l'activation de NIK et par suite bloque le clivage de p100 en p52 entrainant ainsi une suppression de l'expression des gènes impliqués dans l'inflammation. L'efficacité de cet inhibiteur a été également vérifié *in vivo* en confirmant une diminution des lésions hépatiques (Ren et al., 2016).

En plus, une étude appliquée sur le lupus érythémateux disséminé (LED), une maladie autoimmune chronique, a apporté un soutien supplémentaire à la découverte des inhibiteurs de NIK. En effet, les auteurs ont montré que l'inhibition de NIK par SMI1 dans un modèle murin de lupus, améliore la survie et la fonction rénale des animaux traités. SMI1 est un inhibiteur chimique qui se lie au site de liaison d'ATP et inhibe ainsi l'activité enzymatique de NIK. Dans le cadre du LED, cet inhibiteur bloque la signalisation de BAFF nécessaire à la maturation des LB du centre germinatif à l'origine du développement de la pathologie. SMI1 diminue également l'expression des gènes codant pour les protéines pro-inflammatoires et inhibe l'activation des cellules Tfh auto-réactives (Brightbill et al., 2018).

À ce jour, aucun inhibiteur de NIK n'est passé en essai clinique. En 2016, TRACON Pharmaceuticals a annoncé une collaboration avec Janssen Pharmaceutica pour l'obtention d'une licence pour un nouvel inhibiteur de NIK (TRC694) découvert par Janssen. TRC694 inhibe l'accumulation nucléaire de p52 et RelB et inhibe la transcription des gènes cibles dans des lignées de myélome multiple. En plus, son application *in vivo* inhibe la croissance tumorale et ne montre aucun signe de toxicité (Versele et al., 2017). TRC694 est actuellement en cours de développement préclinique.

Pour conclure, la voie NF- κ B est devenue depuis sa découverte un point central dans l'étude du cancer et des maladies inflammatoires. Des centaines d'inhibiteurs ont été décrits dans la littérature pour cibler l'activation non contrôlée de cette voie de signalisation. Pourtant, comme pour la plupart des traitements anti-cancéreux actuels, les inhibiteurs de la voie NF- κ B présentent certaines limites qui empêchent leur application en clinique. Tout d'abord, l'effet anti-tumoral de la plupart de ces inhibiteurs, principalement les anti-inflammatoires et les glucocorticoïdes, proviennent de leur capacité à sensibiliser les cellules tumorales aux traitements chimiothérapiques, montrant que ces inhibiteurs n'ont pas un effet anti-tumoral qui leur est propre. En plus, une résistance à ces inhibiteurs peut se produire par plusieurs mécanismes. Par exemple, dans le cas du bortezomib, des mutations dans les sous-unités protéasomales peuvent affecter la sensibilité de l'inhibiteur et inhiber sa liaison à son site actif. Enfin, des études supplémentaires sont nécessaires pour déterminer si ces inhibiteurs feront partie de l'ensemble des traitements anticancéreux appliqués en clinique.



Figure 13 : Représentation des différents inhibiteurs proposés pour cibler l'activation constitutive de la voie NF-kB

V. Lymphome T angio-immunoblastique

Les cancers hématologiques ou également appelés cancers du sang, sont les cancers développés à partir des cellules sanguines : globules blancs, globules rouges ou plaquettes. Ils sont dus à des altérations de ces cellules survenant à différents stades de leur maturation. On distingue trois grandes familles de cancers hématologiques :

- Les leucémies, caractérisées le plus souvent par la présence de cellules anormales circulant dans le sang.
- Les myélomes, qui se manifestent notamment par des lésions osseuses
- Les lymphomes qui touchent princiapelemnt les ganglions.

Dans cette étude, nous nous sommes intéressées particulièrement à la famille des lymphomes qui représente près de la moitié des cancers hématologiques. Les lymphomes, appelés également maladies du système lymphatique, se caractérisent par la prolifération excessive des lymphocytes B ou T au niveau des ganglions, du foie et de la rate. Il existe deux grands types de lymphomes : les lymphomes hodgkiniens et les lymphomes non hodgkiniens (LNH) qui se différecient princiapelement par la présence de la cellule de Reed-Sternberg. C'est une cellule dérivée d'un lymphocyte B et qui n'est présente que dans les lymphomes hodgkiniens.

Les lymphomes T représentent un sous-groupe d'hémopathies malignes rares appartenant à la famille des lymphomes non-hodgkiniens. Ils correspondent à la prolifération clonale des lymphocytes T matures post-thymiques (TCR $\alpha\beta$ et $\gamma\delta$). Il existe trois sous-groupes de lymphomes T périphériques : les lymphomes T de présentation leucémique, les lymphomes T de localisation extraganglionnaire et les lymphomes T de localisation ganglionnaire. Les trois sous-groupes sont classés en fonction de leur caractéristiques cliniques, histologiques et immunophénotypiques ainsi que leurs anomalies moléculaires et chromosomiques.

Dans cette introduction, je détaillerai les caractéristiques cliniques et moléculaire du lymphome T angioimmunoblastique. La pathologie actuellement reconnue sous le nom de « lymphome T angio-immunoblastique ou AITL » a été décrite pour la première fois dans les années 1970 comme étant un syndrome clinique caractérisé par une lymphadénopathie généralisée. L'histoire de l'AITL semble être plus longue et plus profonde de ce que la littérature suggère, vu les différentes nomenclatures qui lui ont été attribuées. Il a été initialement décrit en tant qu'une lésion prémaligne ou une hyperplasie lymphoïde atypique, reconnue sous les noms de « lymphadénopathie immunoblastique », ou de « lymphogranulose X ». Plus tard dans les années 1980, l'identification des mutations cytogénétiques et des réarrangements clonaux des récepteurs des cellules T a établi la nature néoplasique de la maladie, ainsi le terme de

« lymphome T angio-immunoblastique » a été introduit dans la classification de Kiel en 1988. Par la suite, l'AITL a été reconnu dans la classification de l'Organisation Mondiale de la Santé comme étant la forme la plus courante des lymphomes T périphériques (Dogan et al., 2003; Leval et al., 2010).

A. Epidémiologie et facteurs de risque

Le lymphome T angioimmunoblastique est une pathologie rare, agressive et faiblement accessible aux traitements. Il ne représente que 1 à 2% de tous les cas de lymphomes nonhodgkiniens, mais à peu près un cas sur cinq du total des lymphomes T périphériques (PTCL). L'AITL touche principalement les personnes âgées avec un âge moyen entre 59 et 65 ans sans aucune prédisposition liée au sexe (Alizadeh and Advani, 2008; Lunning and Vose, 2017). Sur le plan géographique, l'AITL est plus répandu en Europe où il représente 28% des cas de PTCL, qu'en Amérique du Nord (16%) ou en Asie (17%). Aucun facteur de risque n'a été spécifiquement associé à l'AITL. Cependant, des suggestions ont lié le développement de cette pathologie à l'administration de certains médicaments comme les antibiotiques. Par contre il est probable que ces médicaments soient des facteurs provoquant une réaction inflammatoire favorisée par une dérégulation immunitaire associée à l'AITL, plutôt qu'un facteur de risque. D'autre part, un certain nombre de maladies ou d'agents infectieux ont été associés à l'AITL, notamment les infections bactériennes comme la tuberculose ou les infections virales comme le cytomégalovirus, le virus de l'hépatite C, le virus de l'immunodéficience humaine ou le virus de l'Epstein-Barr (EBV). Le virus de l'EBV, en particulier, a été retrouvé chez plus de 90% des personnes atteintes d'AITL (Dogan et al., 2003). Cependant la relation entre l'infection par l'EBV et le développement de la pathologie n'est toujours pas claire et nous ignorons si les cellules EBV⁺ sont impliquées dans la lymphomagénèse de l'AITL ou ne sont que des conséquences de l'immunodéficience induite par le lymphome. Ainsi, il a été décrit que la présence de l'EBV est un phénomène secondaire, en relation avec un déficit de l'immunité cellulaire et un taux élevé de cytokines pro-inflammatoires, et non un fait causal (Huang et al., 2012).

B. Aspects cliniques de l'AITL

D'un point de vue clinique, l'AITL se manifeste par une lymphadénopathie généralisée, souvent associée aux symptômes imitant un processus infectieux (fièvre, sueurs nocturnes et

perte de poids). La plupart des patients présente une splénomégalie, un élargissement modéré des ganglions lymphatiques, une hépatomégalie et une infiltration tumorale de la moelle osseuse. D'autres signes cliniques comme des épanchements pleuraux, des ascites, des manifestations neurologiques ou des atteintes gastro-intestinales sont moins fréquents, avec une grande variation entre les patients.

Des symptômes d'éruption cutanée peuvent être observés chez 20 à 50% des patients atteints d'AITL, mais ces symptômes peuvent être cliniquement atypiques, ressemblant aux dermatoses inflammatoires et peuvent être confondus avec une éruption virale, ce qui rend le diagnostic peu spécifique dans les stades précoces de la pathologie. Les patients présentent le plus souvent des lésions dermatologiques de type « skin rash » (lésions maculo-papuleuses), qui peuvent s'aggraver progressivement formant des plaques érythémato-squameuses, des lésions papulo-vasculaires ou également des nodules tumoraux cutanés (Figure 14) (Botros et al., 2015).

D'autre part, des tests de laboratoire révèlent une variété d'anomalies hématologiques, biochimiques et immunologiques. Ainsi, une anémie, un taux élevé de lactate déshydrogénase (LDH) ainsi qu'une augmentation de la vitesse de sédimentation des globules rouges semblent être les altérations sanguines les plus courantes observées au diagnostic. Une hyperlymphocytose sanguine est rarement détectée ; cependant un examen attentif des frottis sanguins peut révéler une petite population de lymphocytes atypiques, dont la présence est généralement confirmée par un immunophénotypage (Baseggio et al., 2006)

Le diagnostic de l'AITL met en évidence également des résultats d'auto-immunité positifs reflétant la présence des auto-anticorps y compris le facteur rhumatoïde, les auto-anticorps nucléaires, et des anticorps anti-muscles lisses, ainsi que des cryoglobulines et des agglutinines froides. Une hypergammaglobulinémie est régulièrement observée chez les patients à des stades avancés de la maladie (Alizadeh and Advani, 2008; Dogan et al., 2003; Leval et al., 2010; Lunning and Vose, 2017).

Enfin, le syndrome clinique de l'AITL chevauche avec de nombreux processus inflammatoires et néoplasiques, d'où les analyses du sang périphérique sont généralement non spécifiques. Pour ces raisons, le diagnostic de l'AITL ne peut être confirmé que par biopsie et par examen histologique mettant en cause les cellules d'origine.

C.Pathogenèse de l'AITL

Plusieurs analyses immunohistochimiques et génétiques ont suggéré que les cellules à l'origine du développement de l'AITL présentent un profil d'expression superposable aux LT folliculaires helper Tfh. Comme détaillé précédemment, les Tfh correspondent à une sous population de lymphocytes T CD4⁺ effecteurs, présentant un phénotype particulier qui les distinguent des autres LT. Ces cellules sont caractérisées par l'expression des marqueurs de surface (PD-1, ICOS, CXCR5, Bcl-6) leur permettant d'accomplir leur fonction au sein des centres germinatifs (voir Partie I.C.1e). Vu le rôle des Tfh dans le recrutement et l'expansion des cellules B, il n'a pas été surprenant de détecter au sein de la tumeur d'autres composants cellulaires associés aux Tfh permettant de maintenir leur survie et formant un microenvironnement tumoral (TME) de l'AITL. Ainsi, des analyses histologiques sur une biopsie de ganglions lymphatiques ont mis en évidence que l'architecture ganglionnaire est caractérisée par la présence d'un infiltrat cellulaire riche polymorphe diffus au sein duquel sont identifiées les cellules tumorales, ainsi que d'autres types cellulaires tels que les LB, les cellules



folliculaires dendritiques, les plasmocytes, les macrophages, les cellules T non néoplasiques qui peuvent tous interagir avec les cellules Tfh. La relation entre les cellules tumorales et le TME est intrigante suggérant que la présence de l'ensemble des composants cellulaires est essentielle pour la mise en place et le maintien de la pathologie. En plus l'incapacité d'établir une lignée cellulaire du lymphome AITL *in vitro* suggère que les cellules tumorales dépendent de leur TME pour leur survie et leur prolifération. Une autre caractéristique importante de l'architecture ganglionnaire des tumeurs de l'AITL est la présence d'un réseau vasculaire constitué de veinules post-capillaires qui représentent les points de passage privilégiés des Tfh vers la circulation sanguine et assurent une communication cytokinique entre les différents composant de la tumeur (Figure 14).



Figure 15 : Pathogenèse de l'AITL

Représentation schématique de la pathogenèse de l'AITL, mettant en évidence les interactions cellulaires qui ont lieu entre les cellules néoplasiques Tfh et les cellules du microenvironnement.

D. Immuno-phénotypage des cellules tumorales de l'AITL

L'architecture et la composition cellulaire des ganglions lymphatiques de l'AITL ont permis de les classer en trois modèles qui peuvent prédire le stade de progression tumorale (Attygalle et al., 2002)

- Modèle I : le ganglion lymphatique préserve l'architecture d'un ganglion normal et contient des follicules hyperplasiques de cellules B bordurées de cellules tumorales Tfh.
- Modèle II : caractérisé par une absence progressive de l'architecture ganglionnaire avec la conservation occasionnelle de certains follicules.
- Modèle III : correspond à un stade tardif de la maladie. L'architecture normale du ganglion est complètement effacée et aucun follicule n'est identifié. Par contre, les cellules folliculaires dendritiques représentent dans ce modèle un composant majeur de l'infiltrat tumoral.

Les cellules tumorales sont des cellules de taille moyenne, avec des noyaux ronds ou légèrement irréguliers et un cytoplasme abondant et clair. Elles ont tendance à former de petites grappes autour des veinules post-capillaires. L'immunophénotypage a montré qu'il s'agit des LT matures CD4⁺CD8⁻TCR $\alpha\beta^+$ qui présentent fréquemment une perte aberrante ou une expression réduite des marqueurs CD3, CD5 et CD7. Les marqueurs des cellules Tfh (PD-1, CXCR5, ICOS) sont également détectés à la surface des cellules néoplasiques (Figure 15). En effet, ils représentent actuellement les marqueurs immunohistochimiques les plus utiles et les plus robustes pour le diagnostic de l'AITL. En outre, une expression cytoplasmique abondante de la chimiokine CXCL13, principalement sécrétée par les Tfh, est identifiée dans la plupart des cas de l'AITL. De manière intéressante, il a été démontré que les LT néoplasiques qui envahissent l'AITL peuvent être identifiés par une expression aberrante de CD10 (Attygalle et al., 2002). C'est une métallo-endo-peptidase exprimée à la surface des cellules B durant les stades précoces de leur différenciation et utilisée comme marqueur de diagnostic dans les leucémies. Elle a été identifiée dans 90% des cas d'AITL alors qu'elle est totalement absente dans les autres lymphomes T périphériques. Ultérieurement, un nouveau marqueur a été identifié dans 30% des cas d'AITL. Il s'agit du CD30, une protéine membranaire appartenant à la famille des récepteurs TNF. C'est une molécule de co-stimulation exprimée lors d'une activation lymphocytaire et contrôle la survie et la réponse cytotoxique des LT. CD30 est également un marqueur des cellules tumorales retrouvées dans les lymphomes de Hodgkin ou le lymphome anaplasique à grandes cellules (Sabattini et al., 2013).

Par ailleurs, les cellules dendritiques folliculaires, typiquement associées aux vaisseaux, peuvent être facilement identifiés par leur morphologie ou plus spécifiquement par un marquage immunohistochimique reconnaissant les marqueurs classiques CD21, CD23 ou CD35 (Troxell, 2005). L'atteinte de la moelle est souvent subtile, se manifestant sous forme de petits foyers nodulaires composés à la fois de cellules T et B prolifératives (Ghani and Krause, 1985).

Enfin, ces particularités morphologiques et cliniques sont à l'origine d'un schéma anormal de sécrétion des cytokines produites à la fois par les cellules Tfh néoplasiques ou les cellules du microenvironnement tumoral. Par exemple, la lymphotoxine bêta est sécrétée par les cellules B permettant une prolifération importante des cellules dendritiques folliculaires (Foss et al., 1995). L'IL-21 par contre est sécrétée par les Tfh et peut exercer soit un effet autocrine soit réguler positivement les cellules B du centre germinatif. Les cellules dendritiques à leur tour sécrètent le facteur de croissance endothéliale VEGF qui agit en synergie avec l'angiopoïtine 1 permettant la formation des vaisseaux sanguins, ce qui potentialise l'angiogenèse des AITL (Konstantinou et al., 2009). D'autres cytokines telles que l'IL-6, l'IL-1β ou l'IL-4 sont

produites par les cellules tumorales et agissent sur les plasmocytes, macrophages ou éosinophiles (Ohshima KHaraoka et al., 2000).

E. Caractéristiques moléculaires et génétiques de l'AITL

1. Clonalité

Des études moléculaires basées sur des techniques de PCR effectuées sur des biopsies d'AITL ont montré que la grande majorité entre eux (90%) présentent des réarrangements clonaux des chaînes gamma du TCR, avec une configuration monoclonale observée dans plus de 75% des cas (Kawano et al., 2005; Tan et al., 2006; Vrsalovic et al., 2004; Weiss et al., 1986; Willenbrock et al., 2001). Plus tard, il a été démontré que ces cellules Tfh néoplasiques présentent une clonalité du segment V de la chaîne β du TCR (Mao et al., 2015).

En outre, un réarrangement clonal des gènes codant pour les chaînes d'immunoglobulines Ig a également été retrouvé (25 à 30% des cas) dans les LB associés aux Tfh (Tan et al., 2006). Cette double clonalité a confirmé une participation importante des LB dans la pathogenèse de l'AITL et a été confirmé plus tard par des observations montrant que des patients atteints d'AITL présentent un risque majeur de développer des lymphomes B principalement les lymphomes B diffus à grandes cellules (Willenbrock et al., 2007).

2. Mutations génétiques observées dans les AITL

Récemment, de nombreuses découvertes ont permis de disséquer le répertoire des altérations génétiques qui peuvent être à l'origine de la pathogenèse des tumeurs AITL. Ces études ont notamment mis en évidence une mutation du gène codant pour la petite protéine GTPase de la famille Rho (*RhoA*) ainsi que des mutations dans des régulateurs épigénétiques *Tet2*, *DNMT3a*, *IDH2* et les gènes de la signalisation du TCR (Couronné et al., 2012; Palomero et al., 2014; Vallois et al., 2016).

a. Mutation Rhoa G17V

RhoA (ras homolog gene A) est une petite protéine de la famille GTPase, qui régule de nombreux processus biologiques y compris le remodelage du cytosquelette, la migration, la prolifération et la survie cellulaire. Comme toutes les autres protéines G, RhoA cycle entre deux états conformationnels interconvertibles, une forme inactive liée au GDP et une forme active liée au GTP. Il a été montré que RhoA joue un rôle essentiel au cours du développement thymique régulant les signaux de survie et de prolifération des thymocytes (Henning et al., 1997). De plus, une activité altérée de RhoA a été liée à un défaut d'activation et de prolifération des LT CD4⁺ matures ainsi qu'au développement de l'auto-immunité, l'une des caractéristiques cliniques de l'AITL (Pernis, 2009). Ainsi le rôle central de RhoA dans la pathogenèse de l'AITL a été établi par l'identification d'une mutation hétérogène très répandue (RHOA G17V), retrouvée dans environ 70% des cas d'AITL. Cette mutation entraîne la conversion de la glycine en position 17 en valine et affecte le site de liaison à la GTP. Des analyses biochimiques et cellulaires ont démontré que cette mutation bloque la liaison de RhoA à son substrat et perturbe donc son activation. *In vitro*, cette mutation est associée à une augmentation de la prolifération et de la capacité d'invasion des cellules tumorales (Sakata-Yanagimoto et al., 2014; Yoo et al., 2014).

Récemment une nouvelle mutation de RhoA (K18N) a été identifiée dans 3% des cas d'AITL. Bien que la mutation G17V a été caractérisée comme étant un dominant négatif, la nouvelle mutation identifiée affecte également le site de liaison à la GTP mais en augmentant son affinité de liaison au substrat et en stabilisant sa forme active ce qui induit une augmentation de la transcription des gènes cibles (Vallois et al., 2016).

b. Mutation de Tet2

La protéine Tet2 (ten-eleven translocation 2) est une dioxygénase dépendante du fer, impliquée dans un processus actif de déméthylation d'ADN par oxydation des résidus méthylcytosine (5mC) en résidus de 5-hydroxymethylcytosine (5hmC). Tet2 a été d'abord identifié comme étant un suppresseur de tumeurs dans les cas de leucémies myéloïdes aigues (Abdel-Wahab et al., 2009). Des analyses ultérieures ont montré que les mutations acquises inactivant *Tet2* surviennent très tôt au cours des processus de transformation hématopoïétique. En effet, dans un modèle murin, l'inactivation de *Tet2* confère aux cellules souches un avantage de croissance et de prolifération et est à l'origine des anomalies de la différenciation des lignées myéloïdes et lymphoïdes (Rasmussen et al., 2015). Dans les AITL, des mutations induisant une perte de fonction du gène *Tet2* sont observées dans 70 à 80% des cas (Lemonnier et al., 2012; Wang et al., 2015). Ces mutations sont réparties sur la totalité du gène et peuvent être de type délétion, insertion ou mutation ponctuelle. Le rôle de cette mutation dans les AITL a été étudié dans des modèles murins présentant une invalidation du gène. Les souris *Tet2^{-/-}* développent un lymphome T ayant les caractéristiques des cellules Tfh. D'une manière intéressante cette étude

a mis en évidence un mécanisme de développement du lymphome dans leur modèle de souris en montrant que l'invalidation de *Tet2* entraîne une hyperméthylation du gène *Bcl-6* qui pourrait être à l'origine du développement aberrant des Tfh (Muto et al., 2014).

c. Mutation d'IDH2

Les protéines IDH1 (isocitrate déshydrogénase) et IDH2 sont situées respectivement dans le cytoplasme et dans les mitochondries. Elles codent pour les enzymes mitochondriales qui convertissent l'isocitrate en α -kétoglutarate. Dans les AITL, les mutations affectant ce gène apparaissent dans 30 à 40% des cas. Ces mutations touchent particulièrement l'isoforme IDH2 en position R172. Elles correspondent à un remplacement de l'arginine par une lysine (R172K), une sérine (R172S), une thréonine (R172T) ou une glycine (R172G) (Cairns et al., 2012). L'enzyme mutée a la capacité de transformer l' α -kétoglutarate en (R)-2-hydroxyglutarate, qui interfère avec la fonction des enzymes dépendante de l' α -kétoglutarate, comme c'est le cas de Tet2. Plus spécifiquement, les mutations d'IDH2 perturbent la fonction de Tet2 et affectent indirectement la production de 5-hydroxyméthylcytosine et conduisent à une augmentation de la méthylation des cytosines de certaines régions du génome impliquant une répression des gènes cibles (Wang et al., 2015).

d. Mutation de DNMT3a

Les DNMT3a sont des enzymes qui catalysent le transfert d'un groupement méthyl sur des résidus cytosines localisés au niveau des dinucléotides CpG induisant une répression de l'expression des gènes cibles. Dans les AITL, des mutations inactivatrices du gène *DNMT3a* ont été identifiées dans 10 à 25% des cas (Couronné et al., 2012). Ce sont des mutations hétérozygotes de type faux-sens ou frameshift et touchent principalement les régions codantes pour le domaine méthyltransférase. Dans 80% des cas, les doubles mutations Tet2/DNMT3a coopèrent dans le processus de transformation maligne (Scourzic et al., 2016).

e. Mutations dans les gènes du TCR

D'autres anomalies moléculaires ont été rapportées dans les AITL telles que les mutations affectant les gènes liés à la signalisation et l'activation du TCR. L'ensemble de ces mutations semble être impliqué dans l'induction d'une activation aberrante des LT à l'origine de leur transformation maligne (Vallois et al., 2016).

C'est ainsi que plusieurs mutations de CD28 ont été décrites dans 10% des cas. CD28 est en effet un récepteur de co-stimulation exprimé à la surface des LT, et qui après interaction avec ses ligands fournit aux LT un signal nécessaire à leur activation. Les mutations identifiées affectent particulièrement les deux résidus D124 et T195, et induisent une activation prolongée des LT. En effet, il a été démontré que le récepteur CD28 muté présente une affinité accrue à son ligand induisant une interaction irréversible et activant une cascade de voies de signalisation impliquée dans la prolifération des LT et la production des cytokines (Lee et al., 2015; Rohr et al., 2016). En outre, un gène de fusion CD28-CTLA-4 a été récemment identifié dans 50% des cas d'AITL. Ce gène code pour le domaine cytoplasmique de CD28 associé au domaine extracellulaire de CTLA-4, et est capable de transformer les signaux inhibiteurs déclenchés par la stimulation de CTLA-4 en signaux d'activation des LT (Yoo et al., 2016).

D'autre part, Fyn est une tyrosine kinase appartenant à la famille des SRC et jouant également un rôle prédominant dans l'activation des LT. Plusieurs mutations de FYN ont été identifiées dans les AITL. Ces mutations perturbent l'interaction inhibitrice du domaine SH2 de FYN, induisant une activation constitutive de la tyrosine kinase (Palomero et al., 2014).

De la même manière, plusieurs altérations génétiques affectant *PLCG1, CARD11, CTNNB1* ainsi que les protéines de la voie PI3K ou MAPK ont été décrites dans les AITL. Ces mutations sont impliquées dans la stabilisation des signaux stimulateurs des LT ce qui favorise leur prolifération et leur survie. De manière intéressante, une relation entre les mutations de *CARD11* et la signalisation NF- κ B a été mise en évidence comme décrit dans la figure 12. Ces mutations semblent être à l'origine d'une activation constitutive de NF- κ B qui pourrait favoriser la croissance tumorale (Vallois et al., 2016).

3. Anomalies cytogénétiques

Différentes anomalies cytogénétiques ont été décrites chez 90% des patients atteints d'AITL. Les plus fréquentes sont les trisomies 3, 5, 21, la présence d'un chromosome X supplémentaire et la perte du bras long du chromosome 6 (Leval et al., 2010). Des analyses d'hybridation génomique comparative effectuées sur une série de 39 patients ont mis en évidence que les gains de fragments chromosomiques sont plus récurrentes dans les AITL que les pertes. Ces études ont identifié de nouvelles altérations cytogénétiques y compris la trisomie 19, le gain d'un bras long supplémentaire du chromosome 22 et une amplification chromosomique du locus 11q13 (Thorns et al., 2007). Cette dernière altération semble avoir un intérêt particulier

parce qu'elle induit une surexpression des deux gènes (*CCND1* et *GSTP1*) impliqués dans la lymphomagenèse B et la résistance aux traitements (Bennaceur-Griscelli et al., 2004).

F. Options thérapeutiques

Malgré les caractéristiques cliniques et pathologiques uniques de l'AITL, les approches thérapeutiques sont actuellement similaires à celles appliquées pour l'ensemble des lymphomes T périphériques (Tableau 3). Par contre, en raison de la rareté de ce lymphome, les meilleures données disponibles pour guider le traitement sont basées sur des études prospectives. Le traitement de première ligne de l'AITL repose sur l'utilisation des agents chimiothérapiques de type CHOP (cyclophosphamide, doxorubicine, vincristine, prednisone). Bien que cette approche ne soit pas curative, elle offre la possibilité d'un meilleur contrôle de la maladie avec un maintien de la qualité de vie. Cependant, malgré des taux de réponse assez élevés, les résultats à long terme sont généralement faibles, principalement dus à un niveau de rechute élevé.

Plusieurs tentatives visant à améliorer ce traitement ont notamment consisté à combiner les agents chimiothérapiques avec d'autres molécules anticancéreuses. D'après les données obtenues suite à une étude réalisée sur une cohorte de patients atteints de lymphomes T périphériques, la chimiothérapie combinée avec l'étoposide (CHOEP) est devenue l'une des options thérapeutiques les plus couramment appliquées chez les patients jeunes (< 60 ans) ayant un taux de LDH relativement normal. Cette étude a montré que l'association de l'étoposide à CHOP pourrait conduire à des rémissions significatives avec une amélioration de la survie globale de 50% à 67.5 % pour les patients atteints d'AITL (Schmitz et al., 2010). D'autres agents qui ont été évalués en association avec le CHOP dans le traitement des AITL comprennent l'Alemtuzumab, la Romidepsine, le Bortezomib et le bélinostat (Gallamini et al., 2007; Johnston et al., 2015; Kim et al., 2012). Malgré les résultats prometteurs obtenus dans des études précliniques, une toxicité accrue a été observé chez les patients, soulignant ainsi la nécessité de mettre en place des études supplémentaires pour réduire la toxicité de ces agents thérapeutiques.

D'autre part, plusieurs molécules ont été testées pour leurs capacités à cibler un composant cellulaire particulier au sein de la tumeur dans le but de perturber le microenvironnement de l'AITL. Par exemple l'augmentation de la vascularisation tumorale a attiré plusieurs unités pharmaceutiques qui ont mis en place un anticorps monoclonal contre le facteur de croissance endothéliale vasculaire VEGF « le Bévacizumab » (Ganjoo et al., 2014). Cette approche

thérapeutique n'a malheureusement pas montré une amélioration des résultats obtenus par la chimiothérapie seule et a été associée à une toxicité importante. De manière similaire, le Rituximab (anticorps monoclonal anti-CD20) a été combiné aux traitements chimiothérapiques pour sa capacité à cibler les LB du centre germinatif, et comme pour les médicaments anti-angiogéniques, ce traitement n'a pas montré de réels bénéfices pour les patients (Delfau-Larue et al., 2012). Par ailleurs, un nouveau médicament a été testé sur des patients AITL en rechute au traitement de première ligne. Il s'agit de l'anticorps anti-CD30 (Brentuximab-védetonie) qui cible principalement les cellules Tfh à l'origine de l'AITL. Les premiers résultats satisfaisants ont mené à la mise en place de la phase III d'un essai clinique qui pourrait aboutir prochainement à déterminer une nouvelle norme pour le traitement de l'AITL (Horwitz et al., 2014).

De manière intéressante, les inhibiteurs des modifications épigénétiques émergent récemment comme ayant une efficacité importante dans des études cliniques. Ainsi le Romidepsine, un inhibiteur des histones désacétylases, a montré des réponses complètes et de longue durée chez des patients AITL en rechute (Pro et al., 2017). Les mêmes résultats étaient observés suite à l'application des agents hypométhylants comme le 5'-Azacytidine (Delarue et al., 2016) qui compensent la perte de fonction de *Tet2*.

Une autre option thérapeutique est la transplantation des cellules souches hématopoïétiques chez des patients jeunes en cas de rechute au traitement. En effet, une analyse réalisée par la société française de Greffe de Moelle et de Thérapie Cellulaire a révélé un taux de rémission à 80% post-greffe avec une survie globale à 5 ans chez les patients AITL (Le Gouill et al., 2008). Enfin, une grande hétérogénéité clinique et moléculaire a été observée chez les patients AITL nécessitant ainsi des options thérapeutiques individuelles. À l'heure actuelle, des études supplémentaires sont en cours pour permettre l'intégration de nouveaux agents dans le traitement des patients atteints d'AITL.

Agents anti-tumoraux	Mode d'action
Cyclophosphamide	C'est un agent alkylant qui, par liaison à l'ADN, inhibe sa transcription et sa réplication, aboutissant à la destruction cellulaire.

Tableau 3: Agents anti-tumoraux utilisés dans le traitement de l'AITL et leur mode d'action

Is 'intercale entre les paires de bases adjacentes boxorubicine de l'ADN dans la double hélice ce qui empêche son déroulement et sa réplication. Vincristine Il entraîne un arrêt en métaphase de la division cellulaire. Prednisone II a un effet anti-inflammatoire. Etoposide Il inhibe l'entrée en mitose des cellules tumorales. Alemtuzumab C'est un anticorps monoclonal ciblant CD52, un antigène exprimé à la surface des LB et LT et qui provoque leur lyse par ADCC. Bortezomib C'est un inhibiteur du protéasome. Il entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose. Belinostat et Romidepsine Deux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales. Rituximab C'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC. C'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des Th dans les acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales. Brentuximab védotine C'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Th dans les active exprimé à la surface des Th dans les active exprimé à la surface des th dans les atriène exprimé à la surface des th dans les active exprimé à la surface des th dans les active exprimé à la surface des th dans les active exprimé à la surface des th dans les actie un agent hypométhylant. Il régule les gènes			
Doxorubicinede l'ADN dans la double hélice ce qui empêche son déroulement et sa réplication.VincristineII entraîne un arrêt en métaphase de la division cellulaire.PrednisoneII a un effet anti-inflammatoire.EtoposideII inhibe l'entrée en mitose des cellules tumorales.AlemtuzumabC'est un anticorps monoclonal ciblant CD52, un antigène exprimé à la surface des LB et LT et qui provoque leur lyse par ADCC.BortezomibC'est un inhibiteur du protéasome. II entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Berntuximab védotineC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des TB et qui provoque leur lyse par ADCC.S-AzacytidineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Th dans les AITL et qui provoque leur apoptose.S-AzacytidineC'est un agent hypométhylant. II régule les gènes du cycle cellulaire et des voies d'apoptose et peut entraîner une réexpression des gènes suppresseurs de tumeurs.		Il s'intercale entre les paires de bases adjacentes	
son déroulement et sa réplication.VincristineIl entraîne un arrêt en métaphase de la division cellulaire.PrednisoneIl a un effet anti-inflammatoire.EtoposideIl inhibe l'entrée en mitose des cellules tumorales.AlemtuzumabC'est un anticorps monoclonal ciblant CD52, un antigène exprimé à la surface des LB et LT et qui provoque leur lyse par ADCC.BortezomibC'est un inhibiteur du protéasome. Il entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un 	Doxorubicine	de l'ADN dans la double hélice ce qui empêche	
VincristineIl entraîne un arrêt en métaphase de la division cellulaire.PrednisoneII a un effet anti-inflammatoire.EtoposideIl inhibe l'entrée en mitose des cellules tumorales.EtoposideC'est un anticorps monoclonal ciblant CD52, un antigène exprimé à la surface des LB et LT et qui provoque leur lyse par ADCC.BortezomibC'est un inhibiteur du protéasome. Il entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.S-AzacytidineC'est un agent hypométhylant. II régule les gènes du cycle cellulaire et des voies d'apoptose et peut entraîner une réexpression des gènes suppresseurs de tumeurs.		son déroulement et sa réplication.	
Vittersturecellulaire.PrednisoneII a un effet anti-inflammatoire.EtoposideII inhibe l'entrée en mitose des cellules tumorales.AlemtuzumabC'est un anticorps monoclonal ciblant CD52, un antigène expriné à la surface des LB et LT et qui provoque leur lyse par ADCC.BortezomibC'est un inhibiteur du protéasome. II entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène expriné à la surface des LB et qui provoque leur lyse par ADCC.Berntuximab védotineC'est un anticorps monoclonal ciblant CD20, un antigène expriné à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène expriné à la surface des Th dans les AITL et qui provoque leur apoptose.S-AzacytidineC'est un agent hypométhylant. II régule les gènes du cycle cellulaire et des voies d'apoptose et peut entraîner une réexpression des gènes suppresseurs de tumeurs.	Vier entration of	Il entraîne un arrêt en métaphase de la division	
Prednisone II a un effet anti-inflammatoire. Etoposide II inhibe l'entrée en mitose des cellules tumorales. Alemtuzumab C'est un anticorps monoclonal ciblant CD52, un antigène exprimé à la surface des LB et LT et qui provoque leur lyse par ADCC. Bortezomib C'est un inhibiteur du protéasome. II entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose. Belinostat et Romidepsine Deux inhibiteurs des histones désacétylases, qui entraînen un actétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales. Rituximab C'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC. Bentezomib C'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC. Brentuximab C'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Th dans les AITL et qui provoque leur apoptose. S-Azacytidine C'est un agent hypométhylant. II régule les gènes du cycle cellulaire et des voies d'apoptose et peut entraîner une réexpression des gènes suppresseurs de tumeurs.	vincristine	cellulaire.	
EtoposideIl inhibe l'entrée en mitose des cellules tumorales.AlemtuzumabC'est un anticorps monoclonal ciblant CD52, un antigène exprimé à la surface des LB et LT et qui provoque leur lyse par ADCC.BortezomibC'est un inhibiteur du protéasome. Il entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Th dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et put entraîner une réexpression des gènes suppresseurs de tumeurs.	Prednisone	Il a un effet anti-inflammatoire.	
Luposucetumorales.AlentuzumabC'est un anticorps monoclonal ciblant CD52, un antigène exprimé à la surface des LB et LT et qui provoque leur lyse par ADCC.BortezomibC'est un inhibiteur du protéasome. II entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.S-AzacytidineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des CB et qui provoque leur apoptose.5-AzacytidineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les gènes du cycle cellulaire et des voies d'apoptose et peut entraîner une réexpression des gènes suppresseurs de tumeurs.	Etoposido	Il inhibe l'entrée en mitose des cellules	
AlemtuzumabC'est un anticorps monoclonal ciblant CD52, un antigène exprimé à la surface des LB et LT et qui provoque leur lyse par ADCC.BortezomibC'est un inhibiteur du protéasome. Il entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. II régule les gènes du cycle cellulaire et des voies d'apoptose et peut entraîner une réexpression des gènes suppresseurs de tumeurs.	Etoposide	tumorales.	
Alemtuzumabantigène exprimé à la surface des LB et LT et qui provoque leur lyse par ADCC.BortezomibC'est un inhibiteur du protéasome. Il entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entraîner une réexpression des gènes suppresseurs de tumeurs.		C'est un anticorps monoclonal ciblant CD52, un	
qui provoque leur lyse par ADCC.BortezomibC'est un inhibiteur du protéasome. Il entraîne un arêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entraîner une réexpression des gènes suppresseurs de tumeurs.	Alemtuzumab	antigène exprimé à la surface des LB et LT et	
BortezomibC'est un inhibiteur du protéasome. Il entraîne un arrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entraîner une réexpression des gènes suppresseurs de tumeurs.		qui provoque leur lyse par ADCC.	
Bortezomibarrêt du cycle cellulaire et une sensibilisation des cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes		C'est un inhibiteur du protéasome. Il entraîne un	
Image: cellules à l'apoptose.Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes	Bortezomib	arrêt du cycle cellulaire et une sensibilisation des	
Belinostat et RomidepsineDeux inhibiteurs des histones désacétylases, qui entraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes		cellules à l'apoptose.	
Belinostat et Romidepsineentraînent une accumulation des histones acétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes		Deux inhibiteurs des histones désacétylases, qui	
Definition and expressionacétylées et induit l'arrêt du cycle cellulaire et l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20 , un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30 , un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes suppresseurs de tumeurs.	Belinostat et Romidensine	entraînent une accumulation des histones	
l'apoptose des cellules tumorales.RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes suppresseurs de tumeurs.	Demostat et Romuepsine	acétylées et induit l'arrêt du cycle cellulaire et	
RituximabC'est un anticorps monoclonal ciblant CD20, un antigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30, un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes suppresseurs de tumeurs.		l'apoptose des cellules tumorales.	
Rituximabantigène exprimé à la surface des LB et qui provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30 , un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes suppresseurs de tumeurs.		C'est un anticorps monoclonal ciblant CD20, un	
Provoque leur lyse par ADCC.Brentuximab védotineC'est un anticorps monoclonal ciblant CD30 , un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes suppresseurs de tumeurs.	Rituximab	antigène exprimé à la surface des LB et qui	
Brentuximab védotineC'est un anticorps monoclonal ciblant CD30 , un antigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes suppresseurs de tumeurs.		provoque leur lyse par ADCC.	
Brentuximab védotineantigène exprimé à la surface des Tfh dans les AITL et qui provoque leur apoptose.5-AzacytidineC'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes suppresseurs de tumeurs.		C'est un anticorps monoclonal ciblant CD30, un	
AITL et qui provoque leur apoptose.C'est un agent hypométhylant. Il régule lesgènes du cycle cellulaire et des voies d'apoptoseet peut entrainer une réexpression des gènessuppresseurs de tumeurs.	Brentuximab védotine	antigène exprimé à la surface des Tfh dans les	
5-Azacytidine C'est un agent hypométhylant. Il régule les gènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes suppresseurs de tumeurs.		AITL et qui provoque leur apoptose.	
5-Azacytidinegènes du cycle cellulaire et des voies d'apoptose et peut entrainer une réexpression des gènes suppresseurs de tumeurs.		C'est un agent hypométhylant. Il régule les	
et peut entrainer une réexpression des gènes suppresseurs de tumeurs.	5-Azacytidine	gènes du cycle cellulaire et des voies d'apoptose	
suppresseurs de tumeurs.	5-Azacytume	et peut entrainer une réexpression des gènes	
		suppresseurs de tumeurs.	

G. Modèles murins de l'AITL

Comme décrit précédemment, la pathogenèse de l'AITL provient principalement d'un réseau complexe d'interactions entre les cellules tumorales et les différents composants cellulaires du

microenvironnement. En raison de cette complexité, il n'a pas été possible d'établir une lignée cellulaire qui permettra d'analyser cette pathologie *in vitro*. D'où la nécessité de générer des modèles murins qui récapitulent les caractéristiques de la maladie humaine et permettent d'identifier de nouveaux mécanismes impliqués dans sa pathogenèse dans le but de proposer de nouvelles options thérapeutiques. Jusqu'à présent, cinq modèles murins ont été décrites pour mimer la pathologie, dont quatre reposent sur des altérations génétiques et le cinquième constitue une xénotransplantation des cellules de patients (PDX).

1. Modèle Roquin san/+

Roquin est une protéine codée par le gène Rc3h1 et qui par le biais du motif à doigts de Zinc inclu dans sa séquence, régule l'expression de ses gènes cibles. Il a été démontré que Roquin déstabilise l'ARNm du co-stimulateur inductible ICOS suite à une liaison de son domaine ROQ à la région 3'UTR de l'ARNm entraînant sa dégradation. Ce processus régule ainsi l'expansion incontrôlée des cellules T et réduit par la suite l'apparition des maladies auto-immunes (Glasmacher et al., 2010). La perte de cette régulation post-transcriptionnelle a été observée pour la première fois dans le modèle de souris Roquin^{San/San} qui présente une mutation ponctuelle homozygote du gène Rc3h1. Cette mutation augmente l'expression d'ICOS et induit une accumulation des LT à l'origine du développement d'une maladie auto-immune équivalente du lupus érythémateux disséminé (Yu et al., 2007). Plus tard, Ellyard et ses collègues ont montré que la mutation hétérozygote du gène Rc3hl induit un phénotype similaire caractérisé par une dérégulation du développement des Tfh mais avec un phénotype d'autoimmunité moins prononcé. Les souris Roquin^{San/+} présentaient à l'âge de 6 mois une splénomégalie accompagnée d'une adénopathie disséminée. Un examen histologique des ganglions a montré une perte massive de l'architecture ganglionnaire associée à une infiltration importante des cellules T et B, mais sans aucune expansion d'un réseau dendritique folliculaire. Une expansion des cellules Tfh a été confirmée par la présence des cellules PD-1^{high} CXCR5⁺ Bcl-6⁺ accompagnée d'un réarrangement clonal de la chaîne β du TCR. De petits amas de plasmocytes ont été également observés reflétant une interaction entre les Tfh et les LB du centre germinatif. Cette caractéristique a été confirmée par une augmentation de la sécrétion des immunoglobulines dans le sérum des souris malades. Ainsi le modèle Roquin^{San/+} a été décrit comme étant le premier modèle murin récapitulant l'ensemble des caractéristiques immunologiques et histologiques de l'AITL (Ellyard et al., 2012). Néanmoins, une analyse transcriptomique effectuée sur des biopsies de patients n'a pas montré des différences

d'expression de *ROQUIN* entre les cellules tumorales de l'AITL et les Tfh réactifs nontumoraux. En plus aucune mutation du gène n'a été identifié montrant ainsi que *ROQUIN* ne joue probablement pas un rôle majeur dans la pathogenèse de l'AITL (Auguste et al., 2013)

2. Modèle d'invalidation de Tet2 « Tet2^{gt/gt} »

Comme décrit précédemment, des mutations de Tet2 ont été identifiées dans 80% des AITL. Cette mutation induisant une perte de fonction de Tet2 est associée à des processus de méthylation aberrante de certains gènes impliqués dans la progression tumorale. Bien que plusieurs études ont été menées pour clarifier le rôle de ces mutations dans le développement du cancer, Muto et ses collègues étaient les premiers à décrire les caractéristiques de la maladie due à l'invalidation de ce gène. En effet, ils ont généré un modèle de souris Tet2-/- sur un fond génétique C57BL/6 et ils ont laissé vieillir les souris jusqu'à l'âge de 40 à 60 semaines. Les premières analyses du sang périphérique ont montré que ces souris ne présentent aucune différence dans les proportions des populations totales de cellules T ou B en comparaison à des souris témoins sauvages ou hétérozygotes. Par contre, les souris Tet2-/- présentent une splénomégalie associée à un élargissement des ganglions lymphatiques. Les analyses de cytométrie en flux ont montré une augmentation du nombre absolu des lymphocytes T caractérisés par la présence à leur surface des marqueurs spécifiques des cellules Tfh (PD1⁺ $CXCR5^+$) avec un réarrangement identique des segments V et J de la chaîne β du TCR. Ces souris présentaient également une faible proportion de cellules B B220⁺, par contre aucune augmentation prononcée de la sécrétion des immunoglobulines n'a été détectée. En outre, des analyses transcriptomiques ont montré un enrichissement des gènes impliqués dans la différenciation des Tfh ainsi que des gènes codant pour les cytokines pro-inflammatoires. Vu le rôle de Tet2 dans la régulation des processus épigénétiques, une étude des profils de méthylation effectuée chez ces souris a montré une hyperméthylation du premier intron de Bcl-6, qui se traduisait par une surexpression du gène et qui pourrait expliquer l'induction aberrante des Tfh. En conclusion, bien que ce modèle murin a développé un lymphome T avec une prédominance de cellules Tfh et des marqueurs épigénétiques évidentes, il a été incapable de récapituler l'ensemble des caractéristiques de la maladie humaine (Muto et al., 2014).

3. Modèle Swiss Jim Lambert SJL

Les souris Swiss Jim Lambert (SJL) ont fait l'objet de nombreuses études en tant que modèles de tumeurs malignes lymphoprolifératives. Chez ces souris, les cellules B du centre germinatif

expriment un superantigène (vSAg) du virus de la tumeur mammaire des souris (mtv-29) connu pour sa capacité à stimuler les cellules CD4⁺ qui, à leur tour, favorisent la prolifération des cellules B. Grâce à ce double paradigme d'activation, de nombreuses troubles lymphoprolifératifs se développent chez 90% de ces souris, montrant une similarité avec des lymphomes non-hodgkiniens (Tang et al., 1998).

En 2015, Jain et ses collègues étaient les premiers à décrire une similitude entre les troubles lymphoprolifératifs des SJL et les caractéristiques de l'AITL, en mettant en évidence le rôle de l'IL21 dans la pathogenèse de cette maladie. En effet, chez les souris SJL de 2 à 24 mois, un marquage immunohistologique a montré des changements de l'architecture splénique ou ganglionnaire, liés à l'âge. Les souris jeunes présentaient une disposition folliculaire bien structurée qui perdait son organisation progressivement à partir de 12 mois. Des analyses de cytométrie en flux ont montré une augmentation de la fréquence des cellules Tfh (CD4⁺ CXCR5⁺ ICOS⁺ PD-1⁺) et des cellules B du centre germinatif (FAS⁺ B220⁺ Gl-7⁺) dans la rate de ces souris en comparaison à des souris témoins. Le séquençage des chaînes β du récepteur des cellules Tfh a montré un réarrangement clonal des segments V alors que les segments J étaient différents. Des analyses d'expression génique ont révélé une surexpression marquée des gènes impliqués dans la signalisation des Tfh, notamment IL-21 et IL-10, et des gènes codant pour les chimiokines CCL8 et CCL12 jouant un rôle dans l'attraction des macrophages et des cellules dendritiques. Pour analyser l'implication d'IL-21 dans la mise en place de la pathologie ils ont généré un modèle de souris SJL IL21r-/-. Une analyse détaillée de ces souris a montré des réductions significatives de fréquences des cellules CD4+ Tfh associées à une disparition des cellules B. Ces résultats ont identifié un modèle murin présentant les caractéristiques de l'AITL et ont confirmé l'importance de la signalisation induite par l'IL21 dans la mise en place de ce lymphome (Jain et al., 2015).

4. RhoA G17V TET2 fl/fl

Comme décrit précédemment, les mutations génétiques sont fréquemment retrouvées dans les AITL mais leur implication dans la transformation maligne des Tfh et la pathogenèse de la maladie reste à identifier. C'est pour cela que plusieurs équipes de recherche se sont intéressées à étudier le rôle des deux mutations les plus récurrentes dans les AITL (*RhoA* G17V et la perte de fonction de *Tet2*) dans la progression tumorale.

La première observation a été réalisée en 2017 par Zang et ses collègues qui ont mis en évidence que l'invalidation génétique de Tet2, associé à l'expression du mutant RhoA G17V, dans les LT murins matures entraîne des anomalies de développement des LT, caractérisées par une prolifération accrue des Tfh au détriment des autres populations CD4⁺. En effet, les auteurs ont expliqué que ces mutations affectent les modifications transcriptionnelles et posttranscriptionnelles des gènes régulant le développement des LT comme en témoin une double inactivation de FoxO1 par hyperméthylation au niveau de la région promotrice et une augmentation du niveau de phosphorylation induisant sa dégradation. En plus ces altérations génétiques étaient à l'origine de l'apparition d'un phénotype inflammatoire aigu chez les souris mais sans aucun symptôme de transformation maligne probablement dû à une mort précoce des animaux (Zang et al., 2017).

Ces observations ont été suivies en 2018 par deux études successives qui ont mis en place un nouveau modèle d'expression inductible des deux mutants dans les LT CD4⁺ et dans les progéniteurs hématopoïétiques *in vivo* pour pouvoir analyser leur implication à long terme (Cortes et al., 2018; Ng et al., 2018). Tout d'abord, les auteurs ont identifié qu'une expression inductible du mutant RhoA G17V dans les CD4⁺ réoriente leur différenciation vers la lignée Tfh, comme le montre une augmentation de la proportion des cellules CD4⁺ CXCR5⁺ PD-1⁺ ce qui suggère un rôle prédominent de cette mutation dans la mise en place des caractéristiques Tfh de l'AITL. En outre, leurs résultats ont révélé une relation importante entre les deux voies de signalisation de RhoA et ICOS au cours de la différenciation des Tfh. En effet, la génération des Tfh à partir des LT exprimant le mutant RhoA G17V est médiée par un mécanisme dépendant d'ICOS, qui régule la signalisation PI3K-mTOR. Par contre, l'expression unique de ce mutant était incapable de récapituler les caractéristiques d'une transformation maligne de Tfh.

D'autre part, l'induction d'une perte de fonction de Tet2 dans les cellules hématopoïétiques progénitrices, en association avec la mutation de RhoA, a provoqué un phénotype lymphoprolifératif similaire de l'AITL, et caractérisé principalement par des cellules Tfh clonales et fortement prolifératives. Le caractère tumoral de ces cellules a été confirmé par un transfert secondaire dans des souris receveuses qui, après transplantation, ont récapitulé l'ensemble des caractéristiques de la tumeur d'origine. De manière intéressante, vu qu'ils ont identifié un rôle de la voie de signalisation PI3K-mTOR dans la pathogenèse de l'AITL, ils ont proposé une nouvelle option thérapeutique, basée sur une inhibition de cette voie par le Duvelisib (double inhibiteur de PI3K δ et PI3K γ) dans le but de bloquer la différenciation des Tfh. Sur la base de ces résultats, les auteurs ont proposé un nouveau modèle de développement de l'AITL dans lequel les mutations de Tet2 interviennent tôt dans le processus de lymphomagenèse et établissent un clone précancéreux qui, après acquisition d'une mutation secondaire RhoA G17V, se réoriente vers un développement Tfh, entraînant la mise en place de la maladie (Figure 16) (Cortes et al., 2018; Ng et al., 2018).



Des mutations de Tet2 et DNMT3a interviennent dans les cellules hématopoeitiques progénitrices établissant ainsi un clone précancéreux, qui après acquisition d'une mutation secondaire de RhoA se réoriente vers un développement de Tfh, mettant en place le lymphome T angioimmunoblastique.

5. Modèle de xénotransplantation (PDX)

Dans cette étude, les auteurs ont établi un modèle murin d'AITL en injectant des cellules de patients à des souris receveuses NOD-SCID-IL $2\gamma^{null}$ (NOD). 2.5^E7 cellules isolées à partir des ganglions lymphatiques d'un patient AITL ont été injectées par voie intrapéritonéale à des souris NOD qui ont été sacrifiées 34 jours après l'injection. Le moment de sacrifice a été identifié en fonction de l'état général de la souris. Un immunophénotypage des rates et des moelles osseuses des souris greffées a montré une infiltration importante des cellules tumorales LT CD4⁺ ainsi que des cellules B CD19⁺. Les marqueurs spécifiques des Tfh ont été également identifiés à la surface des LT tumoraux. De manière intéressante, l'interaction entre les LT et les LB a été confirmée par la présence des plasmocytes polyclonaux sécrétrices d'immunoglobulines. Les résultats immunohistologiques chez les souris greffées ont été similaires à ceux observés chez le patient donneur.

Par contre, les transplantations en série des cellules isolées à partir des rates des souris greffées ont entrainé une perte progressive des composants cellulaires du microenvironnement tumoral. En effet, les cellules B, les plasmocytes ainsi que les cellules CD8⁺ n'ont pas été retrouvées dans les tumeurs suite à une greffe tertiaire. Ces résultats remettent en question la fiabilité de ce modèle vu que les cellules néoplasiques de l'AITL ne survivent pas en absence des cellules du microenvironnement (Sato et al., 2013a).

VI.L'immunothérapie

Le traitement des cancers a considérablement progressé au cours des deux dernières décennies grâce à la mise au point de multiples nouvelles approches thérapeutiques ciblées y compris l'immunothérapie. Le traitement des tumeurs par immunothérapie consiste à mobiliser nos propres défenses contre les cellules cancéreuses qui acquièrent plusieurs mécanismes de résistance au cours de la progression tumorale induisant ainsi un échec thérapeutique.

En effet, lors d'une première rencontre, le système immunitaire est capable de détecter et d'éliminer les cellules tumorales. Néanmoins, des mécanismes de résistance émergent au sein des tumeurs et constituent un obstacle important pour l'efficacité de la défense antitumorale. C'est d'ici qu'a émergé la théorie de l' « ImmunoEditing » ou la théorie des « 3E », développée par Schreiber 2004 (Dunn et al., 2004) et qui explique le concept d'immunorésistance des tumeurs. La première étape dite « l'Élimination » est la phase de détection et d'éradiction normale des cellules tumorales au fur et à mesure de leur apparition. Elle est suivie par une période d'« Équilibre », durant laquelle les cellules tumorales acquièrent de nouvelles mutations leur conférant une haute résistance aux attaques immunitaires, induisant ainsi leur « Échappement » aux traitements de première ligne.

C'est ainsi que l'immunothérapie a émergé afin de renforcer le système immunitaire pour qu'il soit capable de détecter efficacement les cellules tumorales et les détruire.

Les immunothérapies de première génération se sont basées sur l'utilisation des cytokines proinflammatoires telles que l'IFN ou l'IL-2 dans le but de stimuler les fonctions cytotoxiques des lymphocytes. En effet, l'IFN α -2b a été le premier agent à démontrer un bénéfice significatif dans la survie des patients atteints de mélanome de haut risque (Rosenberg et al., 1998). De manière similaire, l'IL-2 a permis une régression complète de la tumeur chez environ 8% de patients atteints du cancer du rein métastatique ou du mélanome (Rosenberg et al., 1998).

Progressivement, suite à une meilleure compréhension des interactions moléculaires entre les cellules du système immunitaire et les composants tumoraux, de nouvelles stratégies immunothérapiques se sont développées. Nous avons distingué ainsi, les approches d'immunothérapie dite passive qui reposent sur l'utilisation des agents immunologiques qui resensibilisent les cellules tumorales aux attaques immunitaires. Ces stratégies consistent soit à utiliser des anticorps monoclonaux qui ciblent des antigènes exprimés à la surface des cellules tumorales déclenchant ainsi une réponse immunitaire immédiate par activation des cellules NK et du système du complément, soit par un transfert adoptif de lymphocytes. Cette stratégie, reconnue par le nom CAR-T, consiste à modifier *in vitro* les cellules immunitaires du patient

pour les resensibiliser aux antigènes tumoraux et rétablir une attaque immunitaire adéquate (Mhaidly and Verhoeyen, 2019; Titov et al., 2018). D'autres approches immunothérapiques sont dites actives et consistent à solliciter directement le système immunitaire du patient. Elles comprennent la vaccination anti-tumorale, basée sur l'utilisation des antigènes tumoraux chargés sur des cellules présentatrices des antigènes pour induire une réponse immunitaire mémoire de la tumeur ; ou encore les molécules ciblant les points de contrôle du système immunitaire dites « immune checkpoints » qui ont pour rôle initial de s'opposer à l'immunosuppression induite par la tumeur (Figure 17).

En effet, il existe de nombreux points de contrôle qui interviennent dans la régulation de l'activation des lymphocytes (Abril-Rodriguez and Ribas, 2017; Pardoll, 2012). Ce sont des complexes récepteurs-ligands qui agissent sur la stimulation ou l'inhibition de la réponse immunitaire. Ces dernières années, la recherche en immunothérapie s'est concentrée sur deux récepteurs inhibiteurs CTLA-4 et PD-1 qui bloquent les fonctions effectrices des LT et les empêchent de s'attaquer aux tumeurs.



Figure 17 Différentes méthodes d'immunothérapie passive et active

A. Anti-CTLA-4

CTLA-4 est un récepteur inhibiteur exprimé à la surface des LT très tôt au cours du processus d'activation. Il empêche l'acquisition des fonctions effectrices des LT soit par dégradation des

ligands de co-stimulation soit par délivrance des signaux inhibiteurs bloquant la prolifération et la sécrétion des cytokines. De plus, CTLA-4 est exprimé de façon constitutive par les LT régulateurs et est essentiel à leur fonction immunosuppressive. Basée sur son rôle en tant que régulateur négatif de l'activation des LT, cette molécule est devenue une cible intéressante pour les traitements visant à restaurer l'activité effectrice des cellules immunitaires. Dans le contexte de l'immunité anti-tumorale, plusieurs études ont montré que le blocage de CTLA-4 par des anticorps monoclonaux inhibe la suppression immunitaire. Ainsi, le premier essai clinique a démontré en 2011 que les patients atteints d'un mélanome de stade III ou IV présentaient une amélioration de leur survie après un traitement avec un anti-CTLA-4 « l'Ipilimunab ». L'importance de ces résultats a été confirmé par une comparaison avec ceux des patients traités avec un agent chimiothérapique et dont le taux de survie à 3 ans ne dépassait pas 12.2% (Hodi et al., 2010; Robert et al., 2011). Bien qu'il ne soit approuvé que pour le traitement des mélanomes, l'Ipiliminab fait l'objet de plusieurs études pour tester son efficacité sur d'autres types de cancers, notamment le cancer des poumons ou de la prostate, et les résultats actuels ont montré des améliorations modestes de la survie des patients (Reck et al., 2013; Yang et al., 2007).

B. Anti-PD-1/PD-L1

Après le succès observé avec l'anti-CTLA4, d'autres inhibiteurs des rétrocontrôles immunitaires se sont développés, ciblant le récepteur PD-1 et son ligand PD-L1.

Le récepteur PD-1 est une glycoprotéine transmembranaire exprimée à la surface d'un grand nombre de cellules immunitaires y compris les LT (CD4⁺ et CD8⁺), les LB, les cellules NK, les monocytes et les cellules dendritiques. Son expression augmente au niveau des LT activés, 24h après leur stimulation pour limiter une réponse immunitaire excessive. Il interagit avec deux ligands PD-L1 et PD-L2, présentés à la surface des cellules présentatrices d'antigènes. En effet, la liaison de PD-1 à ses ligands génère un signal de rétrocontrôle négatif qui inhibe la signalisation émise en aval du TCR et résulte ainsi en une anergie des LT (Jin et al., 2011). Ce phénomène est physiologiquement très important au quotidien pour limiter les risques des maladies auto-immunes. Par ailleurs, les cellules tumorales peuvent exploiter ce récepteur inhibiteur afin de s'échapper aux défenses immunitaires par une surexpression du ligand PD-L1 à leur surface de façon constitutive (Azuma et al., 2008; Dong et al., 2002). C'est pour ça que des anticorps monoclonaux ont été développés pour bloquer ce système de contrôle négatif exercé par les cellules tumorales et rétablir ainsi la réponse immunitaire (Figure 18). Parmi ces
anticorps, le « Nivolumab » est une immunoglobuline de type G4, ciblant PD-1 et qui a montré dès sa phase I, une efficacité satisfaisante. Il a été d'abord autorisé aux USA en Décembre 2014 puis en Europe Avril 2015 pour le traitement du mélanome avancé ou métastatique, suite aux résultats de l'essai clinique qui a révélé une amélioration des taux de réponse au Nivolumab en comparaison aux agents chimiothérapiques (40% vs 13.9%) (Weber et al., 2015). En plus de son efficacité dans le traitement du mélanome, il s'est révélé bénéfique contre plusieurs types de cancers avec des résultats meilleurs que ceux obtenus avec l'anti-CTLA-4 (Brahmer et al., 2015; Ferris et al., 2016; Motzer et al., 2015). Le Nivolumab a également présenté une action thérapeutique importante chez les patients atteints d'un lymphome de Hodgkin en rechute à des traitements de première ligne ce qui offre de nouvelles options thérapeutiques pour les hémopathies malignes (Ansell et al., 2015; Younes et al., 2016).

Le « Pembroluzimab » est un deuxième anticorps anti-PD1 appliqué en traitement de deuxième ligne pour la prise en charge du mélanome métastatique.

En plus de bloquer le récepteur PD-1, le ciblage de PD-L1 avec des inhibiteurs immunitaires s'est également présenté comme étant une approche utile pour améliorer l'activité anti-tumorale des LT. Des études récentes effectuées sur des patients atteints de cancer urothélial n'ont pas révélé une amélioration de la survie par rapport aux traitements chimiothérapiques de base par contre, cet inhibiteur apparaissait avec moins d'effets indésirables sur les patients (Powles et al., 2018). Deux autres inhibiteurs de PD-L1 «l'Avelumab et le Durvalumab » ont été récemment approuvés dans plusieurs contextes notamment les cancers de vessie, le cancer de la cellule de Merkel ou également les cancers de poumons (Antonia et al., 2017; Kaufman et al., 2016; Patel et al., 2018)

Vu l'importance de ces deux inhibiteurs, le prix Nobel de médecine en 2018 a été attribué aux deux chercheurs James Alisson et Tasuku Honjo pour la découverte de ces immunothérapies qui ont permis de lever les freins des réponses immunitaires.



En condition de progression tumorale, les cellules cancéreuses se lient aux cellules immunitaires par la liaison PD-1-PD-L1 et les désactivent. Les immunothérapies bloquent cette interaction et permettent de réactiver le système immunitaire.

C.L'immunothérapie dans les lymphomes T

Comme décrit précédemment, les anticorps monoclonaux « immune checkpoints » ont montré des résultats prometteurs dans les tumeurs solides, ce qui encouragé les chercheurs et les cliniciens à tester leur efficacité sur des hémopathies malignes (Ghione et al., 2018; Khan et al., 2018). Le nivolumab (anti-PD-1) a fait l'objet d'une première étude clinique chez des patients atteints de lymphome T de l'âge adulte (ATLL). Par contre les résultats obtenus ont montré que l'inhibition de PD-1 provoquait une progression tumorale. Il semble que PD-1 agit en tant que suppresseur de tumeur dans les ATLL et par la suite son inhibition peut favoriser la croissance tumorale (Ratner et al., 2018). Cependant, dans d'autres sous-groupes de lymphomes T comme le lymphome T/NK extra-nodal, le traitement avec l'anticorps monoclonal anti-PD-1 a montré une efficacité satisfaisante avec des toxicités limitées. En se basant sur ces résultats,

le nivolumab a été administré dans plusieurs essais cliniques pour les cas de lymphome T périphérique en rechute aux chimiothérapies et a montré une efficacité modeste avec une amélioration de la survie de 40% (Lesokhin et al., 2016). Actuellement de nouvelles combinaisons de traitement sont en cours de développement pour les patients réfractaires aux chimiothérapies. Parmi ces combinaisons, le nivolumab est associée soit au Brentuximab Vedotin (anti-CD30 ; NCT02581631) soit à l'isatuximab (anti-CD38 ; NCT03769181).

Objectif

L'ensemble des données bibliographiques présentées dans la partie introductive souligne l'implication de plusieurs protéines et agents moléculaires dans la différenciation des LT.

Nos travaux de recherche se situent dans la continuité des données actuelles décrivant l'effet de la surexpression de l'enzyme métabolique « la GAPDH » sur le développement des LT et son influence sur la croissance tumorale.

Sur la base de cette question, plusieurs hypothèses se sont proposées :

- Vu que la GAPDH est un régulateur de la mort cellulaire qui intervient dans les stades précoces de développement des LT, son surexpression pourrait induire la génération des thymocytes immatures.
- La GAPDH pourrait également bloquer l'activation des LT effecteurs par le biais de sa liaison à l'IFN-γ.
- Et finalement, quel est l'effet de cette surexpression sur le métabolisme des cellules immunitaires et plus particulièrement des LT.

Afin d'adresser cette question, nous avons généré un modèle de souris transgéniques surexprimant la GAPDH uniquement dans les cellules T. Alors qu'aucune modification phénotypique n'a été détectée dans les stades précoces de développement, nous avons observé que ces souris développent dès l'âge de 18 mois un phénotype tumoral.

Vous retrouverez dans la partie suivante de ce manuscrit, les différentes questions posées et les réponses apportées par notre étude.

Résultats

Article 1

La surexpression de l'enzyme glycolytique « la GAPDH » induit le développement d'un lymphome T angioimmunoblastique par l'intermédiaire de la voie NF-KB

Il a été récemment établi que l'activation des lymphocytes T pourrait être étroitement contrôlée par le métabolisme cellulaire. Parmi les voies métaboliques, la glycolyse est la principale voie utilisée par toutes les cellules pour produire l'énergie nécessaire à leur croissance. C'est une succession de dix réactions enzymatiques qui permettent la conversion du glucose en pyruvate. Parmi les enzymes catalysant cette voie, des études récentes ont fait émerger la glycéraldéhyde 3-phosphate déshydrogénase (GAPDH) comme un acteur principal de la survie et de la différenciation des LT. Afin de mieux caractériser le rôle de la GAPDH dans le développement lymphocytaire T, nous avons généré un modèle de souris transgéniques surexprimant la GAPDH exclusivement dans les LT (plck-GAPDH).

Une première caractérisation de ces souris a montré que la surexpression de la GAPDH n'a pas d'effet sur les stades précoces de développement des LT. De façon intéressante, nous avons observé chez les souris âgées des changements phénotypiques évidents, notamment : une splénomégalie, des ganglions lymphatiques hypertrophiés et une infiltration lymphocytaire majeure au niveau du foie et de la moelle osseuse. Un immunophénotypage précis de ces tissus a montré une augmentation de la proportion des LT folliculaires helpers (Tfh) CD4⁺ Bcl-6⁺CXCR5⁺ PD-1^{high}. De plus, ces cellules, fortement prolifératives et clonales, interagissent avec les lymphocytes B CD95⁺GL7⁺ du centre germinatif, eux-mêmes associés à des cellules dendritiques folliculaires, formant ainsi un microenvironnement tumoral.

À la recherche d'un équivalent humain à notre modèle, nous avons découvert que les caractéristiques pathologiques et immunologiques des souris plck-GAPDH correspondaient étroitement au lymphome T angio-immunoblastique humain (AITL). Il s'agit d'une hémopathie maligne rare pour laquelle aucune stratégie thérapeutique n'a été clairement décrite. Nous avons également mis en évidence dans les souris plck-GAPDH une activation de la voie NF-KB non canonique, également activée chez les patients AITL. Le ciblage de cette voie par un nouvel inhibiteur de la kinase activatrice NIK associé à une immunothérapie anti-PD-1 a augmenté la survie des souris. Ce traitement permet une induction efficace de la réponse immunitaire anticancéreuse et représente une nouvelle perspective thérapeutique pour les patients atteints d'AITL.

Cancer Cell

GAPDH Overexpression in the T Cell Lineage Promotes Angioimmunoblastic T Cell Lymphoma through an NF-kB-Dependent Mechanism

Graphical Abstract



Highlights

- Overexpression of GAPDH in T cell lineage in a mouse model recapitulates AITL disease
- AITL tumors are characterized by a GAPDH induction of the NF- κ B pathway
- plck-GAPDH mice allow to model AITL disease for testing of new therapeutic strategies

Authors

Laura Mondragón, Rana Mhaidly, Gian Marco De Donatis, ..., Thierry Passeron, Jean-Ehrland Ricci, Els Verhoeyen

Correspondence

els.verhoeyen@unice.fr (E.V.), ricci@unice.fr (J.-E.R.)

In Brief

Mondragón et al. find that transgenic mice overexpressing GAPDH in T cells develop lymphoma that recapitulates key features of human angioimmunoblastic T cell lymphoma (AITL), including activation of the non-canonical NF- κ B pathway. Blocking NF- κ B activity with an NIK inhibitor reduces human and mouse AITL growth.

Mondragón et al., 2019, Cancer Cell 36, 1–20 September 16, 2019 © 2019 Elsevier Inc. https://doi.org/10.1016/j.ccell.2019.07.008



Cancer Cell Article

CellPress

GAPDH Overexpression in the T Cell Lineage Promotes Angioimmunoblastic T Cell Lymphoma through an NF-κB-Dependent Mechanism

Laura Mondragón,^{1,16} Rana Mhaidly,^{1,16} Gian Marco De Donatis,¹ Marie Tosolini,² Pascal Dao,³ Anthony R. Martin,³ Caroline Pons,¹ Johanna Chiche,¹ Marie Jacquin,¹ Véronique Imbert,¹ Emma Proïcs,¹ Laurent Boyer,¹ Anne Doye,¹ Frédéric Luciano,¹ Jaap G. Neels,¹ Frédéric Coutant,^{4,5} Nicole Fabien,⁴ Laura Sormani,¹ Camila Rubio-Patiño,¹ Jozef P. Bossowski,¹ Florian Muller,¹ Sandrine Marchetti,¹ Elodie Villa,¹ Jean-François Peyron,¹ Philippe Gaulard,^{6,13} François Lemonnier,^{6,7} Vahid Asnafi,⁸ Laurent Genestier,⁹ Rachid Bennida,³ Jean-Jacques Fournié,^{10,14}

Thierry Passeron,^{1,11} Jean-Ehrland Ricci,^{1,15,*} and Els Verhoeyen^{1,12,15,17,*}

¹Université Côte d'Azur, INSERM, C3M, 06204 Nice, France

²Pôle Technologique du CRCT – Plateau Bioinformatique INSERM-UMR 1037, Toulouse, France

³Institut de Chimie de Nice UMR UNS-CNRS 7272, Université Nice Sophia Antipolis, Parc Valrose, 06108 Nice, France

⁴Immunology Department, Lyon-Sud Hospital, Hospices Civils de Lyon, Pierre-Bénite, France

⁵Immunogenomics and Inflammation Research Unit EA 4130, University of Lyon, Edouard Herriot Hospital, Lyon, France

⁶Université Paris-Est Créteil, Institut Mondor de Recherche Biomédicale, INSERM U955, Créteil, France

⁷Unité hémopathies lymphoïdes, Hôpitaux Universitaires Henri Mondor, Assistance publique des Hôpitaux de Paris, Créteil, France ⁸Université Paris 5, Institut Necker-Enfants Malades (INEM), Institut National de Recherche Médicale (INSERM) U1151, and Laboratory of Onco-Hematology, Assistance Publique-Hôpitaux de Paris (AP-HP), Hôpital Necker-Enfants Malades, Paris, France ⁹CRCL, INSERM U1052-CNRS UMR5286, Centre Léon Bérard, Faculté de Médecine Lyon Sud, Université Claude Bernard Lyon I, 69921

Oullins Cedex, France ¹⁰CRCT, INSERM U1037 – Université Paul Sabatier – CNRS ERL5294, Université de Toulouse, Laboratoire d'Excellence TOUCAN,

Programme Hospitalo-Universitaire en Cancérologie CAPTOR, Toulouse, France

¹¹Université Côte d'Azur, Centre Hospitalier Universitaire de Nice, Department of Dermatology, 06204 Nice, France

¹²CIRI, Université de Lyon, INSERM U1111, ENS de Lyon, Université Lyon 1, CNRS, UMR 5308, 69007 Lyon, France

¹³Département de Pathologie, Hôpitaux Universitaires Henri Mondor, Assistance publique des Hôpitaux de Paris, Créteil, France ¹⁴IUCT, 31037 Toulouse, France

¹⁵Senior author

¹⁶These authors contributed equally

¹⁷Lead Contact

*Correspondence: els.verhoeyen@unice.fr (E.V.), ricci@unice.fr (J.-E.R.)

https://doi.org/10.1016/j.ccell.2019.07.008

SUMMARY

GAPDH is emerging as a key player in T cell development and function. To investigate the role of GAPDH in T cells, we generated a transgenic mouse model overexpressing GAPDH in the T cell lineage. Aged mice developed a peripheral Tfh-like lymphoma that recapitulated key molecular, pathological, and immunophenotypic features of human angioimmunoblastic T cell lymphoma (AITL). GAPDH induced non-canonical NF- κ B pathway activation in mouse T cells, which was strongly activated in human AITL. We developed a NIK inhibitor to reveal that targeting the NF- κ B pathway prolonged AITL-bearing mouse survival alone and in combination with anti-PD-1. These findings suggest the therapeutic potential of targeting NF- κ B signaling in AITL and provide a model for future AITL therapeutic investigations.

Significance

Availability of preclinical mouse models for rare cancers is a prerequisite for revealing important pathways and targets to identify and evaluate possible treatment regimens for these patients lacking treatment options. Our GAPDH transgenic mouse model developed a Tfh-like lymphoma with a strong germinal center B cell component, mimicking angioimmunoblastic T cell lymphoma (AITL), a rare aggressive lymphoma. Our data confirmed that GAPDH-induced AITL disease depends mechanistically on activation of the non-canonical NF- κ B pathway, which we also confirmed to be activated in human AITL. Targeting this pathway with an NF- κ B pathway-inhibiting drug was an effective treatment for murine as well as human AITL cells. This mouse model allows testing of therapeutic options for AITL including immunotherapy.

INTRODUCTION

Immunometabolism has recently become an intense field of research. It shapes the functions and the differentiation of immune cells and reveals anti-cancer immunotherapies (Pearce and Pearce, 2018). Fundamental changes from mitochondrial to glycolytic metabolism are implemented by immune cells as well as cancer cells to meet their high energy requirement (Warburg effect) (Galluzzi et al., 2013; Kroemer and Pouyssegur, 2008). However, the role of specific metabolic enzymes in T cells and cancer is just starting to be unraveled. Aerobic glycolysis converts glucose to pyruvate through a series of ten enzymes, of which one is of particular interest: glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Recent studies have demonstrated that GAPDH could have a rate-limiting function in the context of Warburg effects (Liberti et al., 2017). In addition to its role in glycolysis, GAPDH is a multifunctional protein with numerous non-glycolytic functions (Colell et al., 2007, 2009; Jacquin et al., 2013; Lavallard et al., 2009). GAPDH is overexpressed in many cancers (Chiche et al., 2015; Liberti et al., 2017; Revillion et al., 2000), but is role in tumorigenesis is still unclear (Revillion et al., 2000). Moreover, we previously revealed GAPDH overexpression in chronic myeloid leukemia (CML) cells, which participated in CML resistance to chemotherapeutic treatment (Lavallard et al., 2009). Of note, overexpression of GAPDH, but not other glycolytic enzymes tested, protected cells from caspaseindependent cell death (CICD) through persistence of a few intact mitochondria that might facilitate tumor cell survival and chemotherapy resistance (Colell et al., 2007).

At present, very little is known about GAPDH expression levels and its role in T cell differentiation and T cell cancers such as peripheral T cell lymphomas (PTCLs), but GAPDH has recently emerged as a key factor in T cell survival, activation, and function (Balmer et al., 2016; Chang et al., 2013; Xu et al., 2016). For example, GAPDH expression was implicated in T cell responses under hypoxia (Dimeloe et al., 2016; Xu et al., 2016). Acetylation of GAPDH enhances its activity, boosting rapid memory CD8⁺ T cell responses (Balmer et al., 2016). Interestingly, GAPDH can also modulate T cell effector function (Chang et al., 2013). We and others established a clear connection between high GAPDH expression and the activation of the nuclear factor kB (NF-κB) pathway (Chiche et al., 2015; Gao et al., 2013). We recently showed that elevated GAPDH expression was found to promote NF-kB activation in a non-Hodgkin's B cell lymphoma by binding to tumor necrosis factor receptor-associated factor 2 (TRAF2), which enhanced the transcription and the activity of hypoxia-inducible factor 1α (Chiche et al., 2015). NF-κB activation occurs via two major signaling pathways. The canonical pathway mediates the activation of NF-kB1 p50, RelA, and c-Rel, whereas the non-canonical NF-kB pathway selectively activates p100-sequestered NF-kB members, predominantly NF-kB p52 and RelB. The canonical NF-kB pathway responds to engagement of immune receptors and results in rapid but transient NF-kB activation (Sun, 2017). In contrast, activation of the non-canonical pathway is characteristically slow and persistent, and ligands of the tumor necrosis factor (TNF) receptor superfamily are inducers of the non-canonical pathway. Both NF-kB pathways are involved in malignancy and inflammatory diseases (Cildir et al., 2016; Hoesel and Schmid, 2013;

2 Cancer Cell 36, 1-20, September 16, 2019

Sun, 2017). Importantly, NF- κ B activation is associated with many T cell cancers (Balmer et al., 2016; Cildir et al., 2016; Gerondakis et al., 2014; Imbert and Peyron, 2017; Sun, 2017) and plays an essential role in T cell development and function (Gerondakis et al., 2014; Grinberg-Bleyer et al., 2017; Long et al., 2009). It is, for example, implicated in differentiation and function of regulatory T cells (Grinberg-Bleyer et al., 2017; Long et al., 2009; Vasanthakumar et al., 2017) (Jimi et al., 2008). However, the contribution of GAPDH to T cell function, differentiation, and, possibly, malignancy remains to be comprehensively elucidated.

RESULTS

Overexpression of GAPDH Exclusively in the T Cell Lineage Does Not Affect Early T Cell Differentiation

To uncover GAPDH function in T cells, we generated a transgenic mouse model in which GAPDH was overexpressed under the control of the T cell-specific promoter, plck. A V5 tag was fused to GAPDH to distinguish it from the endogenous GAPDH enzyme, and three founder animals (F16, F07, and F35) were identified (Figure 1A). Overexpression of GAPDH was validated in the plck-GAPDH transgenic mice by western blot (Figure 1B). We confirmed exclusive and strong expression in the T cell lineage (Figure 1C). GADPH-V5 was significantly overexpressed in different thymic subpopulations of the founder plck-GAPDH mice at 4 weeks of age compared with wild type (WT) (Figure 1D). Higher overall GAPDH expression was detected in F07 and F35 thymocytes than in F16 thymocytes (Figure 1E). Notably, although GAPDH was described to inhibit a non-apoptotic cell death named CICD (Colell et al., 2007), which might play a role in thymocyte development and selection, no significant differences in thymic subpopulations were revealed in 4-week-old mice (Figure 1F).

Aged plck-GAPDH Mice Develop a Peripheral T Cell Lymphoma-like Pathology

Upon aging, the plck-GAPDH mice developed tumors (Figure 2A). Tumor frequency coincided with the GAPDH overexpression levels (Figures 1D and 2A), as the high GAPDH-expressing F07 and F35 mice all developed tumors, while tumor frequency for F16 was much lower and with delayed onset of disease (Figure 2A and data not shown). Therefore, we focused our study on F07 and F35. These plck-GAPDH mice showed onset of disease at 18 months or older (Figure 2B). Aged plck-GAPDH mice developed a pathology characterized by a generalized lymphadenopathy (Figure 2C). These mice generally developed splenomegaly, and frequently developed hepatomegaly and enlarged mesenchymal lymph nodes (MES LNs) and LNs at ectopic sites such as around the kidney, heart, or intestines (Figures 2D-2G). The spleens and LNs showed a greatly altered architecture, and the follicular structures visible in the WT were destroyed in the plck-GAPDH mice, marked by large polymorphic infiltration of T cells and B cells (Figures 2H and S1A). These pathological features pointed toward a PTCL. Remarkably, aged plck-GAPDH mice also developed a typical rash and showed a strong LN vascularization with detection of 35-fold increased expression level of vascular endothelial growth factor in murine PTCL (mPTCL) LN versus WT and the presence of venules (CD31⁺) in the LN (Figures S1B-S1D). Moreover, 50% of

CellPress



Figure 1. Overexpression of GAPDH in the T Cell Lineage Does Not Affect Early T Cell Development (A) Schematic presentation of the GAPDH expression construct to generate three founder mice (F16, F35, F07). Plck, lymphocyte-specific protein tyrosine kinase promoter; V5, tag for fluorescence-activated cell sorting (FACS) and western blot detection.

> (legend continued on next page) Cancer Cell 36, 1–20, September 16, 2019 3

plck-GAPDH mice developed ascites in the abdominal region (data not shown). Finally, the splenic tumors of the transgenic mice were characterized by no changes in CD19⁺ B cell counts but a 2-fold higher cellularity of T cells and an increased ratio of CD4⁺-to-CD8⁺ T cells (Figures 2I and 2J). The combination of all these histopathological symptoms suggests that the plck-GAPDH mice developed a PTCL.

Plck-GAPDH Mice Develop a T Cell Lymphoma with a T Follicular Helper Dominance

Since tumors in plck-GAPDH mice manifested a higher presence of CD4⁺ over CD8⁺ T cells, we characterized the CD4⁺ T cells. They were positive for programmed cell death 1 (PD-1) and to a lesser extent for the chemokine receptor, CXCR5 (Figure 3A). The percentage of CD4⁺PD-1^{high}CXCR5⁺ T follicular helper (Tfh)-like cells was significantly higher in plck-GAPDH than in WT spleens, and the increase in total CD4⁺PD-1^{high} was even more pronounced (80%; Figure 3B).

Additionally, two Tfh markers, ICOS (Hu et al., 2011) and Bcl-6, were upregulated on the tumor CD4⁺ T cells (Figure 3A). They also had an activated memory phenotype (Figure 3C) and showed increased expression of the death receptor FAS (CD95) but only slight augmentation of FAS ligand (FASL, CD178) compared with CD4⁺ WT T cells (Figure 3D), both characteristics of Tfh cells. Ki67 staining indicated that these CD4⁺ T splenocytes were strongly cycling in contrast to WT splenocytes (Figure 3E), indicating the aggressiveness of these malignant T cells. When we activated CD4⁺ plck-GAPDH tumor cells by phorbol 12-myristate-13-acetate (PMA)/ionomycin, strong expression of the inflammatory cytokines (TNFa and interferon- γ [IFN γ]) and the anti-inflammatory cytokine interleukin-10 (IL-10) was revealed (Figure 3F). IL-10 is known to promote B cell expansion. In contrast, IL-4 (T helper cell [Th2] marker) and IL-17 (Th17 marker) expression was not increased (data not shown). Finally, we clearly detected a T cell receptor β (TCR β) and TCR γ receptor oligoclonality in the CD4⁺ T cells from different plck-GAPDH tumor-bearing mice (Figures 3G and 3H).

The above results together indicate that the plck-GAPDH mice developed a PTCL with a pronounced Tfh-like phenotype.

Plck-GAPDH Tfh-like Lymphoma Is Associated with Germinal Center and Plasma B Cells

Tfh reside in germinal centers (GCs) and normally interact with GC B cells, ultimately yielding plasma cells (PCs) and memory B cells (Cildir et al., 2016). Indeed, B cell phenotyping of cells in the enlarged spleens and LNs of the plck-GAPDH mice demonstrated significantly increased percentages of CD95⁺GL-7⁺ GC B cells compared with WT (Figures 4A and 4B). Additionally, plck-GADPH

B cells demonstrated increased levels of FAS and FASL compared with WT (Figure 4C), underlining the importance of Tfh FAS and GC B FASL interaction for cell survival. PD-1 expression on Tfh not only regulates GC survival but also affects PC differentiation (Hu et al., 2011; Xu et al., 2014). Identification of PCs by CD138⁺CD19⁻B220^{low} surface expression showed that 60% of the plck-GAPDH tumor mice showed more PCs than WT mice (PC WT < 5%), varying from 6% to 80% of total mononuclear cells (Figures 4D and 4E).

Co-residence of Tfh, GC, and plasma B cells in the plck-GAPDH tumors stresses that this mouse model closely mimics a human Tfh PTCL cancer (Ahsanuddin et al., 2011; Nagoshi et al., 2013). We also detected cycling B cells, hypergammaglobulinemia, and presence of autoantibodies in the sera of mPTCLbearing plck-GAPDH (Figures S2A–S2C). This notion of a PTLC is further reinforced by increased numbers of tumor-associated myeloid cells, specifically follicular dendritic cells (FDCs) (CD35⁺) and total DCs (CD11c⁺), in the enlarged plck-GAPDH LNs compared with WT mice (Figures S1C and S2D). Of note, strong infiltration of the CD4⁺PD-1⁺ Tfh cells and GC B cells in the bone marrow was confirmed in all tumor-bearing plck-GAPDH mice (Figure S2E).

To confirm that the plck-GAPDH mouse developed a rare T cell lymphoma, we transferred the tumor cells from enlarged LN or spleens into NOD/SCID $\gamma c^{-/-}$ (NSG) recipient mice (Traggiai et al., 2004). Engrafted NSG mice recapitulated the disease characteristics of the plck-GAPDH donor mice (Figure S2F). Tumors showed a strong increase of CD4⁺/CD8⁺ T cell ratio (Figure S2G), high expression of PD-1 on CD4⁺ T cells, and the co-residence of GC B cells in the tumors (Figure 4F). We confirmed Tfh and GC B cell colonization of the spleen in three independent NSG recipients per donor (Figure 4G). We also detected infiltration of Tfh and GC B cells in the bone marrow of recipient NSG mice (Figure S2H) and confirmed that engrafted T cells conserved oligoclonality for the TCR β chain (Figure S2I).

Co-residence of Tfh, GC, and plasma B cells and tumor-associated cells in the plck-GAPDH tumors stresses that this mouse model strongly mimics a human Tfh PTCL.

PIck-GAPDH PTCLs Have a Gene-Expression Profile Resembling that of AITL Patient PTCL

To identify the human PTCL (hPTCL) counterpart of the PTCL developed by plck-GAPDH mice, we compared the gene-expression profile (GEP) of our mPTCL with those of hPTCLs. The mPTCLs contained on average $30.0\% \pm 6.2\%$ CD4⁺ cells. Initially, we compared GEPs of murine splenic PTCLs and normal WT splenocytes, identifying 471 up- and 847 downregulated genes. Gene set enrichment analysis (GSEA) for a Tfh-upregulated

(C) Intracellular GAPDH-V5 and GAPDH detection in T cells (CD3⁺) and B cells (CD19⁺) from the spleen analyzed by FACS in 5-week-old mice.

(D) Ectopic GAPDH-V5 expression levels (mean fluorescence intensity [MFI]) detected by FACS using anti-V5 antibody in thymocyte subpopulations: double-negative, DN (CD4⁻CD8⁻); double-positive, DP (CD4⁺CD8⁺); CD4⁺ (CD8⁻CD4⁺) and CD8⁺ (CD4⁻CD8⁺) cells from WT (n = 18) and three founder plck-GAPDH mice at 5 weeks (F07, n = 7; F16, n = 15, and F35, n = 13; mean \pm SD, p < 0.001, one-way ANOVA for all groups).

(E) Total GAPDH expression levels (MFI) detected by FACS using anti-GAPDH antibody in the DN, DP, $CD4^+$, and $CD8^+$ thymocytes for WT (n = 18) and three founder plck-GAPDH mice (F07, n = 7; F16, n = 15, and F35, n = 13; mean \pm SD, $^{**}p < 0.0$, one-way ANOVA) at 5 weeks of age.

(F) Distribution of DN, DP, CD4⁺, and CD8⁺ thymocytes analyzed by FACS in WT and plck-GAPDH mice, and thymocyte cell numbers, are summarized in a histogram (mean ± SD, n = 5).

4 Cancer Cell 36, 1-20, September 16, 2019

⁽B) Western blot using an anti-V5 antibody for GAPDH-V5 detection and anti-GAPDH for detection of endogenous GAPDH and GAPDH-V5 in plck-GAPDH transgenic (Tg 1 and Tg 2) or wild-type (WT 1 and WT 2) 5-week-old mice.

CellPress



Figure 2. Aged plck-GAPDH Mice Develop a PTCL-like Lymphoma

(A) Frequency of tumor appearance among the different plck-GAPDH founder mice (20/20 for F07; 20/20 for F35; 6/16 for F16; 2/23 for WT). (B) Cumulative incidence of tumor development in plck-GAPDH mice from the founders F07 and F35 according to their age (plck-GAPDH, n = 40; WT, n = 30).

(legend continued on next page)

Cancer Cell 36, 1–20, September 16, 2019 5

gene set (18 genes), based on previous studies (de Leval et al., 2007; Jain et al., 2015), showed upregulation of Tfh signature genes and signature enrichment in the murine lymphomas compared with WT (Figures 5A and 5B). This was already suggested by their immunophenotype (CD4⁺CXCR5⁺ ICOS⁺PD-1^{high}, Figures 3A–3E). To further validate the Tfh characteristics, we confirmed the upregulated expression of CXCL13, IL-21, and Bcl-6, all Tfh regulators, in mPTCL isolated CD4⁺ T cells compared with WT CD4⁺ splenocytes (Figure 5C). In contrast, BLIMP, an antagonist regulator of Tfh cell differentiation, was not upregulated (Johnston et al., 2009).

Additionally, GSEA for a GC-plasma B cell signature (24 genes) (Recaldin and Fear, 2016) showed an upregulation of GC- and PC-enriched gene expression in mPTCLs (Figures 5D and 5E), indicating a mixed GC and PC B cell signature.

One of most prominent hPTCL with Tfh dominance associated with GC/plasma B cells is angioimmunoblastic T cell lymphoma (AITL), a rare devastating cancer (Federico et al., 2013). The described pathological and immunophenotypic characteristics of the plck-GAPDH mPTCL closely match human AITL (hAITL) (Table S1). Indeed, when we compared the GEP data of LNs from 60 AITL patients with healthy LNs, we found increased expression of CXCR5, PD-1, CXCL13, Bcl-6, ICOS, and IL-21, confirming the Tfh neoplastic expression pattern in the analyzed AITL patient cohorts (de Leval et al., 2007; Iqbal et al., 2014; Roth et al., 2006), similar to the plck-GAPDH mPTCLs (Figure S3A). The GC and PC B cell signature in the AITL patients (Figure S3B) also matched the plck-GAPDH mPTCL. Finally, an even more specific AITL gene signature was confirmed in the mPTLCs (Figure S3C).

To further validate the AITL pathology, we decided to compare the cytokine and chemokine mRNA expression profiles of plck-GAPDH mouse LNs (Figure S3E) with AITL patient LNs. AITL is characterized by strong autoimmune inflammatory signals (de Leval et al., 2007) such as increased IFN γ , IL-1 receptor α (IL1R α), IL-6, and TNF α (Figure S3F). We confirmed the mRNA expression data in Figure S3E by quantification of secreted cytokines and chemokines in PMA/ionomvcin-stimulated plck-GAPDH mPTCL cells (Figure S3G). Increased levels of inflammatory (IFN $_{\gamma},$ IL1R $_{\alpha},$ IL-6, and TNF $_{\alpha})$ and anti-inflammatory (IL-10 and IL-6) cytokines were comparable with an AITL gene-enriched inflammation signature (Figures S3F and S3G). In AITL, Tfh and GC B cell and microenvironment interaction is based on cytokine-chemokine crosstalks (Mourad et al., 2008). GEP of mPTCL versus WT splenocytes revealed an upregulated expression of multiple cytokines but also of chemokines corresponding to those of AITL patient LNs (Figures S3H–S3K). Moreover, our mPTCL clearly showed a prominent upregulation of humoral immune response genes (Table S2) confirming the presence of PCs in our tumors, and vascularization in the LNs was

confirmed by upregulation of vascularization-related genes (Table S2). All these data confirmed again the matching characteristic features between mPTCL and AITL (de Leval et al., 2007, 2010). Interestingly, 1-year old premalignant plck-GAPDH mice showed no prominent skewing in the T and B cell populations (Figures S4A–S4D) except for the fact that a population of Tfhlike CD4⁺ PD-1^{high} T cells and GC B cells were starting to emerge (Figures S4E and S4F). However, no increased cytokine secretion, TCR signaling, T cell activation, nor increased proliferation were detected in plck-GAPDH versus WT CD4⁺ T cells from 1-year-old mice (Figures S4G–S4J).

We confirmed that the glycolytic enzyme GAPDH was still strongly overexpressed in the mPTCLs at the mRNA and protein level compared with age-matched WT animals (Figure 5F). Strikingly, GAPDH expression was higher in AITL patient LNs than in healthy LNs (Figure 5G). Metabolic pathway analysis did not show pronounced upregulation of glycolysis in the plck-GAPDH lymphoma (Figure 5H). In contrast, CD4⁺ splenocytes from premalignant 1-year-old plck-GAPDH mice showed upregulated glycolysis compared with WT, especially when activated through the TCR as detected by increased extracellular acidification rate, glucose uptake, and lactate production (Figures S4K–S4M). In plck-GAPDH mPTCL, we detected increased mitochondrial respiration (Figure 5H), which was already observed in CD4⁺ T cells from 1-year-old plck-GAPDH upon TCR engagement (Figure S4N).

AITL frequently carries a G17V (p.Gly17Val) mutation in RHOA (Palomero et al., 2014; Sakata-Yanagimoto et al., 2014), which encodes the small guanosine triphosphatase (GTPase) RHOA. In plck-GAPDH CD4⁺ T cells isolated from tumors, we found two mutations in the effector domain of RHOA, one point mutation at position 37 (T37M) present in all 15 plck-GAPDH tumors and an INDEL frameshift mutation consisting of a 5-bp insertion into codon 44 present in 4 of 15 plck-GAPDH tumors (Figure 6A). Sanger and whole-exome sequencing of the WT C57BL6JOIaHsd strain used to generate the plck-GAPDH mice indicated that the T37M mutation and INDEL were not present in the germline (Figures S5A and S5B). The point mutation T37M reduced RHOA activity as measured by the reduced affinity for GST-rhotekin-RBD compared with the WT or the Q63L active mutant of RHOA (Figure 6B), identifying T37M as an inactivating mutation in RhoA. Additionally, the effect of the T37M mutation on RHOA was equivalent to the T37A mutation found in follicular lymphoma and gastric cancer (Figures 6B and 6C), and the activity of RHOA T37M was even lower than that for a well-established mutation (T19N) in the guanosine triphosphate/guanosine diphosphate binding domain (Figure 6B). The INDEL frameshift mutation in RHOA is a truncating mutation, which would likely result in a truncated protein (Figure S5C).

(C) Frequency of tumor localization in plck-GAPDH mice. Ectopic LN were localized at kidney, thymus, and intestines.

See also Figure S1.

6 Cancer Cell 36, 1-20, September 16, 2019

⁽D–F) Weight of spleens (D), mesenchymal LNs (MES LN) (E), and ectopic LNs (F) of WT mice and plck-GAPDH mice presenting tumors (means ±SD, n = 24; ***p < 0.001; **p < 0.001;

⁽G) Representative pictures of spleen, MES LN, and liver of WT and plck-GAPDH mice presenting tumors.

⁽H) Hematoxylin and eosin staining patterns and immunohistochemistry staining for CD3⁺ T cells and B220⁺ B cells of WT and plck-GAPDH spleens.

⁽l and J) Numbers of B (CD19⁺) and T (CD3⁺) cells (l) and the ratio of CD4⁺/CD8⁺ T cells (J) in the spleens of WT and plck-GAPDH tumor-bearing mice (means \pm SD, n = 6; ***p < 0.001, *p < 0.05, Student's t test).

CellPress



Figure 3. plck-GAPDH Mice Developed a T Cell Lymphoma with a Pronounced Tfh Phenotype

(A) Comparison of Tfh cell (CD4⁺CXCR5⁺ PD-1^{high}, CD4⁺CXCR5⁺Bcl-6⁺, and CD4⁺ICOS⁺) infiltration in the spleen of plck-GAPDH and WT mice analyzed by FACS. Representative of n = 10. Initial gating on CD4⁺ cells is performed.

(B) Percentages of CD4⁺ T cells that are PD-1^{high} CXCR5⁺PD-1^{high} T cells in WT and plck-GAPDH spleens (means \pm SD, n = 10; ***p < 0.001, Student's t test). (C) CD4⁺ T cell staining for CD62L and CD44 in plck-GAPDH tumors and WT spleens by FACS. Representative of n = 6.

(D) Expression of FAS receptor (CD95) and FAS ligand (CD178) on CD4 $^+$ T cells by FACS. Representative of n = 6.

(E) Intracellular staining of Ki67 in CD4 $^+$ splenocytes by FACS. Representative of n = 3.

(F) Intracellular marking of IFN_Y, IL-10, and TNF α in CD4⁺ splenocytes upon PMA/ionomycin stimulation in WT and plck-GAPDH mice. Representative of n = 4. (G) Expression of the different TCR- β chains on WT (WT 1, WT 2) and plck-GAPDH (GAPDH 1, 2, 3) CD4⁺ T cells analyzed by FACS.

(H) Detection of different TCR-γ chains by PCR in CD4* splenic cells isolated from WT (lanes 1–3) or plck-GAPDH lymphoma-bearing mice (lanes 4–8).



Figure 4. Germinal Center B Cells Are Strongly Increased in plck-GAPDH Tumors (A) GC B cells identified by FACS using staining for FAS (CD95) and GL-7 gated on CD19⁺B220⁺ B cells. Representative of n = 10.

(B) Percentage of GC B cells on CD19⁺B220⁺ cells in WT and plck-GAPDH spleens and MES LN (mean \pm SD; spleen, n = 38; LN, n = 20; ***p < 0.001, Student's t test).

8 Cancer Cell 36, 1-20, September 16, 2019

(legend continued on next page)

CellPress

Whole-exome sequencing revealed additional mutations in the plck-GAPDH lymphoma CD4⁺ cells (Tables S3–S6). Of note, however, many of the identified mutations in epigenetic modifiers and TCR signaling genes that were also mutated in hAITL were intronic or intergenic in our mPTCL and likely would not lead to loss of function (Tables S3 and S4).

The above data support our suggestion that mPTCL resembles in great detail hAITL, indicating that the plck-GAPDH mouse model represents a valid model for this lymphoma.

PIck-GAPDH Develop an AITL-like PTCL via a GAPDH-Dependent Activation of the NF-kB Pathway

Constitutive activation of NF- κ B signaling has been observed in various tumors including lymphomas and leukemia (Imbert and Peyron, 2017), and we have shown that GAPDH enhances B cell lymphoma aggressiveness via activation of NF- κ B (Chiche et al., 2015). Moreover, our GEP data showed that many NF- κ B targets are upregulated in our plck-GAPDH tumors such as IFN γ , ICOS, IL-6, CCL5, CCL2, CCL4, CXCL9, CXCL10, CXCL11, immunoglobulin G heavy chains, CD40, CD44, ICAM-1, and IRF4. We confirmed an upregulation of the NF- κ B canonical pathway target genes in thymocytes from 4-week-old plck-GAPDH mice (Figure S6A). Activation of the canonical pathway was established by phosphorylation of p65 (Figure S6B) and the binding of TRAF2 to GAPDH (Figure S6C).

GSEA for an NF- κ B gene signature (Gerondakis et al., 2014) showed upregulation of the NF- κ B canonical and non-canonical pathway as well as of their target genes in the plck-GAPDH mPTCL compared with WT splenocytes (Figures 7A and 7B), which was confirmed by qPCR analysis of isolated CD4⁺-cells from mPTCL (Figures 7C and 7D). Activation of the non-canonical pathway was confirmed by increased phosphorylation of p100 and its cleavage into p52 in CD4⁺ mPTCL cells as compared with WT splenocytes (Figure S6D).

We also detected in hAITL a slight upregulation of the canonical NF- κ B pathway (Figure 7E) and a strong activation of the non-canonical NF- κ B pathway (Figure 7F) characterized by increased RelB expression and strong upregulation of the noncanonical target genes EZH2 and IRF4, while other non-canonical targets, SP-1 and ENPP2, were not or slightly upregulated, equivalent to the mPTCL (Figures 7E and 7F).

Finally, to establish whether the modulation of NF- κ B pathway is a central event in mPTCL induction, we aged plck-p65^{-/-} mice, unable to activate the canonical NF- κ B pathway in T cells, and plck-I κ B^{-/-} mice, characterized by a constitutive activation of the NF- κ B pathway in the T cell compartment. At 18 months, the plck-I κ B^{-/-} mice developed a Tfh T cell lymphoma with CXCR5⁺PD-1⁺CD4⁺ T cells and CD95⁺GL-7⁺ GC B cells, an increased CD4⁺/CD8⁺ T cell ratio, and higher GAPDH, IL-21, CXCL13, and Bcl-6 expression levels (Figures 7G and 7H). Upregulation of the non-canonical NF-kB pathway was also detected in CD4⁺ cells from the plck-I κ B^{-/-} mPTCL (Figure S6E). All these features are identical to mPTCL developed by plck-GAPDH mice. Additionally, CD4⁺ splenocytes from 1-year-old premalignant plck-GAPDH mice had upregulated the canonical NF-kB pathway and initiated the upregulation of the non-canonical pathway (Figure S6F). To further confirm the role of GAPDH in NF- κ B activation, we generated plck-GAPDH/p65^{-/-}, in which the canonical NF-kB pathway is inactivated. Since the development of plck-GAPDH tumors can take over 2 years, we isolated splenic CD4⁺ cells from age-matched WT, plck-GAPDH, plckp65^{-/-}, and plck-GAPDH/p65^{-/-} mice and cultured them under Tfh-inducing conditions. Only the plck-GAPDH-derived CD4⁺ cells expressed Tfh markers at 3 weeks of culture (Figure S6G), accompanied by upregulation of the canonical and non-canonical NF- κB pathway (Figure S6H), while plck-GAPDH/p65 $^{-\!/-}$ CD4⁺ T cells were equivalent to WT. This underlined the importance of induction of the NF-kB pathway by GAPDH in accelerated Tfh development.

Finally, we evaluated whether GAPDH is still required for mPTCL growth once established. Ten NSG mice were engrafted with plck-GAPDH lymphoma and were treated upon tumor establishment with a GAPDH-specific inhibitor, koningic acid (KA), or vehicle (Figure S6I). No difference in survival of the two groups of mice was detected (Figure S6J). Despite a reduction in GAPDH activity in the KA-treated mice (Figure S6K), both groups developed splenomegaly (Figure S6L), and the KA-treated group did not show a change in B, T, or Tfh CD4+PD-1^{high} cells nor in NF- κ B activity (Figures S6M–S6P). This suggests that while GAPDH can drive mPTCL development, it is not required to maintain mPTCL tumor growth once it is established.

This indicates that GAPDH-induced constitutive activation of the NF- κ B pathway in T cells results in a dominant Tfh PTCL equivalent to AITL, underlined by a shift toward the NF- κ B non-canonical pathway in the tumor cells.

Plck-GAPDH Mice Developing AITL Respond to Noncanonical NF-kB Inhibition and Anti-PD-1 Treatment

Activation of the non-canonical pathway depends on the activation of the NF- κ B-inducing kinase (NIK) and IKK α phosphorylation (Xiao et al., 2001). We confirmed a 9-fold and 3-fold increased NIK expression in the mPTCL CD4⁺ T cells and CD19⁺ GC B cells compared with WT counterparts, respectively (Figure 8A), suggesting that NIK could represent a therapeutic target in AITL. PD-1 and its ligand PD-L1, which play a central role in downmodulation of anti-tumor immunity (Topalian et al., 2016), might also represent AITL therapeutic targets. High PD-1 expression was characteristic for CD4⁺ Tfh cells in our plck-GAPDH AITL-like tumors (Figures 3A and 3B). Therefore,

⁽C) Representative FACS plot showing the expression levels (MFI) of FAS (CD95⁺) and FAS ligand (CD178⁺) in WT versus plck-GAPDH B cells (CD19⁺B220⁺). Representative of n = 5.

⁽D) FACS histogram showing the quantity of plasmocytes (CD19-B220 low, CD138⁺) per total nucleated cells in the spleen.

⁽E) Summary of the percentage of plasma B cells from (D) (mean \pm SD, n = 22; **p < 0.01, Student's t test).

⁽F and G) Total splenocytes from tumor-bearing plck-GAPDH mice were transferred to NSG mice, which were sacrificed 6–8 weeks post injection. PD-1⁺ CD4⁺ T cells and GC B cells (CD95⁺GL-7⁺) in donor plck-GAPDH mice and in NSG mice after engraftment were analyzed by FACS (F), and the percentages of CD4⁺ T cells that were PD-1⁺ and B cells that were CD95⁺GL-7⁺ were quantified for three independent tumors (G).



Figure 5. PIck-GAPDH mPTCL Transcriptional Profile Corresponds to that of AITL Patients

(A) mRNA expression (log₂) of Tfh signature genes based on gene-expression analysis (GEA) of RNA-sequencing (RNA-seq) data in total splenocytes of lymphoma developing plck-GAPDH relative to WT splenocytes.

(legend continued on next page)

10 Cancer Cell 36, 1–20, September 16, 2019

CellPress

we opted to investigate treatments with an NIK inhibitor alone or in combination with anti-PD-1 in our AITL model.

Due to a lack of specific NIK inhibitors, we developed $\ensuremath{\mathsf{NIK}^{\mathsf{inh}}}\xspace$, a substituted halo-quinoline derivative characterized by potent anti-NIK activity (patent PCT/EP2017/067306; Figure S7A). NIK^{inh} was able to inhibit NIK's ability to phosphorylate a substrate protein in vitro in an ATP-dependent manner (ADP-Glo assay; Figure S7A), demonstrating inhibition of NIK ATPase activity. Using a biotinylated version of the drug (NIK^{inh}-biotin) compared with biotin alone or mock conjugated biotin (phenylbiotin and aminoquinolinyl-biotin), we confirmed that NIK^{inh}biotin was able to affinity precipitate NIK (Figure S7B). Increasing the ratio of free NIK^{inh} and NIK^{inh}-biotin (1:1 and 10:1) reduced the amount of NIK precipitated (Figure S7B), which indicates that the observed interaction between the NIK^{inh} and NIK is specific. $\ensuremath{\mathsf{NIK}}^{\ensuremath{\mathsf{inh}}}$ also reduced EZH2 expression, a downstream target of NIK, in a dose-dependent manner in a melanoma cell line characterized by upregulation of non-canonical pathway (A375), and concomitantly increased the expression of CDKN1A/p21 (Figure S7C). Additionally, we showed that this NIK^{inh} decreased cleavage of p100 into p52, and EZH2 at the protein level in A375 cells, lung cancer cells (A549), and freshly isolated patient melanoma cells (C12.06) (Figure S7D). We then compared the effects of our NIK^{inh} with those of NF- κ B2-siRNA on the proliferation of the A375, C12.06, and A549 cancer cells. The treatments had similar inhibitory effects on the proliferation of the cancer cells and no effect on normal human melanocytes or fibroblasts (Figure S7E), emphasizing that the inhibitor is specific for cancer cells that have the non-canonical NF-KB pathway upregulated. Moreover, we have also confirmed these data in three different diffuse large B cell lymphoma cell lines (U2932, Karpas 422, and SUDHL-6; Figure S7F).

We next evaluated treatment with this NIK^{inh} alone or in combination with an anti-PD-1 *in vivo* in our AITL transplant model. Both NIK^{inh} single treatment and combined with anti-PD-1 resulted in significantly increased survival of up to 60% and 70% of the plck-GAPDH tumor-engrafted mice, respectively, compared with control-treated mice (Figure 8B). At sacrifice, we detected a significant decrease in spleen size for the singleand double-treated mice compared with controls (Figure S8A). For both treatment conditions, we confirmed a decrease in B cells in the spleen while a reduction in T cells was more prominent for the NIK^{inh} single treatment alone than in the combination treatment group (Figures 8C and 8D). CD4⁺ T cells were depleted completely for the Tfh PD-1^{high} population in the combination treatment group, while a residual subpopulation of PD-1^{high}CD4⁺ cells was detected in mice treated with NIK^{inh} only (Figure 8E). The CD4⁺ clonality for TCR-β7 detected in the initial tumor was not detected as dominant in the NIK^{inh} + anti-PD-1-treated mice, but TCR- β 7 dominance was still present in residual CD4⁺ cells for the single NIK^{inh} treatment (Figure S8B). Additionally, expression of non-canonical NF-kB pathway components and targets was reduced in residual CD4⁺ T cells following combined NIK^{inh} and anti-PD-1 treatment, demonstrating the efficacy of NIK^{inh} to inhibit the non-canonical NF- κB pathway in vivo (Figure S8C). In the double-treated group, anti-PD-1-mediated induction of immune response was confirmed by increased production of IFN γ , granzyme B, and perforin by cytotoxic CD8⁺ cells, while the NIK^{inh} alone was capable of increasing IFN γ release but not granzyme B and perforin (Figure 8F). Of note, PD-1 was highly expressed in CD4⁺ but not CD8⁺ T cells in the primary mPTCL, while CD4 Tfh, CD8 T cells, and GC B cells expressed PD-L1 at high levels (Figure S8D), suggesting that anti-PD-1 treatment may not only disrupt T-T cell interactions but also T-B cell interactions, known as drivers of T and B cell proliferation in PTCL. Therefore, we evaluated anti-PD-1 treatment alone. Anti-PD-1-treated plck-GAPDH mPTCL-engrafted mice had significantly increased survival compared with control-treated animals, and two mice survived long term upon continuous treatment (Figure S8E). A strong depletion of CD4⁺ T cells was detected in the spleen (Figure S8F), while CD8⁺ cells were unaffected (data not shown), resulting in a reduced CD4⁺/CD8⁺ ratio (Figure S8G). In contrast, anti-PD-1 treatment increased the percentage of B cells in the spleen but decreased the percentage of GC B cells compared with control treatment (Figures S8H and S8I). As detected for the combination treatment (NIK^{inh} + anti-PD-1), anti-PD-1 treatment stimulated an immune response, confirmed by increased production of IFN_Y, granzyme B, and perforin by cytotoxic CD8⁺ T cells (Figure S8J).

To further emphasize the relevance of NIK inhibition in hAITL, we isolated CD4⁺ T cells from human AITL tumor samples. As detected in our mAITL tumors, the hAITL CD4⁺ T cells showed a Tfh phenotype (PD-1^{high}, ICOS⁺, CXCR5⁺) and B cells demonstrated a GC B cell (GL7⁺) phenotype (data not shown). Both cell types showed strong upregulation of NIK (up to 8-fold) in most cases compared with their healthy counterparts (Figures 8G and 8H). We clearly demonstrated that NIK^{inh} treatment *ex vivo* reduced the growth of CD4⁺ Tfh cells from hAITL biopsies

See also Figures S3 and S4; Tables S1 and S2.

⁽B) GSEA for 18 Tfh-related genes indicated in (A). For all genes with enrichment score >0 (black bars in the pink zone), expression is upregulated. Kolmogorov-Smirnov (KS) test.

⁽C) mRNA expression levels by qPCR analysis of CXCL13, IL-21, BcI-6, and BLIMP in CD4⁺ T splenocytes isolated from plck-GAPDH mPTLC and WT mice. Expression for WT was set to 1 (mean ± SD, n = 3; *p < 0.05, **p < 0.01, Student's t test).

⁽D) mRNA expression levels (log₂) of GC/plasma B cell signature genes based on the GEA of RNA-seq data in plck-GAPDH relative to WT splenocytes.

⁽E) GSEA for GC/plasma B cell related genes presented in (D). For all genes with enrichment score >0 (black bars in the pink zone) expression is upregulated. KS test.

⁽F) mRNA expression level (RNA-seq data) of GAPDH (log₂) in CD4⁺ T splenocytes isolated from plck-GAPDH mPTLC compared with WT splenocytes (box plots represent the 25th and 75th percentiles with midlines indicating the median values; whiskers extend to the maximum and minimum values of the data samples) (**p < 0.01, Student's t test), and GAPDH intracellular staining in CD4⁺ mPTCL and healthy splenocytes analyzed by FACS.

⁽G) mRNA expression levels (RNA-seq data) of GAPDH in LN from AITL and healthy donors (box plots represent the 25th and 75th percentiles with midlines indicating the median values; whiskers extend to the maximum and minimum values of the data samples; ***p < 0.001, Student's t test).

⁽H) Metabolic pathway analysis from RNA-seq data of the plck-GAPDH lymphoma versus WT splenocytes was performed using the KEGG database. *p < 0.05; ns. not significant.



Figure 6. RHOA Mutations and Other Mutations Identified in plck-GAPDH Tfh mPTCL

(A) Sanger sequencing of isolated mPTCL lymphoma CD4⁺ cells identified a *RHOA* single bp mutation (T37M) and INDEL (frameshift insertion) mutation. The T37M mutation was detected in 15 out of 15 different plck-GAPDH mice and the INDEL in 4 out of 15 plck-GAPDH mice.

(B) Active RHOA (Rho-GTP) was compared using a GST-rhotekin RBD pull-down assay using lysates of HEK293T transfected with WT and different RHOAinactivating mutants (T37M, T37A, T19N) or an RHOA-activating mutant (Q36L).

(C) *RHOA* mutations in patients according to published data for AITL (Vallois et al., 2016), PTCL (Palomero et al., 2014), adult T cell leukemia/lymphoma (ATL [Nagata et al., 2016]), follicular lymphoma (Zehir et al., 2017), Burkitt lymphoma (Rohde et al., 2014) and gastric carcinoma (Kakiuchi et al., 2014). Each symbol represents a patient for the indicated mutation and the number of symbols represent the frequency of that mutation found per total number of patients (n) analyzed. See also Figure S5 and Tables S3–S6.

12 Cancer Cell 36, 1-20, September 16, 2019

CellPress



Figure 7. Pick-GAPDH AITL Tumors Show a Strong Activation of the Non-canonical NF-*κ***B Pathway in Tfh Cells** (A) Heatmap for GEA data of canonical (blue) and non-canonical (green) NF-*κ*B pathway and target genes comparing total splenocytes from WT and lymphoma cells from pick-GAPDH mice.

> (legend continued on next page) Cancer Cell 36, 1–20, September 16, 2019 13

upon TCR stimulation, whereas healthy CD4⁺ T cells remained unaffected (Figure 8I). The response of each donor sample correlated with the level of NIK expression over healthy CD4⁺ T cells detected, as the hAITL sample expressing low levels of NIK was not affected by the inhibitor (AITL-1 in Figures 8G and 8I). Moreover, the GC B cells in the hAITL samples showed decreased growth upon NIK^{inh} treatment, in agreement with their increased NIK expression (Figures 8H and 8J).

These results support the proposal that drugs targeting the non-canonical NF- κ B pathway represent a potential treatment option for AITL.

DISCUSSION

By overexpressing GAPDH into the T cell lineage, we established here a preclinical plck-GAPDH mouse model of hAITL, a rare T cell lymphoma. AITL is highly resistant to conventional chemotherapy, and prognosis is usually poor with a survival rate of only 30% at 5 years (de Leval et al., 2010; Federico et al., 2013). AITL pathology develops at a later age and is characterized by splenomegaly, hepatomegaly, and enlarged LNs. Moreover, a large number of patients develop a rash and ascites in the abdomen (de Leval et al., 2010). All these features were detected in our mPTCL. Clonal Tfh cell tumor infiltration is one of the hallmarks of hAITL, and Tfh are possibly at the origin of this disease (Crotty, 2014; de Leval et al., 2007; Dupuis et al., 2006; Grogg et al., 2006). However, this T cell component represents only a fraction of the tumor mass and is always accompanied by GC B and/or plasma B cells and FDCs (Gaulard and de Leval, 2014; Mourad et al., 2008). Thus, hAITL is a complex malignancy, in which the tumor microenvironment is a key element. Tfh cells associate with B cells in the GC, where they sustain GC B cell survival and T-B cell connection through ligand-receptor interactions (PD-1-PD-L1, ICOS-ICOSL, FAS-FASL). Remarkably, a cytokine-chemokine secretion pattern was revealed in hAITL (Heissmeyer and Vogel, 2013; Stone et al., 2015; Weber et al., 2015a) and confirmed in our mPTCL. Moreover, our mPTCL manifests autoimmunity-like symptoms consistent with hAITL (de Leval et al., 2007). Most strikingly, GAPDH was increased in AITL patient LNs, stressing the equivalence of our model with AITL. How GAPDH is modulated in such a lymphoma still needs to be unraveled. GAPDH overexpression strongly induced the canonical NF-κB pathway in the plck-GAPDH thymocytes. This might lead to aberrant T cell development, since NF-kB is implicated in thymocyte development (Gerondakis et al., 2014; Xing et al., 2016). Moreover, constitutive canonical NF-κB activation creates an inflammatory context (Hoesel and Schmid, 2013), which might be prosperous for induction of genetic mutations.

In 50%–70% of AITL patients, the Ras homolog gene family member A (*RHOA*) bears a G17V mutation (Palomero et al., 2014; Sakata-Yanagimoto et al., 2014). This results in the loss of function of the RHOA GTPase and is often associated with TET2 loss-of-function mutations in hAITL (Palomero et al., 2014; Yoo et al., 2014). Genetic mouse models have been proposed to mimic hAITL, such as the TET2-KD mouse (Muto et al., 2014) and the SJL mouse driving T cell lymphoma due to elevated IL-21 levels (Jain et al., 2015). However, both of the models only recapitulate part of hAITL. Recently, two double $TET2^{-/-}$ RHOA^{G17V} mouse models were generated, recapitulating AITL disease more closely (Cortes et al., 2018; Ng et al., 2018). Strikingly, we also revealed *RHOA* mutations in our murine plck-GAPDH AITL disease model.

The connection between GAPDH overexpression and NF- κ B activation was revealed previously by us (Chiche et al., 2015). NF- κ B deregulation is found in many hematological malignancies (Imbert and Peyron, 2017) and other cancers (De Donatis et al., 2016; Fradet et al., 2004; Karin et al., 2002; Madonna et al., 2012). Importantly, increased NF- κ B activation may induce tumor immune escape and chemotherapy resistance (Burstein and Duckett, 2003; Fujioka et al., 2012). Therefore, NF- κ B is currently considered an ideal target for cancer therapy, and various NF- κ B inhibitors targeting different pathway components are under development (Shen and Tergaonkar, 2009).

The constitutive canonical NF- κ B activation might have contributed to the activation of the non-canonical pathway in premalignant and malignant plck-GAPDH T cells. This was previously described in LN formation, underlining the interdependency of these two NF- κ B pathways (Alcamo et al., 2002; Lo et al., 2006; Shih et al., 2011). Genetically manipulating the NF- κ B pathway in mice showed the interdependence of the canonical and non-canonical pathways and the importance of the initial induction of the canonical NF- κ B pathway by GAPDH followed by the non-canonical pathway in accelerated Tfh development.

EZH2 is increasingly expressed during the progression of hematopoietic and solid cancers (Bachmann et al., 2006; Chen et al., 2018; Karakashev et al., 2018; Patnaik et al., 2018; Sudo et al., 2005), and overexpression of mutant EZH2 has been recognized in a wide selection of B and T cell lymphoproliferative disorders (Morin et al., 2010; Raaphorst et al., 2000; Sasaki et al., 2011; Sneeringer et al., 2010; Visser et al., 2001). Therefore, considerable effort has been directed toward development of EZH2 inhibitors, but current specific EZH2 inhibitors can

See also Figure S6.

14 Cancer Cell 36, 1-20, September 16, 2019

⁽B) GSEA for the NF-kB related genes in (A). For all genes with enrichment score >0 (black bars in the pink zone), expression is upregulated. KS test.

⁽C and D) mRNA expression levels in CD4⁺ T cells of plck-GAPDH versus WT mice for the canonical pathway genes (NF- κ B1, RelA) and target genes (NF- κ B1, GADD45) (C) and non-canonical pathway genes (NF- κ B2, RelB) and target genes (EZH2, SP-1, IRF4, ENPP2) (D) (mean ± SD, n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test).

⁽E and F) Heatmaps of transcriptomic data comparing healthy (n = 8) and hAITL (n = 52) LNs for their NF- κ B canonical pathway genes (NF- κ B1, ReIA) and target genes (NF- κ B1, GADD45) (E) and non-canonical pathway genes (NF- κ B2, ReIB) and target genes (EZH2, SP-1, IRF4, ENPP2) (F) (healthy, n = 8; AITL, n = 60).

⁽G) plck-lkB^{-/-}, plck-p65^{-/-}, and WT mice were sacrificed at 18 months and splenocytes were analyzed by FACS for Tfh markers (PD-1⁺CXCR5⁺ gated on CD4⁺), GC B markers (CD95⁺, GL-7⁺ gated on CD4⁺), CD4⁺/CD8⁺ ratio (gated on CD3⁺), and GAPDH expression.

⁽H) mRNA expression levels of Tfh signature genes by qPCR in CD4⁺ T splenocytes isolated from plck-l κ B^{-/-} or WT mice. Expression of WT was set to 1 (mean ± SD, n = 3; *p < 0.05, **p < 0.01, Student's t test).

CellPress



Figure 8. Combining NIK Inhibition and Anti-PD-1 Prolongs Survival of the AITL Mouse Model

(A) mRNA expression levels by qPCR of NIK in CD4⁺ T and CD19⁺ B cells from lymphoma developing plck-GAPDH mice compared with cells from WT spleens, set to 1 (mean ± SD, n = 3; **p < 0.01, ***p < 0.001, Student's t test).

(legend continued on next page) Cancer Cell 36, 1–20, September 16, 2019 15

result in either pro-oncogenic or tumor-suppressive effects (Lund et al., 2014). EZH2 inhibitors are catalytic inhibitors that only act on the enzymatic activity of EZH2. By acting on NIK, we can decrease EZH2 at the transcriptional level. Thus, NIK inhibitors would inhibit the catalytic and non-catalytic effect of EZH2. Indeed, EZH2 inhibits IFN γ production at the transcriptional level (Wee et al., 2014), which is essential for the anti-PD-1 response. Moreover, the non-canonical pathway has pro-oncogenic effects by itself, and targeting NIK will decrease EZH2 but also the non-canonical NF- κ B pathway.

We demonstrated that the non-canonical pathway is highly activated in hAITL, equivalent to our mAITL model. Of note, a major component in our murine and human AITL are GC B cells, which also have the non-canonical NF- κ B pathway constitutively activated (Gardam et al., 2008; Sun, 2017; Xie et al., 2007). Of importance, NIK is required for maintaining high expression of ICOSL on B cells, important for Tfh generation (Hu et al., 2011). We confirmed overexpression of NIK in hAITL Tfh cells and GC B cells compared with their healthy counterparts, and the NIK^{inh} strongly increased survival of our mAITL preclinical model. Altogether, this argues strongly for interference with the non-canonical NF- κ B pathway as a treatment for AITL-like disease.

PD-1 is a checkpoint receptor that upon engagement by PD-L1 dampens T cell effector functions and protects cancer cells from immune-mediated attack (Kleffel et al., 2015; Topalian et al., 2012). Thus, antibodies targeting the interaction between PD-1 and PD-L1 have been developed and are currently used clinically in treating cancer patients (Postow et al., 2015; Topalian et al., 2016; Weber et al., 2015b). Anti-PD-1 treatment led to a decrease in CD4⁺ T cells in engrafted plck-GAPDH tumors and boosted CD8⁺ T cell activity. As for melanoma, the Tfh cancer cells might also be directly targeted by anti-PD-1 by a cellintrinsic mechanism (Kleffel et al., 2015).

Treatment with NIK^{inh} induced IFN_Y secretion, which might augment the response to the anti-PD-1 immunotherapy (Fu et al., 2014; Guo et al., 2015). Moreover, IFN_Y derived from cytotoxic lymphocytes enhances their motility and cytotoxicity (Bhat et al., 2017). However, combining NIK^{inh} with anti-PD-1 treatment did not further increase survival compared with NIK^{inh} alone. The double treatment did result in CD4⁺ T cells, which were devoid of PD-1 expression, in contrast to the residual CD4⁺ T cell upon NIK^{inh} alone, which still expressed PD-1.

Importantly, NIK^{inh} treatment inhibited growth of hAITL CD4⁺ Tfh and GC B cells *ex vivo*, further emphasizing the role of the non-canonical NF- κ B pathway in hAITL and the relevance of NIK inhibition for this disease that is in need of better optimized therapies.

In summary, we have established that plck-GAPDH mice develop AITL, recapitulating the pathology, the transcriptional profile and genetic and immune-phenotypic features of hAITL. This mAITL model will help to unravel the possible origin of the disease and pathways implicated and will permit us to test therapeutic options. Here, we provide evidence for the potential utility of immunotherapies and small-molecule inhibitors of NIK, and combinations thereof, for treating AITL.

STAR*METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 O Mice
 - Cells
- METHOD DETAILS
 - Western Blot Analysis
 - Immunoprecipitation
 - Flow Cytometry and Antibodies
 - Isolation of Primary Mouse T Cells
 - Detection of TCR-Signaling
 - Immunohistochemistry Analysis and Antibodies
 - Detection of T Cell Clonality
 - Cytokine-Chemokine Array
 - Quantification of Cytokines in Mouse Serum
 - Transcriptomic Analysis
 - PCR Amplification and Sanger Sequencing
 - O Determination of GTP Levels Loaded on RHOA
 - In Vitro Tfh Differentiation
 - Germinal Center Induction In Vivo
 - Reverse Transcriptase Quantitative- PCR (RT-qPCR) Analysis
 - Genomic DNA Isolation
 - Whole Exome Sequencing
 - Lactate and Glucose Measurement
 - Metabolic Assavs
 - 2-NBDG Uptake
 - O IgG Determination in Mouse Serum

16 Cancer Cell 36, 1-20, September 16, 2019

⁽B) Splenic lymphoma cells from plck-GAPDH mice were injected intravenously into recipient NSG mice (n = 18), which were treated with vehicle (n = 5), an NIK inhibitor (NIK^{inh}; n = 6), and NIK^{inh} + anti-PD-1 (n = 7). Survival curves for mice are shown. Mice were sacrificed at 90 days post transplant (**p < 0.01, *p < 0.05, Mantel-Cox test).

⁽C) Percentage of B cells (CD19⁺) and T cells (CD3⁺) in the spleen per total mononuclear cells for the indicated treatment groups at sacrifice (mean ± SD, n = 4; ***p < 0.001, **p < 0.05, one-way ANOVA).

⁽D and E) Ratio of CD4⁺/CD8⁺ T cells (D) and percentage of PD-1^{high} CD4⁺ T cells (E) in the spleen for the indicated treatment groups at sacrifice (mean ± SD, n = 4; ***p < 0.001, **p < 0.001, one-way ANOVA).

⁽F) Splenocytes isolated from NIK^{inh}-, NIK^{inh} + anti-PD-1-, and control-treated NSG mice at sacrifice were activated for 5 h with PMA/ionomycin, then surfacestained for CD8 followed by intracellular staining for IFN_Y, perforin, and granzyme B (mean \pm SD, n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA). (G and H) Fold-change mRNA expression levels by qPCR analysis of NIK in human CD4⁺ T cells (G) and GC B cells (H) isolated from LN biopsies from five different AITL patients and in healthy human counterparts, for which expression level was set to 1 (mean \pm SD, analysis was performed in triplicate for each AITL sample). (I and J) Effect of NIK^{inh} (10 μ M, 48 h) on the cell number of CD4⁺ T cells (I) and GC B cells (J) of LN biopsies from five different AITL patients upon TCR stimulation compared with TCR-stimulated healthy CD4⁺ human T cells and healthy B cells. Cell numbers are normalized to the corresponding DMSO-treated control cells. See also Figures S7 and S8.

CellPress

- Measurement of GAPDH Activity
- Cell Proliferation Detection
- Anti-nuclear Antibody Detection
- ADP-Glo Experiments
- O Biotin Conjugated Drug Affinity Precipitation
- Comparison of NF-kB and NIK Knock-down by siRNA Treatment and NIK Inhibitor Treatment
- DATA AND CODE AVAILABILITY
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j. ccell.2019.07.008.

ACKNOWLEDGMENTS

We acknowledge animal and confocal facilities at the C3M (INSERM U1065, Nice, France). This work was supported by the 'Fondation ARC pour la Recherche sur le Cancer' and the 'Agence Nationale de la Recherche' (Labex SIGNALIFE ANR-11-LABX-0028-01), the Canceropôle PACA, Institut National Du Cancer (INCA) and Conseil Régional PACA, and the French Ministry of Research. L.M. was supported by 'la Fondation pour la Recherche Médicale (FRM)' and 'Aides individuelles aux jeunes chercheur ville de Nice'. R.M. was supported by a PhD fellowship from the French Ministry of Research. RNA-seq experiments were performed at the UCAGenomiX (IPMC), Sophia-Antipolis, Nice, France. Sequencing at UCAGenomiX (IPMC), a partner of the National Infrastructure France Génomique, was supported by Commissariat aux Grands Investissements (ANR-10-INBS-09-03, ANR-10-INBS-09-02) and Canceropôle PACA. We thank the platform of Experimental Histopathology at INSERM/UPS US006 CREFRE, Toulouse Purpan, France. For bioinformatics analysis we thank Nicolas Nottet (C3M, INSERM U1065, Nice, France). For cloning we thank Patrick Munro. hAITL cells were obtained from the INCA labeled Tumorotek of Necker-Enfants Malades Hospital, Paris, France.

AUTHOR CONTRIBUTIONS

E. Verhoeyen coordinated the project, designed and performed experiments, analyzed and discussed the data, and wrote the manuscript; J.-E.R. designed experiments and wrote the manuscript; L.M., R.M., and G.M.D.D. designed and performed experiments, analyzed and discussed the data, and wrote the manuscript; M.T., C.P., L.B., A.D., F. Luciano, J.G.N., L.S., N.F., F.C., J.C., M.J., V.I., E.P., C.R.-P., J.P.B., F.M., S.M., and E. Villa performed experiments, analyzed the data, and discussed results. V.A., P.G., and F. Lemonnier provided AITL patient cells and discussed results; J.-F.P., L.G., R.B., J.-J.F., and T.P. provided critical discussions and reading of the manuscript.

DECLARATION OF INTERESTS

NIK inhibitor compounds are under licensing by YUKIN therapeutics. R.B. and T.P. are co-founders of YUKIN therapeutics. The remaining authors declare no competing interests.

Received: May 1, 2018 Revised: April 17, 2019 Accepted: July 26, 2019 Published: August 22, 2019

REFERENCES

Ahsanuddin, A.N., Brynes, R.K., and Li, S. (2011). Peripheral blood polyclonal plasmacytosis mimicking plasma cell leukemia in patients with angioimmunoblastic T-cell lymphoma: report of 3 cases and review of the literature. Int. J. Clin. Exp. Pathol. *4*, 416–420. Alcamo, E., Hacohen, N., Schulte, L.C., Rennert, P.D., Hynes, R.O., and Baltimore, D. (2002). Requirement for the NF-kappaB family member RelA in the development of secondary lymphoid organs. J. Exp. Med. *195*, 233–244.

Algul, H., Treiber, M., Lesina, M., Nakhai, H., Saur, D., Geisler, F., Pfeifer, A., Paxian, S., and Schmid, R.M. (2007). Pancreas-specific RelA/p65 truncation increases susceptibility of acini to inflammation-associated cell death following cerulein pancreatitis. J. Clin. Invest. *117*, 1490–1501.

Bachmann, I.M., Halvorsen, O.J., Collett, K., Stefansson, I.M., Straume, O., Haukaas, S.A., Salvesen, H.B., Otte, A.P., and Akslen, L.A. (2006). EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J. Clin. Oncol. *24*, 268–273.

Balmer, M.L., Ma, E.H., Bantug, G.R., Grahlert, J., Pfister, S., Glatter, T., Jauch, A., Dimeloe, S., Slack, E., Dehio, P., et al. (2016). Memory CD8(+) T cells require increased concentrations of acetate induced by stress for optimal function. Immunity 44, 1312–1324.

Bhat, P., Leggatt, G., Waterhouse, N., and Frazer, I.H. (2017). Interferongamma derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. Cell Death Dis. *8*, e2836.

Burstein, E., and Duckett, C.S. (2003). Dying for NF-kappaB? Control of cell death by transcriptional regulation of the apoptotic machinery. Curr. Opin. Cell Biol. *15*, 732–737.

Chaffin, K.E., Beals, C.R., Wilkie, T.M., Forbush, K.A., Simon, M.I., and Perlmutter, R.M. (1990). Dissection of thymocyte signaling pathways by in vivo expression of pertussis toxin ADP-ribosvltransferase. EMBO J. 9. 3821–3829.

Chang, C.H., Curtis, J.D., Maggi, L.B., Jr., Faubert, B., Villarino, A.V., O'Sullivan, D., Huang, S.C., van der Windt, G.J., Blagih, J., Qiu, J., et al. (2013). Posttranscriptional control of T cell effector function by aerobic glycolysis. Cell *153*, 1239–1251.

Chen, Z., Yang, P., Li, W., He, F., Wei, J., Zhang, T., Zhong, J., Chen, H., and Cao, J. (2018). Expression of EZH2 is associated with poor outcome in colorectal cancer. Oncol. Lett. *15*, 2953–2961.

Chiche, J., Pommier, S., Beneteau, M., Mondragon, L., Meynet, O., Zunino, B., Mouchotte, A., Verhoeyen, E., Guyot, M., Pages, G., et al. (2015). GAPDH enhances the aggressiveness and the vascularization of non-Hodgkin's B lymphomas via NF-kappaB-dependent induction of HIF-1alpha. Leukemia *29*, 1163–1176.

Cildir, G., Low, K.C., and Tergaonkar, V. (2016). Noncanonical NF-kappaB signaling in health and disease. Trends Mol. Med. 22, 414–429.

Colell, A., Green, D.R., and Ricci, J.E. (2009). Novel roles for GAPDH in cell death and carcinogenesis. Cell Death Differ. *16*, 1573–1581.

Colell, A., Ricci, J.E., Tait, S., Milasta, S., Maurer, U., Bouchier-Hayes, L., Fitzgerald, P., Guio-Carrion, A., Waterhouse, N.J., Li, C.W., et al. (2007). GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. Cell *129*, 983–997.

Cortes, J.R., Ambesi-Impiombato, A., Couronne, L., Quinn, S.A., Kim, C.S., da Silva Almeida, A.C., West, Z., Belver, L., Martin, M.S., Scourzic, L., et al. (2018). RHOA G17V induces T follicular helper cell specification and promotes lymphomagenesis. Cancer Cell *33*, 259–273.e7.

Crotty, S. (2014). T follicular helper cell differentiation, function, and roles in disease. Immunity 41, 529–542.

De Donatis, G.M., Le Pape, E., Pierron, A., Cheli, Y., Hofman, V., Hofman, P., Allegra, M., Zahaf, K., Bahadoran, P., Rocchi, S., et al. (2016). NF-kB2 induces senescence bypass in melanoma via a direct transcriptional activation of EZH2. Oncogene 35, 2735–2745.

de Leval, L., Gisselbrecht, C., and Gaulard, P. (2010). Advances in the understanding and management of angioimmunoblastic T-cell lymphoma. Br. J. Haematol. *148*, 673–689.

de Leval, L., Rickman, D.S., Thielen, C., Reynies, A., Huang, Y.L., Delsol, G., Lamant, L., Leroy, K., Briere, J., Molina, T., et al. (2007). The gene expression profile of nodal peripheral T-cell lymphoma demonstrates a molecular link between angioimmunoblastic T-cell lymphoma (AITL) and follicular helper T (TFH) cells. Blood *109*, 4952–4963.

Cancer Cell 36, 1–20, September 16, 2019 17

DePristo, M.A., Banks, E., Poplin, R., Garimella, K.V., Maguire, J.R., Hartl, C., Philippakis, A.A., del Angel, G., Rivas, M.A., Hanna, M., et al. (2011). A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat. Genet. *43*, 491–498.

Dimeloe, S., Mehling, M., Frick, C., Loeliger, J., Bantug, G.R., Sauder, U., Fischer, M., Belle, R., Develioglu, L., Tay, S., et al. (2016). The immune-metabolic basis of effector memory CD4⁺ T cell function under hypoxic conditions. J. Immunol. *196*, 106–114.

Dupuis, J., Boye, K., Martin, N., Copie-Bergman, C., Plonquet, A., Fabiani, B., Baglin, A.C., Haioun, C., Delfau-Larue, M.H., and Gaulard, P. (2006). Expression of CXCL13 by neoplastic cells in angioimmunoblastic T-cell lymphoma (AITL): a new diagnostic marker providing evidence that AITL derives from follicular helper T cells. Am. J. Surg. Pathol. *30*, 490–494.

Federico, M., Rudiger, T., Bellei, M., Nathwani, B.N., Luminari, S., Coiffier, B., Harris, N.L., Jaffe, E.S., Pileri, S.A., Savage, K.J., et al. (2013). Clinicopathologic characteristics of angioimmunoblastic T-cell lymphoma: analysis of the international peripheral T-cell lymphoma project. J. Clin. Oncol. *31*, 240–246.

Fradet, V., Lessard, L., Begin, L.R., Karakiewicz, P., Masson, A.M., and Saad, F. (2004). Nuclear factor-kappaB nuclear localization is predictive of biochemical recurrence in patients with positive margin prostate cancer. Clin. Cancer Res. *10*, 8460–8464.

Fu, J., Malm, I.J., Kadayakkara, D.K., Levitsky, H., Pardoll, D., and Kim, Y.J. (2014). Preclinical evidence that PD1 blockade cooperates with cancer vaccine TEGVAX to elicit regression of established tumors. Cancer Res. 74, 4042–4052.

Fujioka, S., Son, K., Onda, S., Schmidt, C., Scrabas, G.M., Okamoto, T., Fujita, T., Chiao, P.J., and Yanaga, K. (2012). Desensitization of NFkappaB for overcoming chemoresistance of pancreatic cancer cells to TNF-alpha or paclitaxel. Anticancer Res. *32*, 4813–4821.

Galluzzi, L., Kepp, O., Vander Heiden, M.G., and Kroemer, G. (2013). Metabolic targets for cancer therapy. Nat. Rev. Drug Discov. *12*, 829–846.

Gao, X., Wang, X., Pham, T.H., Feuerbacher, L.A., Lubos, M.L., Huang, M., Olsen, R., Mushegian, A., Slawson, C., and Hardwidge, P.R. (2013). NIeB, a bacterial effector with glycosyltransferase activity, targets GAPDH function to inhibit NF-kappaB activation. Cell Host Microbe *13*, 87–99.

Gardam, S., Sierro, F., Basten, A., Mackay, F., and Brink, R. (2008). TRAF2 and TRAF3 signal adapters act cooperatively to control the maturation and survival signals delivered to B cells by the BAFF receptor. Immunity *28*, 391–401.

Gaulard, P., and de Leval, L. (2014). The microenvironment in T-cell lymphomas: emerging themes. Semin. Cancer Biol. 24, 49–60.

Gerondakis, S., Fulford, T.S., Messina, N.L., and Grumont, R.J. (2014). NFkappaB control of T cell development. Nat. Immunol. *15*, 15–25.

Grinberg-Bleyer, Y., Oh, H., Desrichard, A., Bhatt, D.M., Caron, R., Chan, T.A., Schmid, R.M., Klein, U., Hayden, M.S., and Ghosh, S. (2017). NF-kappaB c-Rel is crucial for the regulatory T cell immune checkpoint in cancer. Cell *170*, 1096–1108.e13.

Grogg, K.L., Attygalle, A.D., Macon, W.R., Remstein, E.D., Kurtin, P.J., and Dogan, A. (2006). Expression of CXCL13, a chemokine highly upregulated in germinal center T-helper cells, distinguishes angioimmunoblastic T-cell lymphoma from peripheral T-cell lymphoma, unspecified. Mod. Pathol. 19, 1101–1107.

Guo, Z., Wang, H., Meng, F., Li, J., and Zhang, S. (2015). Combined Trabectedin and anti-PD1 antibody produces a synergistic antitumor effect in a murine model of ovarian cancer. J. Transl. Med. *13*, 247.

Heissmeyer, V., and Vogel, K.U. (2013). Molecular control of Tfh-cell differentiation by Roquin family proteins. Immunol. Rev. 253, 273–289.

Hoesel, B., and Schmid, J.A. (2013). The complexity of NF-kappaB signaling in inflammation and cancer. Mol. Cancer *12*, 86.

Hu, H., Wu, X., Jin, W., Chang, M., Cheng, X., and Sun, S.C. (2011). Noncanonical NF-kappaB regulates inducible costimulator (ICOS) ligand expression and T follicular helper cell development. Proc. Natl. Acad. Sci. U S A *108*, 12827–12832.

18 Cancer Cell 36, 1-20, September 16, 2019

Imbert, V., and Peyron, J.F. (2017). NF-kappaB in hematological malignancies. Biomedicines 5, https://doi.org/10.3390/biomedicines5020027.

Iqbal, J., Wright, G., Wang, C., Rosenwald, A., Gascoyne, R.D., Weisenburger, D.D., Greiner, T.C., Smith, L., Guo, S., Wilcox, R.A., et al. (2014). Gene expression signatures delineate biological and prognostic subgroups in peripheral T-cell lymphoma. Blood *123*, 2915–2923.

Jacquin, M.A., Chiche, J., Zunino, B., Beneteau, M., Meynet, O., Pradelli, L.A., Marchetti, S., Cornille, A., Carles, M., and Ricci, J.E. (2013). GAPDH binds to active Akt, leading to Bcl-xL increase and escape from caspase-independent cell death. Cell Death Differ. *20*, 1043–1054.

Jain, S., Chen, J., Nicolae, A., Wang, H., Shin, D.M., Adkins, E.B., Sproule, T.J., Leeth, C.M., Sakai, T., Kovalchuk, A.L., et al. (2015). IL-21-driven neoplasms in SJL mice mimic some key features of human angioimmunoblastic T-cell lymphoma. Am. J. Pathol. *185*, 3102–3114.

Jimi, E., Strickland, I., Voll, R.E., Long, M., and Ghosh, S. (2008). Differential role of the transcription factor NF-kappaB in selection and survival of CD4⁺ and CD8⁺ thymocytes. Immunity 29, 523–537.

Johnston, R.J., Poholek, A.C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A.L., Craft, J., and Crotty, S. (2009). Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science *325*, 1006–1010.

Kakiuchi, M., Nishizawa, T., Ueda, H., Gotoh, K., Tanaka, A., Hayashi, A., Yamamoto, S., Tatsuno, K., Katoh, H., Watanabe, Y., et al. (2014). Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma. Nat. Genet. *46*, 583–587.

Karakashev, S., Zhu, H., Wu, S., Yokoyama, Y., Bitler, B.G., Park, P.H., Lee, J.H., Kossenkov, A.V., Gaonkar, K.S., Yan, H., et al. (2018). CARM1-expressing ovarian cancer depends on the histone methyltransferase EZH2 activity. Nat. Commun. 9, 631.

Karin, M., Cao, Y., Greten, F.R., and Li, Z.W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit. Nat. Rev. Cancer 2, 301–310.

Kleffel, S., Posch, C., Barthel, S.R., Mueller, H., Schlapbach, C., Guenova, E., Elco, C.P., Lee, N., Juneja, V.R., Zhan, Q., et al. (2015). Melanoma cell-intrinsic PD-1 receptor functions promote tumor growth. Cell *162*, 1242–1256.

Kroemer, G., and Pouyssegur, J. (2008). Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell 13, 472–482.

Lavallard, V.J., Pradelli, L.A., Paul, A., Beneteau, M., Jacquel, A., Auberger, P., and Ricci, J.E. (2009). Modulation of caspase-independent cell death leads to resensitization of imatinib mesylate-resistant cells. Cancer Res. *69*, 3013–3020.

Li, B., and Dewey, C.N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics *12*, 323.

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760.

Liberti, M.V., Dai, Z., Wardell, S.E., Baccile, J.A., Liu, X., Gao, X., Baldi, R., Mehrmohamadi, M., Johnson, M.O., Madhukar, N.S., et al. (2017). A predictive model for selective targeting of the Warburg effect through GAPDH inhibition with a natural product. Cell Metab. *26*, 648–659.e8.

Lo, J.C., Basak, S., James, E.S., Quiambo, R.S., Kinsella, M.C., Alegre, M.L., Weih, F., Franzoso, G., Hoffmann, A., and Fu, Y.X. (2006). Coordination between NF-kappaB family members p50 and p52 is essential for mediating LTbetaR signals in the development and organization of secondary lymphoid tissues. Blood *107*, 1048–1055.

Long, M., Park, S.G., Strickland, I., Hayden, M.S., and Ghosh, S. (2009). Nuclear factor-kappaB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor. Immunity *31*, 921–931.

Lund, K., Adams, P.D., and Copland, M. (2014). EZH2 in normal and malignant hematopoiesis. Leukemia 28, 44–49.

Madonna, G., Ullman, C.D., Gentilcore, G., Palmieri, G., and Ascierto, P.A. (2012). NF-kappaB as potential target in the treatment of melanoma. J. Transl. Med. *10*, 53.

Morin, R.D., Johnson, N.A., Severson, T.M., Mungall, A.J., An, J., Goya, R., Paul, J.E., Boyle, M., Woolcock, B.W., Kuchenbauer, F., et al. (2010).

CellPress

Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nat. Genet. 42, 181–185.

Mourad, N., Mounier, N., Briere, J., Raffoux, E., Delmer, A., Feller, A., Meijer, C.J., Emile, J.F., Bouabdallah, R., Bosly, A., et al. (2008). Clinical, biologic, and pathologic features in 157 patients with angioimmunoblastic T-cell lymphoma treated within the Groupe d'Etude des Lymphomes de l'Adulte (GELA) trials. Blood *111*, 4463–4470.

Muto, H., Sakata-Yanagimoto, M., Nagae, G., Shiozawa, Y., Miyake, Y., Yoshida, K., Enami, T., Kamada, Y., Kato, T., Uchida, K., et al. (2014). Reduced TET2 function leads to T-cell lymphoma with follicular helper T-cell-like features in mice. Blood Cancer J. *4*, e264.

Nagata, Y., Kontani, K., Enami, T., Kataoka, K., Ishii, R., Totoki, Y., Kataoka, T.R., Hirata, M., Aoki, K., Nakano, K., et al. (2016). Variegated RHOA mutations in adult T-cell leukemia/lymphoma. Blood *127*, 596–604.

Nagoshi, H., Kuroda, J., Kobayashi, T., Maegawa, S., Chinen, Y., Kiyota, M., Nakayama, R., Mizutani, S., Shimura, Y., Yamamoto-Sugitani, M., et al. (2013). Clinical manifestation of angioimmunoblastic T-cell lymphoma with exuberant plasmacytosis. Int. J. Hematol. *98*, 366–374.

Ng, S.Y., Brown, L., Stevenson, K., deSouza, T., Aster, J.C., Louissaint, A., Jr., and Weinstock, D.M. (2018). RhoA G17V is sufficient to induce autoimmunity and promotes T-cell lymphomagenesis in mice. Blood *132*, 935–947.

Palomero, T., Couronne, L., Khiabanian, H., Kim, M.Y., Ambesi-Impiombato, A., Perez-Garcia, A., Carpenter, Z., Abate, F., Allegretta, M., Haydu, J.E., et al. (2014). Recurrent mutations in epigenetic regulators, RHOA and FYN kinase in peripheral T cell lymphomas. Nat. Genet. *46*, 166–170.

Patnaik, M.M., Vallapureddy, R., Lasho, T.L., Hoversten, K.P., Finke, C.M., Ketterling, R., Hanson, C., Gangat, N., and Tefferi, A. (2018). EZH2 mutations in chronic myelomonocytic leukemia cluster with ASXL1 mutations and their co-occurrence is prognostically detrimental. Blood Cancer J. 8, 12.

Pearce, E.J., and Pearce, E.L. (2018). Immunometabolism in 2017: driving immunity: all roads lead to metabolism. Nat. Rev. Immunol. *18*, 81–82.

Postow, M.A., Callahan, M.K., and Wolchok, J.D. (2015). Immune checkpoint blockade in cancer therapy. J. Clin. Oncol. 33, 1974–1982.

Raaphorst, F.M., van Kemenade, F.J., Blokzijl, T., Fieret, E., Hamer, K.M., Satijn, D.P., Otte, A.P., and Meijer, C.J. (2000). Coexpression of BMI-1 and EZH2 polycomb group genes in Reed-Sternberg cells of Hodgkin's disease. Am. J. Pathol. *157*, 709–715.

Recaldin, T., and Fear, D.J. (2016). Transcription factors regulating B cell fate in the germinal centre. Clin. Exp. Immunol. *183*, 65–75.

Ren, X.D., and Schwartz, M.A. (2000). Determination of GTP loading on Rho. Methods Enzymol. 325, 264–272.

Revillion, F., Pawlowski, V., Hornez, L., and Peyrat, J.P. (2000). Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer. Eur. J. Cancer *36*, 1038–1042.

Rohde, M., Richter, J., Schlesner, M., Betts, M.J., Claviez, A., Bonn, B.R., Zimmermann, M., Damm-Welk, C., Russell, R.B., Borkhardt, A., et al. (2014). Recurrent RHOA mutations in pediatric Burkitt lymphoma treated according to the NHL-BFM protocols. Genes Chromosomes Cancer *53*, 911–916.

Roth, R.B., Hevezi, P., Lee, J., Willhite, D., Lechner, S.M., Foster, A.C., and Zlotnik, A. (2006). Gene expression analyses reveal molecular relationships among 20 regions of the human CNS. Neurogenetics 7, 67–80.

Rupec, R.A., Jundt, F., Rebholz, B., Eckelt, B., Weindl, G., Herzinger, T., Flaig, M.J., Moosmann, S., Plewig, G., Dorken, B., et al. (2005). Stroma-mediated dysregulation of myelopoiesis in mice lacking I kappa B alpha. Immunity *22*, 479–491.

Sakata-Yanagimoto, M., Enami, T., Yoshida, K., Shiraishi, Y., Ishii, R., Miyake, Y., Muto, H., Tsuyama, N., Sato-Otsubo, A., Okuno, Y., et al. (2014). Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. Nat. Genet. *46*, 171–175.

Sasaki, D., Imaizumi, Y., Hasegawa, H., Osaka, A., Tsukasaki, K., Choi, Y.L., Mano, H., Marquez, V.E., Hayashi, T., Yanagihara, K., et al. (2011). Overexpression of Enhancer of zeste homolog 2 with trimethylation of lysine 27 on histone H3 in adult T-cell leukemia/lymphoma as a target for epigenetic therapy. Haematologica *96*, 712–719. Shen, H.M., and Tergaonkar, V. (2009). NFkappaB signaling in carcinogenesis and as a potential molecular target for cancer therapy. Apoptosis 14, 348–363.

Sherry, S.T., Ward, M.H., Kholodov, M., Baker, J., Phan, L., Smigielski, E.M., and Sirotkin, K. (2001). dbSNP: the NCBI database of genetic variation. Nucleic Acids Res. 29, 308–311.

Shih, V.F., Tsui, R., Caldwell, A., and Hoffmann, A. (2011). A single NFkappaB system for both canonical and non-canonical signaling. Cell Res. *21*, 86–102.

Sneeringer, C.J., Scott, M.P., Kuntz, K.W., Knutson, S.K., Pollock, R.M., Richon, V.M., and Copeland, R.A. (2010). Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. Proc. Natl. Acad. Sci. U S A *107*, 20980–20985.

Stone, E.L., Pepper, M., Katayama, C.D., Kerdiles, Y.M., Lai, C.Y., Emslie, E., Lin, Y.C., Yang, E., Goldrath, A.W., Li, M.O., et al. (2015). ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. Immunity *42*, 239–251.

Sudo, T., Utsunomiya, T., Mimori, K., Nagahara, H., Ogawa, K., Inoue, H., Wakiyama, S., Fujita, H., Shirouzu, K., and Mori, M. (2005). Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma. Br. J. Cancer *92*, 1754–1758.

Sun, S.C. (2017). The non-canonical NF-kappaB pathway in immunity and inflammation. Nat. Rev. Immunol. *17*, 545–558.

Topalian, S.L., Drake, C.G., and Pardoll, D.M. (2012). Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. Curr. Opin. Immunol. *24*, 207–212.

Topalian, S.L., Taube, J.M., Anders, R.A., and Pardoll, D.M. (2016). Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. Nat. Rev. Cancer *16*, 275–287.

Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J.C., Lanzavecchia, A., and Manz, M.G. (2004). Development of a human adaptive immune system in cord blood cell-transplanted mice. Science *304*, 104–107.

Vallois, D., Dobay, M.P., Morin, R.D., Lemonnier, F., Missiaglia, E., Juilland, M., Iwaszkiewicz, J., Fataccioli, V., Bisig, B., Roberti, A., et al. (2016). Activating mutations in genes related to TCR signaling in angioimmunoblastic and other follicular helper T-cell-derived lymphomas. Blood *128*, 1490–1502.

Vasanthakumar, A., Liao, Y., Teh, P., Pascutti, M.F., Oja, A.E., Garnham, A.L., Gloury, R., Tempany, J.C., Sidwell, T., Cuadrado, E., et al. (2017). The TNF receptor superfamily-NF-kappaB axis is critical to maintain effector regulatory T cells in lymphoid and non-lymphoid tissues. Cell Rep. 20, 2906–2920.

Visser, H.P., Gunster, M.J., Kluin-Nelemans, H.C., Manders, E.M., Raaphorst, F.M., Meijer, C.J., Willemze, R., and Otte, A.P. (2001). The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br. J. Haematol. *112*, 950–958.

Wang, K., Li, M., and Hakonarson, H. (2010). ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 38, e164.

Weber, B.S., Ly, P.M., Irwin, J.N., Pukatzki, S., and Feldman, M.F. (2015a). A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. Proc. Natl. Acad. Sci. U S A *112*, 9442–9447.

Weber, J.S., D'Angelo, S.P., Minor, D., Hodi, F.S., Gutzmer, R., Neyns, B., Hoeller, C., Khushalani, N.I., Miller, W.H., Jr., Lao, C.D., et al. (2015b). Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol. *16*, 375–384.

Wee, Z.N., Li, Z., Lee, P.L., Lee, S.T., Lim, Y.P., and Yu, Q. (2014). EZH2-mediated inactivation of IFN-gamma-JAK-STAT1 signaling is an effective therapeutic target in MYC-driven prostate cancer. Cell Rep. 8, 204–216.

Xiao, G., Harhaj, E.W., and Sun, S.C. (2001). NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. Mol. Cell 7, 401–409.

Xie, P., Stunz, L.L., Larison, K.D., Yang, B., and Bishop, G.A. (2007). Tumor necrosis factor receptor-associated factor 3 is a critical regulator of B cell homeostasis in secondary lymphoid organs. Immunity 27, 253–267.

Cancer Cell 36, 1–20, September 16, 2019 19

Xing, Y., Wang, X., Jameson, S.C., and Hogquist, K.A. (2016). Late stages of T cell maturation in the thymus involve NF-kappaB and tonic type I interferon signaling. Nat. Immunol. *17*, 565–573.

Xu, H., Wang, X., Lackner, A.A., and Veazey, R.S. (2014). PD-1(HIGH) follicular CD4 T helper cell subsets residing in lymph node germinal centers correlate with B cell maturation and IgG production in Rhesus macaques. Front. Immunol. *5*, 85.

Xu, Y., Chaudhury, A., Zhang, M., Savoldo, B., Metelitsa, L.S., Rodgers, J., Yustein, J.T., Neilson, J.R., and Dotti, G. (2016). Glycolysis determines dichotomous regulation of T cell subsets in hypoxia. J. Clin. Invest. 126, 2678–2688.

Yoo, H.Y., Sung, M.K., Lee, S.H., Kim, S., Lee, H., Park, S., Kim, S.C., Lee, B., Rho, K., Lee, J.E., et al. (2014). A recurrent inactivating mutation in RHOA GTPase in angioimmunoblastic T cell lymphoma. Nat. Genet. *46*, 371–375.

Zehir, A., Benayed, R., Shah, R.H., Syed, A., Middha, S., Kim, H.R., Srinivasan, P., Gao, J., Chakravarty, D., Devlin, S.M., et al. (2017). Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. Nat. Med. *23*, 703–713.

CellPress

STAR***METHODS**

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD3 APCcy7 (145-2C11)	Miltenyi	Cat#130-102-306 ; AB_2660402
CD19 PE (1D3)	BD Pharmingen	Cat# 553786 ; AB_395050
Anti-V5 APC	Abcam	Cat# Ab72560 ; AB_1271439
CD4 FITC (RM4-4)	Miltenyi	Cat# 130-102-541 ; AB_2659902
CD8 PEcy7 (REA601)	Miltenyi	Cat# 130-119-123; AB_2733250
CD44 PE (IM7)	BD Pharmingen	Cat# 553134 ; AB_394649
CD25 APC (PC61.5)	eBioscience	Cat# 47-0251 ; AB_1272215
B220 FITC (REA755)	Miltenyi	Cat# 130-110-845 ; AB_2658273
PD-1 PE (CD279; REA802)	Miltenyi	Cat# 130-111-800 ; AB_2656934
CXCR5 APC (REA215)	Miltenyi	Cat# 130-103-113; AB_2655792
CD62 L APCcy7 (MEL-14)	BD Pharmingen	Cat# 560514 ; AB_10611861
Ki67 PE (REA183)	Miltenyi	Cat# 130-100-289 ; AB_2652551
CD95 VB (Jo2)	BD Pharmingen	Cat# 562633 ; AB_2737690
CD178 PE (MFL3)	Miltenyi	Cat# 130-102-590 ; AB_2655652
INF G APC (XMG1.2)	BD Pharmingen	Cat# 554413 ; AB_398551
IL10 PE (JES5-16E3)	eBiosciences	Cat# 12-7101-81 ; AB_466175
Perforin PE (eBioOMAK-D)	eBiosciences	Cat# 12-9392-82 ; AB_466243
IL-4 APC (11B11 Ruo)	BD Pharmingen	Cat# 554436 ; AB_398556
IL-17 PE (TC11-18H10)	BD Pharmingen	Cat# 559502 ; AB_397256
Granzyme B PEcy7 (NGZB)	eBiosciences	Cat# 25-8898-82 ; AB_10853339
TNFα FITC (MP6-XT22)	BD Pharmingen	Cat# 557719 ; AB_396828
F4/80 VB (BM8)	eBiosciences	Cat# 48-4801-82 ; AB_1548747
CD11c PE (HL3)	BD Pharmingen	Cat# 560521 ; AB_1727423
GL-7 APC (GL7)	BD Pharmingen	Cat# 561529 ; AB_10716056
CD138 APC (REA104)	Miltenyi	Cat# 130-102-528 ; AB_2655022
PD-L1 PEcy7 (MIH5)	eBioscience	Cat# 25-5982-80 ; AB_2573508
ICOS-VB (CD278; REA192))	Miltenyi	Cat# 130-100-639 ; AB_2656917
ICOSL APC (CD275; HK5.3)	Miltenyi	Cat# 130-102-944 ; AB_2656899
CD19 FITC (6D5)	Miltenyi	Cat# 130-102-494 ; AB_2661108
Ter119 FITC (TER-119)	Miltenyi,	Cat# 130-102-257 ; AB_2660080
NK1.1 FITC (PK 136)	BD Pharmingen	Cat# 553164 ; AB_394676
CD49b FITC (DX5)	BD Pharmingen	Cat# 553857 ; AB_395093
Ly6C FITC (RB6-8C5)	BD Pharmingen	Cat# 553127 ; AB_394643
CD11c FITC (HL3)	BD Pharmingen	Cat# 553801 ; AB_395060
CD4 FITC (clone MT466)	Miltenyi	Cat#130-113-815 ; AB_2726332
CD19 FITC (clone LT19)	Miltenyi	Cat#130-113-165 ; AB_2725993
CD122 FITC (5H4)	BD Pharmingen	Cat# 554452 ; AB_395401
Anti- Lck pY505 APC	Miltenyi	Cat# 130-110-285 ; AB_2652607
Anti-ZAP70 (PY319)/Syk (PY352)	BD Biosciences	Cat# 557817 ; AB_396884
Anti-Erk1/2; Thr202/Tyr204	Cell signaling	Cat# 4370 ; AB_2315112
CD69 APC-efluor 780	eBioscience	Cat# 47-0691-82 ; AB_2573966
CD25-FITC	Miltenyi	Cat# 130-104-275 ; AB_2660257
Rabbit anti-murine IFN γ	Peprotech	Cat# 500-P119 ; AB 147751

(Continued on next page)

Cancer Cell 36, 1-20.e1-e10, September 16, 2019 e1

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit anti-murine IL-4	Peprotech	Cat# 500-P54 ; AB_147969
Mouse anti-TGFBeta (1,2,3) 51D11)	R&D Systems	Cat# MAB1835 ; AB_357931
Mouse V β TCR screening panel (FITC)	BD Biosciences	Cat# 557004 ; AB_647180
Rabbit mAb Hsp90 (C45G5)	Cell signaling	Cat# 4877S ; AB_2233307
Rabbit mAb p NF-kappa B2 p100 (S866)	Cell signaling	Cat# 4810S ; AB_659925
Rabbit mAb p-IKKalpha (S176)/IKKbeta (S177)	Cell Signaling	Cat# 2078S ; AB_2079379
Rabbit mAb anti-GAPDH	Abcam	Cat# ab9485 ; AB_307275
Mouse mAb NF-κB1	Santa Cruz	Cat# sc-166588X ; AB_2236119
Rabbit mAb PD-1 (PMP1-14)	BioXcell	Cat# BE-0146 ; AB_10949053
Mouse mAb V5tag	Invitrogen	Cat# R96025 ; AB_159313
Rabbit Polyclonal anti-GAPDH	Abcam	Cat# Ab9485 ; AB_307275
Anti CD3 (2GV6) Rabbit monoclonal primary antibody	Roche	Cat# 7904341 ; AB_2335978
Rat Anti-mouse CD45R monoclonal antibody (RA3-6B2)	BioRad	Cat# MCA1258GT ; AB_1102070
Ki67 Monoclonal Antibody (SP6)	Thermofisher Scientific	Cat# MA5-14520 ; AB_10979488
Mouse Monoclonal Anti-Bcl-6	Santa Cruz	Cat# sc-7388 ; AB_2063455
Rabbit Polyclonal Anti CD35	Biorbyt	Cat# orb13316 ; AB_10767957
Anti-CD31 (PECAM)	BD biosciences	Cat# 553373 ; AB_394819
Anti-NF-κB2	Santa Cruz	Cat# sc-7386x ; AB_2267131
Rabbit polyclonal anti-p65	Santa Cruz	Cat# sc-8008x ; AB_628017
Anti- RelB	Santa Cruz	Cat# sc-166416x ; AB_2179178
Rabbit mAb anti-pNF-kappaB p65 (S536)	Santa Cruz	Cat# sc-372 ; AB_632037
Rabbit polyclonal anti NF-kappaB p52	Santa Cruz	Cat# sc-298 ; AB_2151277
Rabbit polyclonal anti-TRAF2	Santa Cruz	Cat# sc-7187 ; AB_793340
Anti-human NF-κB2 p100/p52	Cell Signaling	Cat# 4882 ; AB_10695537
Anti-human EZH2 (D2C9) XP	Cell Signaling	Cat# 5246 ; AB_10694683
Anti-human RelB (C1E4)	Cell Signaling	Cat# 4922 ; AB 2179173
Anti-human NIK	Cell Signaling	Cat# 4994 ; AB_2297422
Mouse mAb anti-rabbit IgG Conformational specific coupled to HRP	Cell signaling	Cat# 5127; AB_10892860
Hamster anti-mouse CD3e (NA/LE)	BD Pharmingen	Cat# 553057 ; AB_394590
Hamster anti-mouse CD28 (NA/LE)	BD Pharmingen	Cat# 553294 ; AB_394763
Biological Samples		
AITL patient biopsies	This manuscript Tumorotek Necker –Enfants Malades Hospital	N/A
Datiant Malanaautaa	This manuacrist	NI/A
Patient Melanocytes	This manuscript	N/A
Chemicals, Peptides, and Recombinant Proteins		
	PC1/EP201//06/306	
RPMI medium 1640		Cat# 618/0044
Fetal Bovine Serum	Life Technologies	Cat# 102/0-106
Glucose	Sigma	Cat# G6152
L-glutamine	ThermoFischer	Cat# 25030081
Sodium pyruvate	ThermoFischer	Cat# 11360088
Cell Tack	Fisher Scientific	10317081
PMA (phorbol 12-myristate-13-acetate)	Sigma	Cat# P8139
Ionomycin	Sigma	Cat# 10634
Golgi-Plug protein Transport inhibitor	BD Biosciences	Cat# 555029

(Continued on next page)

e2 Cancer Cell 36, 1-20.e1-e10, September 16, 2019
CellPress

Continued			
REAGENT or RESOURCE	SOURCE	IDENTIFIER	
Oligomycin	Sigma	Cat# 04876	
Koningic acid	Clinisciences	Cat# AG-CN2-0118	
B-Nicotinamide adenine dinucleotide hydrate (NAD)	Sigma	Cat# N1636	
Sodium arsenate	Sigma	Cat# A6756	
D-glyceraldehyde-3-phosphate	Sigma	Cat# G5376	
Sodium pyrophosphate tetrabasic decahydrate $Na_4P_2O_7$	Sigma	Cat# S9515	
CFSE	BD Biosciences	Cat# 565082	
2-desoxyglucose	Toronto Research Chemicals	Cat# D239000	
Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone FCCP	Santa Cruz	Cat# sc-203578	
Rotenone	Sigma	Cat# R8875	
Antimycin A	Sigma	Cat# A8674	
2-NBDG	Fischer Scientific	Cat# N13195	
Recombinant Human IL2	Preprotech	Cat# 200-02	
Recombinant Mouse IL6	Peprotech	Cat# 216-16	
Recombinant Mouse IL21	Peprotech	Cat# 210-21	
Critical Commercial Assays			
G-Sepharose 4B conjugate beads	Life technologies	Cat# 101242	
Cytofix/Cytoperm kit	BD Biosciences	Cat# 554714	
Flexi Tube Gene Solution siRNA-NF-kB2	Qiagen	Cat# GS4791	
Flexi Tube Gene Solution siRNA-NIK	Qiagen	Cat# GS9020	
Negative control siRNA	Qiagen	Cat#SI03650325	
ECL Detection kit	GE Healthcare	Cat# RNP2106	
ADP-Glo [™] kinase assay	Promega	Cat# V6930	
Anti-FITC microbeads kit	Miltenyi	Cat# 130-048-701	
RNeasy mini kit	Qiagen	Cat# 74104	
Omniscript RT kit	Qiagen	Cat# 205113	
Nucleospin Tissue Kit	Macherey Nagel	Cat# 740901	
Power Sybr™ Green PCR Master Mix	Thermofisher Scientific	Cat# 4368577	
Taqman Fast Universal PCR Master Mix	Fisher Scientific	Cat# 4366072	
ELISA Mouse mAb Isotyping Kit	Thermofisher Scientific	Cat# 37503	
PE Mouse Anti-Ki67 set	BD Pharmingen	Cat# 556027	
Proteome Profiler Mouse Cytokine Array Kit, Panel A	R&D systems	Cat# ARY006	
Milliplex MaP Kit 96-well plate assay	Millipore Corporation-Merck	Cat# MCYTOMAG-70K	
Streptavidine Magnetic beads	Roche	Cat# 11 641 778 001	
Pan T cell isolation kit	Miltenyi	Cat #130-096-535	
T cell Transact beads, human	Miltenyi	Cat# 130-111-160	
Deposited Data			
RNAseq- sequencing data	This manuscript	GSE121748; yjmnaqagjnwxrcn	
Whole exome sequencing data	This manuscript	GSE121748; yjmnaqagjnwxrcn; PRJEB11861	
Experimental Models: Cell Lines			
A375	ATTC	Cat# CRL-1619	
C12.06	This manuscript	N/A	
A549	ATTC	Cat# CCL-185	
Karpas 422	ECACC	06101702	
U-2932	DSMZ	ACC 633	

(Continued on next page)

Cancer Cell 36, 1-20.e1-e10, September 16, 2019 e3

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
SU-DHL-6	DSMZ	ACC 572
Experimental Models: Organisms/Strains		
Mouse: plck-GAPDH+/- (C57BL/6)	This manuscript	N/A
Mouse: C57BL/6	ENVIGO France	N/A
Mouse: plck-Cre (C57BL/6)	Jackson labs	003802
Mouse: ΙκΒ ^{flox/flox} (C57BL/6)	Rupec et al., 2005	N/A
Mouse: plck IκB-/-(C57BL/6)	This manuscript	N/A
Mouse: p65 ^{flox/flox}	Algul et al., 2007	N/A
Mouse: plck p65-/-	This manuscript	N/A
Mouse: NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ	Jackson labs	005557
Oligonucleotides		
Primers (See Table S7)	This manuscript	N/A
HIF Taqman gene expression assay	Thermofisher Scientific	Mm00468878_m1
LDH Taqman gene expression assay	Thermofisher Scientific	Mm00495282_g1
Recombinant DNA		
Plck-hGH-GAPDH-V5	This manuscript	N/A
Software and Algorithms		
Flowjo 10.2	Flowjo	https://www.flowjo.com/solutions/flowjo
Macsquantify Version 2.11	Miltenyi	N/A
Multigauge software	Fuji Film	N/A
xPONENT sofware	Luminex Corporation	https://www.luminexcorp.com/xponent/
Prism 7	Graphpad	https://www.graphpad.com/ scientificsoftware/prism/
Pannoramic Viewer	3DHistec	https://www.3dhistech.com/pannoramic_viewer
KEGG data base	N/A	https://www.ncbi.nlm.nih.gov/pubmed/ 10592173.
C5 data base	N/A	http://www.geneontology.org/page/ go-citation-policy
Other		
Sanger sequencing	Biofidal, France	www.biofidal.com
Whole exome sequencing	Novogene, China	http://en.novogene.com

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Els Verhoeyen (els.verhoeyen@unice.fr).

The mouse lines generated in this study are available upon request.

There are restrictions to the availability of the NIK inhibitor compounds due to licensing by YUKIN therapeutics.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Mice were bred and maintained under pathogen-free conditions at the local animal facility (C3M, INSERM U1065, Nice, France). All experimental procedures were carried out in compliance with protocols approved by the local ethical and experimentation committee (SBEA, Nice, France, autorisation N° A06088014 and B0608820). At sacrifice, single cell suspensions were prepared from all the hematopoietic organs (spleen, LNs, liver, blood and bone marrow) for further isolation and analysis.

Generation of plck-GAPDH Transgenic Mice

The cDNA of human GAPDH fused to the V5 tag in the plasmid pcDNA3 was amplified by PCR and inserted in a plasmid under the control of the promoter of the kinase LCK (plck-hGH (Chaffin et al., 1990)) upon BamHI digestion. The plck-GAPDH-V5 cassette was linearized from this plasmid and microinjected into the pronuclei of oocytes from C57BL/6J mice that were subsequently implanted into gestating females (Service d'experimentation animale et de Transgénèse (SEAT), CNRS, Villejuif, France).

e4 Cancer Cell 36, 1-20.e1-e10, September 16, 2019

CellPress

Three transgenic founders (F35, F16, F07) were selected and were bred by serially backcrossing with C57BL/6JOlaHsd mice for at least 12 generations before phenotypic analysis of the mice. C57BL/6J mice for breeding were obtained from ENVIGO (Cannat, France).

Genotyping of the plck-GAPDH mice was performed on genomic DNA isolated from a tail biopsy using the following couples of primers: S1 = 5' GAGGGAACCCAGTCAGGAGC 3' and 5' TTGATTTTGGAGGGATCTCG 3' and S2 = 5' GTCAGTGGTGGACC TGACCT 3' and 5' GCGCTTACCTGTAGCCATTGC 3'. These primers were used with standard IL-2 primers as controls.

Generation of plck-p65^{-/-} and plck-lkB^{-/-} Mice

Plck-p65^{-/-} mice were generated by crossing plck-Cre transgenic mice (Jackson Institute (n°cat #003802) with p65^{flox/flox} mice provided by R. Schmidt (Algul et al., 2007) in our animal facility. Genotyping was performed using following primers: p65loxRV: 5'GAGCGCATGCCTAGCACCAG 3' and p65loxFW: 5'GTGCACTGCATGCGTGCAG 3'; LckCreRV: 5' GCGGTCTGGCAGTAAAAA CTATC 3' and LckCreFW: 5'GTGAAACA GCATTGCTGTCACTT '

Plck-IkB^{-/-} mice were generated by crossing plck-Cre transgenic mice with IkB ^{flox/flox} mice provided by R. Rupec (Rupec et al., 2005). Genotyping was performed using following primers:

IKB-I0X2-FW: GTG GAG TCA GAT GTA GCA CG; IKB-L0X-2-RV: AGA AAG GGA TAA GCC ATG GAG.

Tumor Transplantation into NOD/SCID_Yc-/- (NSG) Mice

NOD/SCID_Yc-/- mice (NSG) were obtained from the Jackson Laboratory (#005557) and housed in our local animal facility (C3M, Nice, France).

Single cell suspensions from spleen or LNs of old (> 18 months) plck-GAPDH mice with splenomegaly were injected intravenously (i.v.) into 6-8 week old NSG mice (1-2x10⁷ splenocytes per mouse). Recipient mice were sacrificed for evaluation when the mice became weak and/or splenomegaly could be detected (30 to 60 days after injection). The different hematopoietic organs (liver, spleen, blood and bone marrow) were isolated and single cell suspensions were prepared for FACS analysis (see below).

Anti-PD-1 Treatment

Two different plck-GAPDH tumors expressing high levels of PD-1 on the CD4⁺ T cells were injected into 10 NSG mice and the mice were treated with anti-PD-1 antibody (clone PMP1-14, BioXcell (#BE-046); 125 µg in PBS/mouse) or an isotope control antibody daily for the first 2 days post-tumor injection, then every other day.

Treatment with NIK Inhibitor

Splenic lymphoma cells from plck-GAPDH mice were injected intravenously into recipient NSG mice (n=18). Six lymphoma engrafted mice were treated with a chemical NIK inhibitor (Figure S7) at 250 μ g in 100 μ l NaCl 0.9% /mouse; patent PCT/EP2017/067306), 5 with vehicle (100 μ l NaCl 0.9% /mouse) and 7 with NIK^{inh} + anti-PD-1 (clone PMP1-14, BioXcell (#BE-046); 125 μ g in PBS/mouse) on day 1 and 2 after tumor cell injection and then every other day.

Recipient mice were sacrificed at endpoint (> 10% weight loss or palpable splenomegaly). Single cell suspensions were prepared from the spleen, BM and liver for immunophenotypic FACS analysis.

Koningic Acid Treatment In Vivo

2x10⁷ splenic lymphoma cells from plck-GAPDH mice were injected intravenously into recipient NSG mice (n=10). After 3 mice were treated daily by intraperitoneal injection with koningic acid (Clinisciences, #AG-CN2-0118) (1mg/kg) or with vehicle. Recipient mice were sacrificed at endpoints (when reaching >10% weight loss or palpable splenomegaly). Cell suspensions were prepared from the spleen and used for FACS immuno-phenotyping.

Cells

Melanoma cell lines were purchased from the American Tissue Culture Collection (ATCC, Molsheim, France); DLBCL cell lines U-2932 and SU-DHL-6 were obtained from DSMZ, (Germany) while the Karpas 422 were obtained from ECACC (England) and cultured as described with the suggested optimal medium. Human primary melanoma cells were extracted from biopsy specimens of metastatic melanoma obtained from the Department of Dermatology and the Biobank of the University Hospital of Nice; France. All the protocols regarding patients' specimen were reviewed and approved by local ethical committee from University Hospital of Nice; all patients provided informed written consent. Biopsy was dissected and digested for 1–2 hr with collagenase A (0.33 U/ml), dispase (0.85 U/ml) and Dnase I (144 U/ml) with rapid shaking at 37°C. Large debris were removed by filtration through a 70-μm cell strainer. Viable cells were obtained by Ficoll gradient centrifugation. All melanoma cells were cultured in RPMI medium or DMEM (Gibco Life Science, Life Technologies, Grand Island, NY), supplemented with 7% FBS and penicillin-streptomycin 50 μM (Gibco) at 37°C in 5% CO₂. Cells extracted from patients were used for a maximum of 10 passages.

Adult healthy blood samples were collected in sterile tubes containing the anti-coagulant, citrate-dextrose (ACD, Sigma, France). Samples of AITL patients were retrospectively obtained from the onco-hematology laboratory of the 'Necker-Enfants Malades' hospital in Paris (France). The cells were suspended after mechanical dissection of the lymph node biopsy and frozen in DMSO. All human blood and tissues were obtained after informed consent and approval was obtained by the ethical committees of the hospitals according to the Helsinki declaration. Human T cells were isolated from peripheral adult blood isolated by negative selection using a Pan T cell enrichment Kit (Miltenyi, #130-096-535) according to manufacturer's instructions. TCR-stimulation was performed using anti-CD2+anti-CD28 coated Transact beads (Miltenyi, # 130-111-160) according to manufacturer's instructions and supplemented with hlL-2 (10 ng/ml).

METHOD DETAILS

Western Blot Analysis

Thymocytes of 5-week old mice or CD4⁺ splenic T cells were isolated from WT or plckGAPDH mice and lysed using Laemmli buffer for western blot analysis. Proteins were immunoblotted with the indicated antibodies. Immunoblots were visualized (FUJIFILM LAS4000) using ECL Western blotting Detection Reagents (GE Healthcare, #RNP2106) and quantification was performed using ImageJ Software. Mouse anti-V5 was purchased from Invitrogen (#R960-25), rabbit anti-GAPDH from Abcam (# ab9485) and rabit anti-Hsp90 (C45G5; #4875), and pospho-NF-kappaB2 p100 (S866/870; #4810T) from Cell signaling (Leiden, The Netherlands).

Antibodies against murin NF- κ B1 (#sc-166588X), NF- κ B2 (#sc-7386x), p65 (#sc-8008x) and RelB (#sc-166416x), pospho-p65 (S536; # sc372) were purchased from Santa Cruz (Heidelberg, Germany). Antibodies against human EZH2 (#5246), NF- κ B2 (#4882), RelB (#4922) and NIK (#4994) were purchased from Cell Signaling (Leiden, The Netherlands).

Immunoprecipitation

Thymocytes of 5-week old mice were lysed and incubated with G-Sepharose 4B conjugate beads (Life technologies, #101242), preabsorbed with either 4 µg rabbit anti-TRAF2 from Santa Cruz (#sc-7187) or 2 µg mouse monoclonal anti-V5 purchased from Invitrogen/ Thermofisher Scientific (#R960-25). Immuno-precipitated proteins were immunoblotted and revealed with mouse anti-rabbit IgG Conformational specific mAb coupled to HRP from Cell Signaling (#5127S).

Flow Cytometry and Antibodies

Antibodies used for detailed phenotyping or intracellular staining by flow cytometry of T, B and myeloid cells are listed in principle table of the STAR Methods.

For intracellular staining of Granzyme B, Perforin, IFN_Y, TNFα, IL-10, IL-4 and IL-17 splenocytes were stimulated for 5 hours in PMA (phorbol 12-myristate-13-acetate; Sigma, # P8139)/ionomycin (Sigma, # I0634) in the presence of Golgi-stop (BD Biosciences, #555029) and upon surface staining (anti-CD4 or anti-CD8) cells were fixed and permeabilized using the Cytofix/Cytoperm kit and protocol (BD Biosciences; #554714). Intracellular staining for V5tag and GAPDH was performed using the same Cytofix/Cytoperm kit.

All stainings were detected using a MACSQuant flow cytometer (Miltenyi Biotec, Paris, France). Analysis of the FACS data was performed using MACSquantify Version 2.11 (Miltenyi) and FlowJo Software.

Isolation of Primary Mouse T Cells

CD4⁺ T cells from the spleen of WT or plck GAPDH mice were isolated by negative selection using FITC coupled antibodies: anti-CD19 (Miltenyi, #130-102-494) anti-B220 (Miltenyi, #130-110-845), anti-CD8b (Miltenyi, #130-111-710), anti-Ter119 (Miltenyi, #130-102-257), anti-NK1.1 (BD Pharmingen, #553164), anti-CD49b (BD Pharmingen, #553857), anti-Ly 6C (BD Pharmingen, #553127), anti-CD122 (BD Pharmingen,# 554452) and anti-CD11c (BD Pharmingen, #553801), followed by anti-FITC microbead (Miltenyi, #130-048-701) incubation according to manufacturer's instructions.

Detection of TCR-Signaling

CD4⁺T cells were activated on NA/LA hamster anti-mouse CD3e (BD Pharmingen, # 553294) coated plates (3 µg) in the presence of NA/LE Hamster anti-mouse CD28 (3 µg/ml; BD Pharmingen, #553294) for 30 min, 6 hr and 24 hr followed by intracellular staining with anti-Lck pY505 APC from Miltenyi (#130-110-285), alexa fluor 647 mouse anti-ZAP70 (PY319)/Syk (PY352) from BD Biosciences (#557817) or anti-phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204) from Cell signaling technology (#4370) and analysis by flow cytometry. Intracellular staining was performed by fixation and permeabilization according to manufacturer's instructions.

Activation markers were detected on the CD4⁺ T cells at 6h and 24h of TCR-stimulation by flow cytometry using surface staining with anti-mCD69APC-efluor 780 purchased from eBioscience (# 47-0691-82) and anti-mCD25-FITC purchase from Miltenyi (#130-104-275). Analysis of the FACS data was performed using Macsquantify Version 2.11 (Miltenyi) and Flojo Software.

Immunohistochemistry Analysis and Antibodies

Hematopoietic tissues were fixed in formaldehyde overnight and subsequently transferred into 70% ethanol and embedded in paraffin. Hematoxylin and eosin staining were performed on the paraffin embedded tissue sections to color the follicular center. Immuno-staining was performed using anti-mouse antibodies against CD3e (#05493315001, SP7) from Roche, B220 (CD45R) from BioRad (# MCA1258GT), Ki67 (SP6, # MA5-14520) from Thermofisher Scientific, BcI-6 (Santa Cruz Biotechnology, # sc-7388), CD31 (PECAM-1; 553373; BD Biosciences) and anti-CD35 (Cliniciences, #13316). Adapted secondary antibodies were used for revelation.

Detection of T Cell Clonality

TCR- β clonality of T cells was determined by surface staining for TCR- β of the CD4⁺ T cells in the tumors with antibodies directed against the different β -chains (Mouse V β TCR screening panel (FITC): mAb against β 2, β 3, β 4, β 5.1, β 6, β 7, β 8.1, β 8.3, β 9, β 10, β 11, β 12, β 13, β 14, β 17; BD Biosciences; #557004).

CellPress

TCR- γ clonality was detected using specific primers for V γ 1: 3'-CCGGCAAAAAGCAAAAAGT-5', for V γ 4: 3'- TG TCCTTGCAACCCCTACCCA-5', for V γ 7: 3'- GTGATACTCACAGAACAGGC-5' combined with reverse primer for C γ 3'- CTTATGG AGATTTGTTTCAGC -5'

Cytokine-Chemokine Array

Mesenchymal lymph nodes from lymphoma developing plck-GAPDH mice and WT mice used as a control were stimulated for 4 hr with PMA and ionomycin in RPMI/10% FCS at a density of 5x10⁶ cells/ml. Then the supernatant was tested for secreted cytokine and chemokine expression levels using the Proteome Profiler Mouse Cytokine Array Kit, Panel A (ARY006, R&D systems) according to manufacturer's instructions. Quantification was performed using Multigauge (Fuji Film, Tokyo, Japan).

Quantification of Cytokines in Mouse Serum

Mouse IL-10, IFN γ , IL-1 α , IL-1 β , IL-6 and TNF α were determined by using the Milliplex MaP Kit 96-well plate assay (MCYTOMA G-70K) according to manufacturer's instructions (EMD Millipore Corporation-Merck, Darmstad, Germany). Detection was performed on the Luminex analyzer MAGPIX® using the acquisition software xPONENT® (Luminex Corporation.

Transcriptomic Analysis

RNA Seq Data for plck-GAPDH versus WT Mouse Splencytes

Total RNA was extracted from splenocytes of tumor developing plck-GAPDH mice and WT mice using the RNA extraction kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Libraries were generated from 500ng of total RNAs using Truseq Stranded Total TNA kit (Illumina). Libraries were then quantified with Qubit dsDNA BR Assay kit (Invitrogen) and pooled. 4nM of this pool were loaded on a high output flow cell and sequenced on a NextSeq500 platform (Illumina) using 2x75bp paired-end chemistry.

RNAseq data were quantified using RSEM sofware 1.2.25 (Li and Dewey, 2011) with bowtie2-2.2.6 and m38 as reference genome. The gene sets were chosen on the basis of known expression patterns in human and mice Tfh and both GC and post-GC B cell

subsets. AITL Patient Data

Public raw data AITL transcriptomes using Affymetrix HGU133 Plus 2.0 microarrays were downloaded from GEO dataset (GSE58445 (lqbal et al., 2014), GSE3526(Roth et al., 2006): GSE7307; https://www.ncbi.nlm.nih.gov/gds) and ArrayExpress (E-TABM-783 (de Leval et al., 2007); https://www.ebi.ac.uk/arrayexpress/), normalized together (RMA) and collapsed to HUGO gene symbols using chipset definition files.

Expression data were normalized with z-score methods when specified and illustrated with heatmaps using R software (3.3.2). Statistical differences were verified using an unpaired two-tailed Wilcoxon signed rank test versus the specified controls.

Metabolic pathway analysis was performed using the data base KEGG: https://www.ncbi.nlm.nih.gov/pubmed/10592173. For other signaling/functional pathway analysis we used the data base C5: http://www.geneontology.org/page/go-citation-policy.

PCR Amplification and Sanger Sequencing

Mutations in the *RHOA*, *TET2*, *IDH2* and *DNMT3A* allele were detected in plck-GAPDH tumor cells by PCR amplification using the KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems, KK2601) and the following primers: RHOA-Fw: CTGCCATCAGGAAGAA ACTGGTG and RHOA-Rv: CTGCTTCCCATCCACCTCGATATC; TET2-Fw: TGGTGGTCACCCTCAATA and TET2-Rv: GAAG ACTTTCCCTTCTCAGTC; IDH2-Fw: GTTCAAGCTGAAGAAAATGTGG and IDH2-Rv: CTGGTCGCCATGGGCGTGCC; DNMT3 A-Fw: GGGTGTTTGGCTTCCCCGTCC; DNMT3A-Rv: CAGTCACAGCAAG

AAACAAAAACC.

The PCR fragments were then sequenced by Sanger sequencing performed at Biofidal (Vaulx en Velin, France; www.biofidal.com).

Determination of GTP Levels Loaded on RHOA

HEK293T cells were transfected using lipofectamine 2000 (Thermofisher) for 24h with pKH3-HA-RhoA, pKH3 HA-RhoAT37M, pKH3 HA-RhoAT19T or pKH3-HA-RhoAQ63L plasmids. Cells were lysed and levels of active Rho (RhoGTP) in each sample were measured using a GST-rhotekin RBD pull down assay as described previously (Ren and Schwartz, 2000).

In Vitro Tfh Differentiation

To induce Tfh differentiation *in vitro*, naive CD4⁺ T cells were co-cultured with irradiated T cell–depleted splenocytes as co-stimulator cells (irradiated antigen presenting cells) on anti-CD3 coated plates using RPMI media supplemented with 25 ng mL⁻¹ IL-6 (#216-16, Peprotech), 50 ng mL⁻¹ IL-21 (#210-21, Peprotech), 4 ng mL⁻¹ anti-IFN γ (#500-P119, Peprotech), 4 mg mL⁻¹ anti-IL-4 (#500-P54, Peprotech) and 20 mg mL⁻¹ anti-TGFB (#1D11, R&D Systems). At several time-points the Tfh differentiation was evaluated by staining with anti-ICOS, anti-PD-1 and anti-CXCR5 antibodies and detection by flow cytometry and also by Q-PCR for Tfh marker (CD21, Bcl-6 and CXCL-13; see STAR Methods principle table).

Germinal Center Induction In Vivo

To generate germinal center responses and T cell activation, WT mice were immunized with 1x10⁹ sheep red blood cells (SRBC; ORGENTEC, #VSE-410) delivered by intraperitoneal injection every 2 weeks for 4 months.

Reverse Transcriptase Quantitative- PCR (RT-qPCR) Analysis

CD4⁺ T cells were isolated from the spleen of tumor bearing plck-GAPDH or WT C57/BL6 mice by incubation of total splenocytes an anti-mouse CD4-FITC antibody (Miltenyi, #130-102-541) following by an incubation with anti-FITC microbeads (Miltenyi, #130-048-701) according to manufacturer's instructions. CD19⁺ B cells were isolated using an anti-mouse CD19-FITC (Miltenyi, 130-102-494) antibody and then following the same procedure as for CD4⁺ cells. Sorting was performed on an autoMACS cell sorter (Miltenyi).

Total RNA was extracted from cells using the RNA extraction kit (Qiagen, #74104, Valencia, CA, USA) according to manufacturer's instructions. Reverse transcription was performed using the Omniscript RT kit (Qiagen, #205113). After reverse transcription-PCR, the relative mRNA expression level of GAPDH, CXCL13, IL-21, BLIMP, BcI-6, ReIA, cReI, GADD45B, E2F1, NF-κBia, HIF-1α, LDHα, NF-κB2, ReIB, EZH2, SP1, IRF4, ENPP2, NOD2, 18S (mouse) were obtained by real-time quantification PCR, using SYBR™ Green PCR Mix (Thermofisher Scientific, France) and the corresponding primers sets (see principle table STAR Methods) on the 7500 Fast and the Step One real-time PCR systems (Applied Biosystems) according to the manufacturer's instructions. All samples were normalized to 18S. The relative mRNA expression level of HIF-1α, and LDHα were obtained by real-time quantification PCR, using the TaqMan PCR Master Mix (Eurogentec, Seraing, Belgium) and TaqMan assay primer set (Thermofisher; HIF-1α,: Mm00468878_m1; LDHα: Mm00495282_g1) on the 7500 Fast and the Step One (Applied Biosystems) according to the manufacturer's instructions. All analyses were performed in triplicate and melting curve analysis was performed for SYBR GREEN to control product quality and specificity.

Genomic DNA Isolation

For whole exome sequencing and Sanger Sequencing genomic DNA was extracted from CD4⁺ T cells, isolated from plck-GAPDH tumors by negative microbead selection (see above), using the Nucleospin Tissue Kit (Macherey Nagel, #740901).

Whole Exome Sequencing

Genomic DNA was extracted from CD4⁺ T cells as mentioned above. Paired-end DNA library was prepared according to the manufacturer's instructions (Agilent). The adapter-modified gDNA fragments were enriched by six cycles of PCR. Whole exome capture was carried out using Agilent's SureSelect Kit. After DNA quality evaluation, pooled samples were sequenced on Illunima Hiseq 2000.

Raw data were first filtered using an in house quality control. Paired-end clean reads are aligned to the reference genome (UCSC hg19) using Burrows–Wheeler Aligner (BWA) software(Li and Durbin, 2009). If a read or reads pair is mapped to multiple positions, BWA will choose the most likely placement. While if two or more most-likely placements are present, BWA will choose any one randomly. Aligned reads were realigned to the genome. Genome Analysis Toolkit (GATK)(DePristo et al., 2011) was used to ignore those duplicates resulted from PCR amplification with Picard-tool. We utilized the INDELrealigner and RealignerTargetCreator in GATK do realignment around the INDELS according to GATK best practice. Furthermore, we performed base quality score recalibration with GATK to avoid system bias. After realignment to genome, we identified and filtered variants (SNP, INDELs) using GATK HaplotypeCaller and variantFiltration to guarantee meaningful analysis. Variants obtained from previous steps were compared based on the dbSNP (Sherry et al., 2001) and annotated with ANNOVAR(Wang et al., 2010).

Th RhoA genomic regions of interest was amplified by PCR with flanking HTS primer pairs. PCR amplification was carried out with Phusion High-Fidelity DNAPolymerase (Thermo Fisher), according to the manufacturer's instructions. Purified DNA was amplified by PCR with primers containing sequencing adaptors. The products were gel purified and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher) and KAPALibraryQuantification Kit (KAPABiosystems). Samples were sequenced on an Illumina MiSeq, as previously described.

Lactate and Glucose Measurement

To determine glucose consumption and lactate production, $CD4^+$ T cells were isolated from the spleen of WT and plck-GAPDH mice using negative selection (see above) and were activated for 48h on NA/LA hamster anti-mouse CD3e (BD Pharmingen, # 553294) coated plates (3 µg mL⁻¹) in the presence of NA/LE Hamster anti-mouse CD28(3 µg mL⁻¹; BD Pharmingen, #553294) in RPMI/ 10%FCS. After pre-activation, culture medium was replaced with fresh RPMI/10%FCS medium and collected 24h later. Glucose and lactate levels were determined electro-enzymatically using the YSI 2950 Biochemistry analyser (Bioprocess systems), which normalized to the number of viable cells.

Metabolic Assays

OCR and ECAR were measured with an WF96 extracellular flux analyzer (Seahorse Bioscience) following protocols recommended by the manufacturer. Freshly isolated naive or activated cells were seeded on Cell Tack (Fisher Scientific, #10317081) pre-coated XF96 microplates ($2x10^5$ cells/well). The plates were quickly centrifuged to immobilize cells. Cells were maintained in a non-buffered assay medium supplemented with 25 mM glucose (Sigma, #G6152), 1 mM sodium pyruvate (ThermoFischer, #11360088) and 2 mM L-glutamine (ThermoFischer, #25030081) for OCR measurement, or with 2 mM L-glutamine for ECAR measurement. Cells were incubated in a non-CO₂ incubator for 30 minutes before starting the measurements. Glycolysis measurements were obtained after three injections, 25 mM glucose followed by 3,75 μ M oligomycin (Sigma, #04876) that inhibits mitochondrial ATP production and shifted the energy production to glycolysis. The increased ECAR revealed the maximum glycolytic capacity of T cells. The third injection was 100 mM 2-DG (Toronto Research Chemicals, #D239000), a glucose analog that inhibits glycolysis. The OCR measurements were

CellPress

obtained under basal conditions and in response to mitochondrial inhibitors, 3.75 μM oligomycin, 2 μM FCCP (Sigma, #C2759) and 1 μM rotenone (Sigma, #R8875) and 2 μM antimycin A (Sigma, #A8674).

2-NBDG Uptake

2x10⁵ cells were incubated in glucose free RPMI/10%FCS/1%Penicilin/streptamidin medium containing 100 μM 2-NBDG (Fischer Scientific, #N13195) for 2 hours before measuring fluorescence by flow cytometry.

IgG Determination in Mouse Serum

IgG isotyping for IgG₁, IgG_{2a}, IgG_{2b}, IgG3, IgA, IgM, Kappa light chain or lambda chain was performed using the Pierce Rapid ELISA Mouse mAb Isotyping Kit from Invitrogen/Thermofisher Scientific (#37503) according to manufacturers' instructions. Sera were diluted 1/15 into TBS buffer provided in the kit.

Measurement of GAPDH Activity

Splenocytes from treated and vehicle mice were lysed in buffer A (10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 10 μ g mL⁻¹ aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride and 10 μ mol/L leupeptin). 10 μ g of proteins were incubated in GAPDH activity buffer (0.25 mmol/L NAD (Sigma, #N1636), 3.3 mmol/L DTT, 13 mmol/L Na₄P₂O₇ (Sigma, #S9515) (pH 8.5), 26 mmol L⁻¹ sodium arsenate (Sigma, #A6756), and 25 mmol/L D-glyceraldehyde-3-phosphate (Sigma, G5376) in a black 96-well plate (Cellstar). GAPDH activity was measured on a fluoroscan at 445 nm after excitation at 340 nm as the increase in fluorescence related to NADH accumulation. GAPDH activity is expressed as the delta absorbance (representing the speed of NADH production) per milligram of protein.

Cell Proliferation Detection

For cell cycle entry analysis 10⁶ CD4⁺ T cells were activated by anti-CD3 and anti-CD28 (see TCR signaling above) for 6h and 24h and intracellular staining was performed using the mouse anti-Ki67-PE kit (BD Pharmingen, # 556027) according to manufacturer's instructions.

CFSE staining was performed on 10⁶ CD4⁺ T cells activated by anti-CD3 and anti-CD28 for 4 days (see TCR signaling above) according to manufacturer's indications (BD Biosciences, # 565082).

Anti-nuclear Antibody Detection

These assays were performed using the NOVA Lite HEp-2 kit (INOVA Diagnostics, Inc.) according to the manufacturer's directions sera were diluted at 1:50 and 1:100 in PBS. Slides were incubated with anti-mouse IgG-FITC conjugate and visualized with a Zeiss Axioplan 2i microscope. Pictures were taken with an AxioCam HRm camera (Carl Zeiss International, Thornwood, NY). Data were analyzed using AxioVision LE.

ADP-Glo Experiments

Kinase inhibition assay for NIK were performed with the use of ADP-Glo[™] Kinase Assay following manufacturer protocol (Promega, Charbonnières-les-Bains, France) and with the following conditions: 10nM NIK, 10 to 100nM MBP (protein substrate), 10 to 100iM ATP, incubation time was 30 min at 25°C. For each experimental condition a blank assay without enzyme was used as a control of ATP non-enzymatic hydrolysis.

Biotin Conjugated Drug Affinity Precipitation

A375 cells were grown in 150 mm dishes until they reached 50% confluence and then lysed with a 10mM phosphate buffer pH=7.5 containing 250mM NaCl, 0.2% Triton-X100. Crude extract was then centrifuged at 5000xg for 10 min to remove cell debris. 1 mL of supernatant with a protein concentration of 7 mg/mL was then incubated with 100µM of one of the following conditions: biotin, phenyl-biotin, aminoquinolinil-biotin, NIK^{inh}-biotin and 1X excess of free drug, NIK^{inh}-biotin and 10X excess of free drug. Solution were incubated overnight at 4°C under stirring conditions and then incubated with streptavidin magnetic beads (Roche, Germany; Cat. No. 11 641 778 001) for 2 hr. At the end of the incubation beads were washed 4 times with 1 volume of lysis buffer with the use of a magnetic rack. Protein attached to the beads were eluted out by adding 100mL of Laemmli sample buffer and used for further analysis.

Comparison of NF-kB and NIK Knock-down by siRNA Treatment and NIK Inhibitor Treatment

Typically, 50000 cells (A375, C12.06, A549) were plated in six-well culture plates and transfected with 30 or 50 nM siRNAs using Lipofectamine RNAimax reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. If not otherwise noted, the cells were incubated with the 3 different siRNAs per target for 4 days prior to harvesting for further experiments. siRNA against NF-kB2 (FlexiTube Genesolution GS4791) and against NIK (FlexiTube Genesolution GS9020) and negative control siRNA were purchased from Qiagen. Under the same conditions these cells and non tumoral cells (melanocytes and fibroblasts) were determined by FACS and normalized to the corresponding DMSO-treated control cells.

Different B cell lymphoma cell lines (u2932, Karpas 422, SU-DHL-6) were treated with NIK^{inh} (10 μM, 96hr) or vehicle (DMSO) and the cell numbers were determined. Healthy human CD4⁺T cells and human AITL patient biopsies were stimulated through the TCR

and treated with NIK^{inh} (10 μ M, 48hr) or vehicle (DMSO) and the CD4⁺ T and B cell numbers were determined. The cell numbers were normalized to the corresponding DMSO-treated control cells.

DATA AND CODE AVAILABILITY

The [datasets/code] for RNAseq and whole exome sequencing generated during this study are available in this paper [GSE121748/ yjmnaqagjnwxrcn; www.ncbi.nlm.nih.gov/geo].

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis were conducted using Microsoft excel 2013 and Prism software v6.0 (GraphPad Software, La Jolla, CA, USA). Results are indicated as means \pm SD (standard deviation) in the figure legends unless otherwise. For statistical testing of significance a student's t-test or one way ANOVA was used and p values are indicated in the figure legends. A p value < 0.05 was considered to indicate statistical significance. Mice survival curves were evaluated using Log-rank test to determine significance. All flow cytometry data shown are representative of at least 3 reproduced experiments. Gene set enrichment analysis was performed as described above.

Cancer Cell, Volume 36

Supplemental Information

GAPDH Overexpression in the T Cell Lineage

Promotes Angioimmunoblastic T Cell Lymphoma

through an NF-κB-Dependent Mechanism

Laura Mondragón, Rana Mhaidly, Gian Marco De Donatis, Marie Tosolini, Pascal Dao, Anthony R. Martin, Caroline Pons, Johanna Chiche, Marie Jacquin, Véronique Imbert, Emma Proïcs, Laurent Boyer, Anne Doye, Frédéric Luciano, Jaap G. Neels, Frédéric Coutant, Nicole Fabien, Laura Sormani, Camila Rubio-Patiño, Jozef P. Bossowski, Florian Muller, Sandrine Marchetti, Elodie Villa, Jean-François Peyron, Philippe Gaulard, François Lemonnier, Vahid Asnafi, Laurent Genestier, Rachid Benhida, Jean-Jacques Fournié, Thierry Passeron, Jean-Ehrland Ricci, and Els Verhoeyen



Figure S1: plck-GAPDH mice are marked by Tfh and GC bone marrow infiltration and DC infiltration in the spleen and strong LN vascularization (related to Figure 2).

(A) Staining of splenic tissue sections by immunohistochemistry for T (CD3), B cells (B220) and Ki67 and hematoxylin and eosin staining of LN of tumor bearing plck-GAPDH mice.

(B) Skin rash and tumorigenic mesenchymal LN with strong vascularisation were exclusively detected for plck-GAPDH mice as compared to WT mice.

(C) Staining of LN tissue sections by immunohistochemistry for CD31 (endothelial cells), Bcl-6 (Tfh marker) and CD35 (follicular dendritic cells).

(D) VEGF mRNA expression levels determined by Q-PCR in LN of PTCL bearing plck-GAPDH mice and WT (mean \pm SD, n=3, **p<0.01, student t-test).



Figure S2: B-cell characterization in plck-GAPDH tumors and transfer of plck-GAPDH tumor cells induce Tfh-like PTCL in NSG mice (related to Figure 4)

(A) Ki67 staining of B cells analyzed by FACS. Representative of n=5.

(B) Measurement of IgGs by ELISA in the sera of lymphoma bearing plck-GAPDH mice. Indicated as fold increase of IgG levels as compared to sera of age-matched WT mice (mean \pm SD, n=5, *p<0.05, student t-test).

(C) plck-GAPDH AITL-like mice produce anti-nuclear antigen antibodies and anti-muscle antibodies. HEp-2 cells were incubated with sera (1/50 diluted) from littermate or plck-GAPDH lymphoma mice and were developed with anti-mouse IgG-FITC. Data shown are representative of sera samples from plck-GAPDH lymphoma mice positive for auto-antibodies (nuclear staining) or possible anti-muscle antibodies (cytoplasmic staining).

(D) FACS analysis of dendritic cell by F4/80⁻ CD11c⁺ staining in the plck-GAPDH tumors. Data are summarized in a histogram (mean \pm SD, n=5, **p<0.01, student t-test).

(E) FACS analysis of GC B cells (GL-7⁺ CD95⁺ gated on B220⁺CD19⁺ cells) and Tfh cells (PD-1⁺ CD4⁺ T cells) in the BM of tumor-bearing plck-GAPDH mice. Data are summarized in histograms (mean \pm SD, n=7; **p<0.01, *** p<0.001, student t-test).

(F-I) Total splenocytes from tumor bearing plck-GAPDH were transfered to NSG mice. 6-8 weeks postinjection mice were sacrificed. Identical to the plck-GAPDH mice, transplanted NSG mice developed splenomegaly, liver infiltration and presented abdominal ascites (F). Splenocytes were analyzed in donor and recipient for CD4⁺ and CD8⁺ T cells (gated on CD3⁺ cells) (G). BM aspirates were analyzed in donor and recipient for PD-1^{high} CD4⁺ T cells and GC B cells by FACS (H). (G,H) Data are representative for n= 3. Conservation of TCR- β clonality in transplanted NSG mice. Surface expression of the different TCR- β chains by FACS on plck-GAPDH (GAPDH 1, GAPDH 2) CD4⁺ T cells and on the respective CD4⁺ T cells from NSG tumor engrafted mice (NSG 1.1, NSG 1.2 ; NSG 2.1 and 2.2) are shown (I).



D







AITL gene	signature
Pon2	Ly96
Stk39	Cebpa
Tox	Myo7a
Sh2d1a	F11r
Rgs3	Ust
Rgs1	F13a1
Dnajc4	Slc7a10
CD69	Tfdp2
ICOS	Man2a1
Ampd3	Rasa1
Ralgds	ld3
Bcl-6	Nab1
Nmb	Maf
Nucb2	Tbc1d4
Pdcd1	Gsr
lghg3	Abcg1
Mafb	Lrmp
Nckap1	Nfatc1
Ptpn13	Trim8
Sypl	Ctsb
Gfod1	Coro1b
Tiam1	lghm





Figure S3: GEA for AITL gene signatures in AITL patient and plck-GAPDH mPTCL (related to Figure 5) (A) Clustering and heatmap based on transcriptomic data of 18 core upregulated genes from a Tfh gene signature (de Leval et al. 2007; Jain et al. 2015) and (B) GC-plasma B cell gene signature (23 genes; Recaldin and Fear; 2016) for AITL patient (n=60) and healthy LNs (n=8). (C) GSEA for AITL related genes from transcriptomic data of AITL patient LN (Cortes et al. 2018, De Leval et al., 2007) and plck-GAPDH LN. All genes (black bars in the pink zone with enrichment score above zero) are upregulated. (Kolmogorov-smirnov test). (D) The genes included in the AITL signature in (C) are listed.

(E) mRNA upregulation of cytokine and chemokine genes based on GEA transcriptomic data comparing total splenocytes of lymphoma developing plck-GAPDH to WT splenocytes. Fold change expression is shown (log²).

(F) Heatmaps of transcriptomic data comparing healthy (n=8) and AITL patient LN (n=52) for their inflammatory cytokine expression patterns. (G) Cells from plck-GAPDH LN or from WT LN were activated with PMA/ionomycin and supernatant was subjected to a cytokine-chemokine array for quantification of the indicated proteins. (H) Heatmaps of transcriptomic data comparing healthy (n=8) and AITL patient LN (n=52) for their chemokine expression patterns. (I) Cell from plck-GAPDH LN or from WT LN were activated with PMA/ionomycin and supernatant was subjected to a cytokine-chemokine-chemokine array for quantification of the indicated chemokine expression patterns. (I) Cell from plck-GAPDH LN or from WT LN were activated with PMA/ionomycin and supernatant was subjected to a cytokine-chemokine array for quantification of the indicated chemokines.

(J) Heatmaps of transcriptomic data comparing healthy (n=8) and AITL patient LN (n=52) for their inflammatory cytokine expression patterns. (K) Cells from plck-GAPDH LN or from WT LN were activated with PMA/ionomycin and supernatant was subjected to a cytokine-chemokine array for quantification of the indicated interleukins.





Figure S4: Pre-maligant plck-GAPDH mice show an upregulation of PD-1^{high} CD4⁺ cells and noncanonical NF-κB activation and their CD4⁺ T cell GAPDH^{high} cells have unchanged TCR signaling and rely on glycolysis (related to figure 5).

(A) Distribution of the different thymic subpopulations DN (CD4⁻CD8⁻), DP (CD4⁺CD8⁺), CD4⁺ (CD4⁺CD8⁻) and CD8⁺(CD4⁻CD8⁺) analyzed by FACS in WT and plck-GAPDH 1-year old mice; data are summarized in a histogram; the ratio of CD4⁺/CD8⁺ T cells in the thymus is indicated (mean \pm SD, n=8; ns: not significant, student t-test).

(B) FACS analysis of the % of T cells (CD3⁺cells), the ratio of CD4⁺/CD8⁺ T cells and the ratio of CD19⁺/CD3⁺ cells in the spleens of 1-year old WT and plck-GAPDH mice. (C) FACS analysis of the % of Th1, Th2 and Th17 T cells identified by intracellular staining of INF γ , IL-4 or IL-17 in CD4⁺ splenocytes upon PMA and ionomycin stimulation for WT and plck-GAPDH 1-year old mice. (D) The percentage of regulatory T cells (Tregs) on total CD4⁺ T cells identified by CD25 and FoxP3 FACS staining. (B to D: mean ± SD; WT, n=6; GAPDH, n=12; ns, non significant, student t-test). (E) Percentages GC B cells gated on total CD19⁺B220⁺ cells and CD4⁺PD-1^{high} T cells in WT and plck-GAPDH spleens of 1-year old mice (mean ± SD, n=10; *p<0.05, student t-test). (F) mRNA expression levels by Q-PCR of Tfh signature genes (CXCL13, IL-21, Bcl-6) in isolated CD4⁺ T splenocytes from WT and plck-GAPDH 1-year old mice (mean ± SD, n=5;*p<0.05, **p<0.01, student t-test).

Figure S4 continued

(G) Concentration of cytokines (pg/ml) by luminex methodology in the sera of WT and plck-GAPDH 1year old mice (mean ± SD, n=5).

(H) CD4⁺ T cells, isolated from 1 year old pre-disease plck-GAPDH and age-matched WT mice, were stimulated through the TCR-receptor by anti-CD3 and anti-CD28 antibodies. FACS analysis of TCR-signaling for WT and plck-GAPDH CD4⁺ T cells at 30 min, 6 hr and 24 hr of stimulation by intracellular staining of phosphorylated proteins: pY505Lck, pY319/Y352ZAP or pY202/Y204ERK. Representative of n=3. (I) FACS analysis of surface staining for CD69 and CD25 for WT and plck-GAPDH CD4⁺ T cells at 6 hr and 24 hr after TCR stimulation (mean \pm SD; n=3). (J) CFSE-labeled freshly isolated T cells were activated with anti-CD3/anti-CD28 antibodies for 48 hr, and then proliferation was measured by FACS. Data are representative of n = 3.

(K) ECAR of naive and TCR-activated WT and plck-GAPDH CD4⁺ splenocytes was detected upon stimulation with anti-CD3/anti-CD28 for 48 hr or determined directly after negative selection for CD4⁺ cells. ECAR at baseline and stressed ECAR are shown (mean \pm SD for n=3; *p<0.05; ***p<0.001, ****p<0.0001, one way ANOVA).

(L) Lactate production and glucose uptake was detected in TCR-activated WT and plck-GAPDH CD4⁺ splenocytes. CD4⁺ splenocytes were activated by in vitro stimulation with anti-CD3/anti-CD28 for 48 hr (means \pm SD for n=3; ****p<0.0001, student t-test).

(M) FACS analysis of glucose uptake using an fluorescently labeled analogue of glucose, 2-NBDG in CD4⁺ splenocytes after stimulation with anti-CD3/anti-CD28 for 48 hr . Data are representative of n=3.

(N) OCR of naive and TCR-activated WT and plck-GAPDH CD4⁺ splenocytes was detected upon stimulation with anti-CD3/anti-CD28 for 48 hr or determined directly after negative selection for CD4⁺ cells. OCR at baseline and stressed OCR are shown (mean \pm SD for n=3; *p<0.05; ***p<0.001, ****p<0.0001, one way ANOVA)

Glu: glucose, oligo: oligomycin, 2DG: 2-deoxy-D-glucose, FCCP: Carbonyl cyanide 4-trifluoromethoxy phenylhydrazone, Rot: Rotenone, Ant: Antimycin.

T37 A. WT strain C57BL6JOlaHsd ____ AC G www.ww M GAPDH 1 40 CAGCAA M T37M GAPDH 2 YG 200 \mathbb{M} WΜ Δ T37M GAPDH 3 40 CAGCAAC 70 Δ hm T37M GAPDH 4 TGAAAA h hΜ T37M GAPDH 5 70 CTATGTGCCC 80 F G T T T G AAAA 1/m WΜ $\langle M \rangle$ GAPDH 6 T37M 40 50 60 70 T CAG CAAG G ACCA G T T CCCAG AG G T C T AT G T G C C C 80 AYGGTGTTTGAAAA M MT37M



C <u>Seq mRhoA WT</u>

atggctgccatcaggaagaaactggtgattgttggtgatggaggtggtggaggacagtgctatgtgccatagtcttcagcaaggaccagttcccaga ggtctatgtgcccacggtgtttgaaaactatgtggcggatatcgaggtggatgggaagcaggtagagttggctttatgggacacagctggacag gaagattatgaccgcctgcggcctctctcttatccagacaccgatgttatattgatgtgttttccattgacagccctgatagtttagaaaactaccca gaaaaatggactccagaagtcaagcatttctgtccaaatgtgcccatcatcctggttgggaacaagaaggaccttcggaatgaggagcacac gagacgggagttggccaaaatgaagcaggagccggtaaaacctgaagaaggcaggagatatggcaaacaggatggcctttgggaagaaaa ggagtgttcagcaaagaccaaagatggagtgaggaggggtttttgagaaggcacggagagtggccacggagagatatggcaaacggggggtggccaacgtgggaagaaaa gtctgggtgcctcatcttgtga

MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDGKQVELALWDTAGQEDYD RLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRNDEHTRRELAKMK QEPVKPEEGRDMANRIGAFGYMECSAKTKDGVREVFEMATRAALQARRGKKKSGCLIL*

Seq mRhoA T37M + bp Indel

MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPMVFENYVADRISRWMGSR*

Alignment mRhoA WT/ mRhoA T37M+ 5bp Indel

QEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRND EHTRRELAKMKQEPVKPEEGRDMANRIGAFGYMECSAKTKDGVREVFEMATRAALQARRGKK KSGCLIL*

Figure S5: *RHOA* mutations revealed in plck-GAPDH CD4⁺ cells as compared to C57BL6JOlaHsd WT strain (related to figure 6).

(A) Results of Sanger sequencing of the *RHOA* locus containing T37M mutation (indicated by a square) in the GAPDH tumors. WT is representative of n=5. For GAPDH tumors 6/15 are shown. (B) *RHOA* locus from whole exome sequencing of the C57BL6JOlaHsd WT strain. The T37 position and INDEL position found mutated in the GAPDH tumors are absent as indicated by the squares. (C) Comparison of *RHOA* DNA and protein sequences for WT and plck-GAPDH tumors (T37M + INDEL).





Figure S6: Implication of NF-κB pathway in the T cell lineage of plck-GAPDH mice (related to Figure 7).

(A) <u>4-week old mice</u> were sacrified and thymocytes isolated for analysis. mRNA expression levels detected by Q-PCR of target genes of the NF- κ B canonical and non-canonical pathway in plck-GAPDH and WT mice (mean ± SD, n=3; *p<0.05, **p<0.01, t-test student).

(B) Immunoblot analysis of p100, P-p100, p65, P-p65, p52, and V5 in WT and plck-GAPDH thymocytes of young mice. (C) Immunoprecipitation (IP) of GAPDH-V5 (IP-V5) and of TRAF2 (IP TRAF2) in thymocytes of young plck-GAPDH mice followed by immunoblotting using anti-GAPDH or anti-TRAF2 antibodies. A non-relevant isotype antibody was used as a control. INPUT: thymocyte lysate before IP.

(D) Immunoblot analysis of p100, P-p100, p65, P-p65, p52, and V5 in CD4⁺ splenic T cells isolated from WT or lymphoma bearing plck-GAPDH mice. For B and D only 1 representative data is shown for each condition and the other 2 lanes left and right are not shown.

(E) mRNA expression levels by Q-PCR in CD4⁺ T cells of lymphoma bearing plck-lkB^{-/-} and WT mice for canonical and non-canonical pathway genes (mean \pm SD, n=3; *p<0.05, **p<0.01, ****p<0.0001, student t-test).

(F) mRNA expression levels by Q-PCR in CD4⁺ T cells of 1 year old pre-disease plck-GAPDH and WT mice for canonical and non-canonical pathway genes (mean ± SD, n=5; *p<0.05, **p<0.01, ***p<0.001, student t-test).

(G, H) CD4⁺ T cells were isolated from 1-year old mice by negative selection from WT, plck-GAPDH, plck-p65^{-/-} and plck-GAPDH/p65^{-/-} mice and were cultured under Tfh differentiation conditions. Representative FACS plot and mRNA expression levels by Q-PCR of the Tfh marker (PD-1, CXCR5, ICOS, IL-21, CXCL13 and Bcl-6) expression in CD4⁺ T cell from wild-type (WT), plck-GAPDH, plck-p65^{-/-} and plck-GAPDH/p65^{-/-} mice after 21 days of culture in Tfh differentiation medium (G) (mean ± SD, n=3; * p < 0.05, *** p<0.001, one way ANOVA). mRNA expression levels by Q-PCR in CD4⁺ T cells of 1-year old plck-GAPDH, plck-p65^{-/-} and plck-GAPDH/p65^{-/-} versus WT mice for canonical and non-canonical pathway genes after 21 d of culture in Tfh differentiation medium (H) (mean ± SD, n=3; *p<0.05, **p<0.01, *** p<0.001, one way ANOVA).

(I) Schematic presentation of KA injections into plck-GAPDH tumor engrafted NSG mice. Splenic lymphoma cells from plck-GAPDH mice were injected intravenously into recipient NSG mice (n= 10). Five lymphoma engrafted mice were treated with KA and 5 with vehicle (PBS). (J) Comparison of survival curves between KA treated (n=5) and vehicle treated (n=5) mice is shown. No significant difference was detected by Mantel-cox analysis.

(K) Verification of *in vivo* inhibition of GAPDH enzymatic activity in splenocytes of KA and vehicule treated mice (mean \pm SD, n=4; *** p<0.001, student t-test). (L) Spleen weight of KA and vehicle treated mice at sacrifice. (M) % B cells (CD19⁺) in the spleen for the indicated treatments groups at sacrifice. (N) % T cells (CD3⁺) and Ratio of CD4⁺/CD8⁺ T cells in the spleens at sacrifice. For (L-N) statistics, mean \pm SD, n=4, ns: not significant, student t-test. (O) % PD-1^{high} CD4⁺ T cells in the spleen and detection of Tfh marker mRNA expression by Q-PCR (CXCL13, IL-21, BcI-6) in CD4⁺ cells for the indicated treatments groups at sacrifice (mean \pm SD, n=4; *p<0.05, **p<0.01, one way ANOVA). (P) mRNA expression levels by Q-PCR for canonical and non-canonical pathway genes in CD4⁺ T cells for KA treated compared to vehicle treated mice and WT CD4⁺ splenocytes, for which mRNA level was set to 1 (mean \pm SD, n=4;*p<0.05, **p<0.01, *** p<0.001, *** p<0.0001, one way ANOVA).



Figure S7: An NIK-specific inhibitor allows inhibition of the non-canonical pathway of NF- κ B (related to Figure 8). (A) *In vitro* inhibition of NIK ATPase activity (ADP-Glo assay) with indicated concentrations of NIK^{inh}. The insert shows the structure of the NIK inhibitor drug. (B) Affinity precipitation of NIK by streptavidin-conjugated magnetic beads and biotinylated NIK^{inh} or mock compounds. (C) mRNA levels by Q-PCR of EZH2 or CDKN1A in A375 cells treated for 96 hr with increasing concentrations of NIK^{inh} (mean ± SD, n=3, *p<0.05, **p<0.01, one way ANOVA) (D) Variation of protein expression level in melanoma (A375, C12.06) or lung carcinoma cells (A549) treated for 96 hr with NIK^{inh}. (E) Effect of NF- κ B2 KD (96 hr) or NIK^{inh} treatment (10µM, 96 hr) on the cell number of different melanoma (A375, C12.06) or lung carcinoma cells (A549). Healthy human melanocytes and fibroblasts were unaffected by the treatment. The cell number is normalized to the corresponding DMSO-treated control cells (mean ± SD, n=3, *p<0.05, student t-test). (F) Effect of NF- κ B2 knock-down (96 hr), NIK knock-down or NIK^{inh} treatment (10µM, 96 hr) on the cell number of different B cell lymphoma cell lines (u2932, Karpas 422, SU-DHL-6). Healthy human T cells were unaffected by the treatment (mean ± SD, n=3, ****p<0.0001, one way ANOVA).



Figure S8: NIK^{inh}, anti-PD-1 and NIK^{inh} + anti-PD-1 treatment have an inhibiting effect on tumor development (related to figure 8)

(A-C) Single NIK^{inh} and NIK^{inh} + anti-PD-1 treatment of plck-GAPDH tumor engrafted mice. Spleen size of NIK^{inh}, double treated and vehicle treated mice at sacrifice (A) (mean ± SD, n=4, **p<0.01, one way ANOVA). Surface expression of the different TCR-β chains analyzed by FACS on the original engrafted plck-GAPDH CD4⁺ T cells (plck-GAPDH) and on the CD4 T-cells in the spleens of NIKinh, NIKinh + anti-PD-1 treated NSG mice at sacrifice (B). mRNA expression levels by Q-PCR for canonical and non-canonical NF-kB pathway genes and target genes in CD4⁺ T cells for NIK^{inh} + anti-PD-1 double treated mice as compared to the vehicle treated group for which mRNA level was set to 1 (C) (mean ± SD, n=4, *p<0.05, **p<0.01, ***p<0.001, student t-test). (D) PD-1 expression on CD4⁺ or CD8⁺ T cells and PD-L1 expression on CD4⁺, CD8⁺ and CD19⁺ cells in primary plck-GAPDH tumors. Representative of n=4. (E-J) Single anti-PD-1 treatment of plck-GAPDH tumor engrafted mice. Survival curve from plck-GAPDH tumor engrafted NSG mice treated with an isotype (n=5) or anti-PD-1 mAb (n=5) (E) (**p<0.01, Mantel-cox analysis). FACS analysis for the CD4+T cells in the spleen of anti-PD-1 isotype mAb treated tumor recipient mice (F). Ratio of CD4⁺/CD8⁺ T cells in both treatment groups (G). FACS analysis for B cells in both treatment groups (H). GL-7 expression levels in B cells in the spleen of tumor recipient mice treated with isotype or anti-PD-1 mAb (I). (E-I) statistics, mean ± SD n=4, *** p<0.001, **p<0.01, student t-test. (J) CD8 T cell cytotoxicity in the spleens of isotype and anti-PD-1 mAb treated tumor recipient mice. Splenocytes were activated with PMA/ionomycin, then stained for CD8, IFNy, perforin and Granzyme B. Representative of n=3.

Table S1: Comparison between disease characteristics of plck-GAPDH tumor lymphoma mice and AITLpatients. Related to Figures 1 to 6.

				AITL patient	Plck GAPDH mice
		Age of tumo	r appearance	59-65 years	18-24 months
		Splenomegaly/ Enlarged MES L	Hepatomegaly/ LN and ectopic N	\checkmark	\checkmark
		Vasculariz	ation of LN	\checkmark	\checkmark
Clinical f	eatures	Architecture a spleen, liv	Ilteration of the ver and LN	\checkmark	\checkmark
		Skin	Rash	1	1
		Abdomin	al Ascites	\checkmark	\checkmark
		Ki67+ prolife	erative T cells	\checkmark	\checkmark
		Tfh phenotype CXCR5⁺ CX	(CD4 ⁺ PD-1 ^{high} CL13 ⁺ Bcl-6 ⁺)	\checkmark	\checkmark
	T cells	Activated mem	nory CD4+ cells	\checkmark	\checkmark
		Inflammatory cytokines production (IFN γ , IL-10, TNF α , IL-1 β , IL-6)		\checkmark	\checkmark
		TCRβ	clonality	\checkmark	\checkmark
	B cells	Appearance of germinal center and plasma B cells		\checkmark	\checkmark
Immuno-		Hyper-immunoglobulinemia/ auto-antibodies detection		\checkmark	\checkmark
phenotype	Follicular Dendritic cells	CD35 ⁺ FDC cells		\checkmark	\checkmark
			PD-1-PD-L1	\checkmark	\checkmark
		Ligand-	Fas-FasL	\checkmark	\checkmark
	Interaction T-	interaction	ICOS-ICOS L	\checkmark	\checkmark
	B cells		IL-21-IL-21R	\checkmark	\checkmark
		Cytokines-Chemokines (CXCL13, CCL5, CCL2, CCL8)		\checkmark	\checkmark
	BM infiltration			\checkmark	\checkmark
	NSG engraftme (Tfh, DC, GCB	ent reproduces th cells)	ne phenotype	√	√
	Activation of no	n-canonical NF-	kB pathway	\checkmark	\checkmark
Mecanisms	Increase of EZI	H2 expression		✓	√
	Increase of NIK	expression		\checkmark	\checkmark

Table S2: Comparison of GEA for genes implicated in humoral immune response, vascularisation and proliferation in splenocytes of WT and plck-GAPDH lymphoma mice. Related to Figure 2.

Gene Symbol	Gene name	Fold Change
Humoral Immune F	Response	
IGHE	Immunoglobulin heavy constant epsilon	301.99
IGLC1	Immunoglobulin lambda constant 1	136.55
IGLV1	Immunoglobulin lambda variable 1	120.32
C1QNF1	Complement C1q Tumor necrosis factor related protein 1	13.10
IGSF3	Immunoglobulin superfamily member 3	11.78
IGSF9B	Immunoglobulin superfamily member 9b	9.49
TNFRSF8	Tumor necrosis factor receptor superfamily member 8	7.72
TNFSF15	Tumor necrosis factor superfamily member 15	7.59
TNFSF17	Tumor necrosis factor superfamily member 17	4.69
SDC1	Syndecan 1 (CD138 antigen)	4.13
TNFSF9	Tumor necrosis factor superfamily member 9	3.40
C3	Complement component 3	3.07
TNFRSF4	Tumor necrosis factor receptor superfamily member 4	2.46
IGHA1	Immunoglobulin heavy constant alpha	2.33
C1R	Complement component 1, r subcomponent a	2.28
TNFSF14	Tumor necrosis factor superfamily member 14	2.15
IGHG2	Immunoglobulin heavy constant gamma 2B	2.13
IGKC	Immunoglobulin Kappa constant	1.20
IGLC2	Immunoglobulin lambda constant 2	1.11
Vascular biology		
VEGFA	Vascular endothelial growth factor A	18.11
SERPINF1	Serpin family F member 1	7.6
EFNB2	Ephrin B2	6.34
SERPING1	Serpin family G member 1	3.87
GJA1	Gap junction Protein alpha 1	2.85
PGF	Placental growth factor	2.11
GAS6	Growth arrest specific 6	1.87
SERPINH1	Serpin family H member 1	1.74
SELP	Selectin P	1.46
EPAS1	Endothelial PAS Domain Protein 1	1.37
	-	
Proliferation-relate	d genes	
GSPT2	G1 to S phase transition 2	3.30
CDK5R1	Cyclin dependant Kinase S regulatory subunit 1	2.12
CDC42BPB	CDC42 binding protein kinase beta	2.04
CCND2	Cyclin D2	1.81
CDC42SE2	CDC42 small effector 2	1.34
CDC25b	Cell division cycle 25B	1.28
CCNI	Cyclin I	1.26
CDC37	Cell division cycle 37	1.08
CCNG2	Cyclin G2	1.04

Table S3: List of genes frequently mutated in AITL found to be altered in 6 different plck-GAPDH T cell lymphomas by whole exome sequencing*. Related to Figure 6.



*Detailed analysis of these mutations, which are intronic or intergenic for the vast majority is available in supplementary table 4.

Table S4 : Detailed analysis of the mutations detected by whole exome sequencing found in the plck-GAPDH tumors and frequently mutated in AITL patient as listed in supplementary Table 3. Related to Figure 6.

	I	1		1		1		
Gene	Tumor	Mutation	Function	chr	Start	End	Ref	Obs
	C1	type						
	GI	-	-	-	-	-	-	-
	GZ	-	-	-	-	-	-	-
DNIATOA	G3	SNP	intronic	12	3903348	3902248	С	Т
DNMT3A	G4	indel	Intergenic « DNMT3A- POMC »	12	3942218	3942218	TG	Deletion of one bp « G »
G5 G6		-	-	-	-	-	-	-
	G6	-	-	-	-	-	-	-
G1		indel	intronic	3	13319977	133199780	TAAGA	Deletion of 4 bp « AAGA »
	G2	SNP	Intergenic « Tet2-Gm23011 »	3	133229268	133229268	С	G
TET2	G3	-	-	-	-	-	-	-
	G4	indel	Intergenic « Tet2-Gm23011 »	3	133241935	133241935	СТ	Deletion of one bp « T »
	G5	-	-	-	-	-	-	-
	G6	indel	intronic	3	133204436	133204438	CTTT	Deletionof 3 bp « TTT »
	G1	indel	ncBNA intronic	7	87241430	87241430	CGTTT	Deletion of 4 bn «GTTT »
	62	indel		7	07241430	07241450	тс	Deletion of one hp "G "
	GZ	indei	IICKINA IIIUTOIIIC		87240450	87240450	10	Deletion of one bp « G »
	63	-	-	-	-	-	-	-
IDH2	G4	-	-	-	-	-	-	-
	G5	indel	Intergenic « IDH2- Gm27592 »	7	87285541	87285543	GAAA	Deletion od 3 bp « AAA »
	G6	-	-	-	-	-	-	-
	G1	-	-	-	-	-	-	-
	G2	-	-	-	-	-	-	-
	63	indel	intropic	9	20746885	20746886	ΤCΔ	Deletion of 2 hn « CA »
DNMT1	63	inder	intronic	5	20740005	20740000	10/1	Deletion of 2 bp « ert »
	64	-	-	-	-	-	-	-
	G5	-	-	-	-	-	-	-
	G6	SNP	UTR3	9	20711871	20711871	Т	G
	61	-						-
	01			6	17506450	17506450		
	G2	Indel	ncRNA intronic	6	47506152	47506153	IGA	Deletion of 2 bp « GA »
		indel	intronic	6	47543582	47543582	GA	Deletion of one bp « A »
EZH2	G3	indel	ncRNA intronic	6	47506152	47506153	TGA	Deletion of 2 bp « GA »
	G4	-	-	-	-	-	-	-
	G5	-	-	-	-	-	-	-
				6		17506450		
	Gb	indel	nckina intronic	6	47506152	47506153	IGA	Deletion of 2 bp « GA »
	G1	indel	ncRNA intronic	16	23972062	23972062	Т	GGAA »
	G2	-	-	-	-	-	-	-
DCLC	G3	-	-	-	-	-	-	-
BCLO	G4	-	-	-	-	-	-	-
	G5	-	-	-	-	-	-	-
	G6	indel	ncRNA intronic	16	23972062	23972062	Т	Insertion of 4 bp « GGAA »
	G1	-	-	-	-	-	-	-
	62	indel	LITR3	1	65205966	65205966	ТΔ	Deletion of one bn « A »
	02	inder	011(5	-	05205500	05205500		
	G3	indel	UTR3	1	65205966	65205966	TA	Deletion of one bp « A »
IDH1			Intronic	1	65221711	65221712	TAA	Deletion of 2 bp « AA »
10111			UTR3	1	65205966	65205966	TA	Deletion of one bp « A »
	G4	indel	Intronic	1	65221711	65221712	TAA	Deletion of 2 bp « AA »
	65		_	-	-	-	-	- '
	65							
	60	-	- inter:-	15	91452004	-	-	Deletion of the time
	GI	Indel	Intronic	15	81453984	81453984	AI	Deletion of one bp « 1 »
	G2	indel	intronic	15	81471922	81471922	G	Insertion of one bp « T »
ED300	G3	indel	intronic	15	81453984	81453984	AT	Deletion of one bp « T »
LF 300	G4	-	-	-	-	-	-	-
	G5	indel	intronic	15	81471922	81471922	G	Insertion of one bp « T »
	G6	indel	intronic	15	81453984	81453984	AT	Deletion of one bn « T »
					01.00007	01.00004		
110.000	61	-	-	-	-	-	-	-
HDAC2	G2	-	-	-	-	-	-	-
1	G3		-	I -	-	-	-	-

	G4	-	-	-	-	-	-	-
	65			10	26042042	26042045	CCATA	Deletion of 4 bp
	65	Indel	Intergenic « HDAC2-Marcks »	10	36812042	36812045	GGATA	« GATA »
	G6	-	-	-	-	-	-	-
	G1	-	-	-	-	-	-	-
	62			_		_	_	
	63	indel	intronic	2	71276946	712769/17	ATG	Deletion of 2 bn « TG »
HAII	G4	-	-	-	-	-	-	-
	G5	-	-	-	-	-	-	-
	G6	-	-	-	-	-	-	-
	G1	SNP	intronic	17	32357287	32357287	С	G
	62	SNP	intronic	17	32357287	32357287	C	G
	02	5111	intronic	17	52557267	52557207	c	5
BRD4	G3	indel	intronic	17	32349714	32349715	TTG	Deletion of 2 bp « TG »
	G4	SNP	intronic	17	32357287	32357287	С	G
	G5	-	-	-	-	-	-	-
	G6	-	-	-	-	-	-	-
	G1	-	-	-	-	-	-	-
	G2	-	-	-	-	-	-	-
	G3	-	-	-	-	-	-	-
CD28	64	in de l	DNA internit	1	0700007	60700007	â	Insertion of 3 bp
	G4	Indel	Incrina Intronic	1	60799807	60799807	А	« AGG »
	G5	-	-	-	-	-	-	-
	G6	-	-	-	-	-	-	-
	G1	-	-	-	-	-	-	-
	G2	indel	intronic	17	57446324	57446325	TTC	Deletion of 2 bp « TC »
	G3	-	-	-	-	-	-	-
VAV1	G4	-	-	-	-	-	-	-
	G5	-	-	-	-	-	-	-
	66	indel	Intergenic « Trin10-Vav1 »	17	57/170/2	57/170/15	TTCTC	Deletion of 4 bp
	00	inder	intergenie « mpio-vavi »	17	57417042	57417045	THEIE	« TCTC »
		11 SNP	intronic	10	39115801	39115873		
	61		Intronic	10	39115830	39115830	A	Insertion of 3 bp « CCT »
	01	indel	Intronic	10	39115838	39115838	С	Insertion of one bp « A »
			intronic	10	39115840	39115842	CTTT	Deletion of 3 bp « TTT »
	62	SNP	intronic	10	39115735	39115735	А	Т
EVN		Intergenic « Wisp3-Fyn »	10	38918520	38918520	G	А	
	G3	-	-	-	-	-	-	-
		SNP	intronic	10	39115735	39115735	А	т
	G4	indel	intronic	10	39246445	39246446	ATG	Deletion of 2 bp « TG »
	G5	-	-	-	-	-	-	-
	<u> </u>	SNP	intronic	10	39115735	39115735	А	Т
	Gb	indel	intronic	10	39246445	39246446	ATG	Deletion of 2 bp « TG »
	G1	-	-	-	-	-	-	-
				2	27281829	27281829	А	G
				2	27281841	27281841	А	G
	G2	5 SNP	exonic	2	27281916	27281916	С	G
VAV2				2	27281931	27281931	G	С
VAVZ				2	27281934	27281934	G	С
1	G3	-	-	-	-	-	-	-
	G4	-	-	-	-	-	-	-
	G5	-	-	-	-	-	-	-
	G6	-	-	-	-	-	-	-
	G1	-		-	-	-	-	-
	G2	-	-	-	-	-	-	-
	G3	SNP	UTR5	18	10181344	10181344	Т	A
		indel	intronic	18	10104324	10104324	TA	Deletion of one bp « A »
ROCK1	G4	indel	intronic	18	10104324	10104324	TA	Deletion of one bp « A »
				18	10079282	10079282	GA	Deletion of one bp « A »
	G5	indel	intronic	18	10104324	10104324	TA	Deletion of one bp « A »
1				18	10150974	10150974	ŤĊ	Deletion of one bp « C »
	Gb C1	-	-	-	-	-	-	-
DIKOD1	62	-	-	-	-	-	-	-
LIVOUT	63	-	-	-	-	-	-	-
L		-	-	1	-	-	-	-

	SNP In		Intergenic « PIK3r1- Gm25808 »	13	102762450	102762450	С	Т
	64	indel	Intergenic « PIK3r1- Gm25808 »	13	102762426	102762427	GTA	Deletion of 2 bp « TA »
	G5		-	-	-	-	-	
	G6	indel	Intergenic	13	101723575	101723662		Deletion of 87 bp
	G1	-	-	-	-	-	-	-
	G2	-	-	-	-	-	-	-
CADD11	G3	indel	intronic	5	141352101	141352106	AACACAC	Deletion od 6 bp « ACACAC »
CARDII	C A	indal	intropio	-	141384723	141384724	TTG	Deletion of 2 bp « TG »
	G4	Indel	Intronic	5	141440277	141440278	AGT	Deletion of 2 bp « GT »
	G5	indel	intronic	5	141384723	141384724	TTG	Deletion of 2 bp « TG »
	G6	-	-	-	-	-	-	-
		18 SNP	intronic	4	129235734	129238176		
	G1	indel	intronic	4	129235696	129236596	А	Insertion of 4 bp « CAGG »
		indel	Intronic	4	129236588	129236588	С	Insertion of one bp « A »
LCK	G2	15 SNP	intronic	4	129235879	129238746		
	G3	9 SNP	intronic	4	129235734	129238176		
	G4	-	-	-	-	-	-	-
	G5	-	-	-	-	-	-	-
	G6	-	-	-	-	-	-	-
	G1	-	-	-	-	-	-	-
	G2	-	-	-	-	-	-	-
	G3	indel	Intergenic « RIK-TRAF6 »	2	101497980	101497985	CTGATGA	Deletion of 6 bp « TGATGA »
TRAF6		indol	Intergonia « DIK TDAFC »	, ,	101497980	101497985	CTGATGA	Deletion of 6 bp « TGATGA »
	04	inder	intergenic « Kik-TKAPO »	2	101514698	101514701	AACAC	Deletion of 4 bp « ACAC »
	G5	-	-	-	-	-	-	-
	G6	-	-	-	-	-	-	-
	G1	-	-	-	-	-	-	-
	G2	-	-	-	-	-	-	-
CTAT2	G3	-	-	-	-	-	-	-
STATS	G4	-	-	-	-	-	-	-
	G5	indel	ncRNA intronic	11	100759644	100759645	GGA	Deletion of 2 bp « GA »
	G6	indel	ncRNA intronic	11	100759643	100759643	G	Insertion of 2 bp « GA »
	G1	indel	Intergenic « Gm5518-KAK2 »	19	29319648	29319662	AAAACCAA ACCAAATC	Deletion of 15 bp
	G2	-	-	-	-	-	-	-
JAK2	G3	indel	intergenic	19	29323534	29323535	TCA	Deletion of 2 bp « CA »
1	G4	-	-	-	-	-	-	-
	G5	-	-	-	-	-	-	-
	G6	-	-	-	-	-	-	-

	94100				
Mice	Gene	Exonic Mutation type	Chr	Ref	Obs
		Incortion	2	G	GA
		Insertion	2	G	GA
		Deletion	3	G	GAA
	POU3F1	Deletion	4	CG	C
	ZFP982*	Deletion	4	GC	G
	01001	Deletion	5	10	1
	ANKRD17	Deletion	5	CG	С
G1	AUTS2	Insertion	5	A	AG
	RIK	Deletion	6	TC	Т
	Gm4978*	Deletion	9	AG	А
	RAB10*	Deletion	12	AG	А
	PROX2*	Deletion	12	Del of 19 bp	
	RCOR1*	Deletion	12	CG	С
	Gm8369*	Deletion	19	CGT	С
	MROH2A*	Deletion	1	GCC	G
	PPIG	Insertion	2	Insertion of 55	
				bp	
	RIK*	Deletion	2	AC	Α
	TOMM34*	Insertion	2	Insertion of 64	
				bp	
	Gm10985	Deletion	3	СТА	С
	MPDZ	Insertion	4	G	GT
	CALR4	Deletion	4	GT	G
	GBP8	Deletion	5	GT	G
	WDR66*	Deletion	5	GT	G
G2	CUX1*	Deletion	5	GCC	G
	Gm10722	Deletion	9	GTTTT	G
	Gm5611*	Deletion	9	GTT	G
	Gm4978*	Deletion	9	AG	Δ
	EYCO1*	Deletion	9	ATTC	Δ
	KIF1BP*	Deletion	10	GCACA	6
	NACAD*	Deletion	11	TG	т
	Gm6316*	Deletion	12	AG	^
	7SW/IM6	Insertion	12	AG G	60
		Insertion	15	G	GCTGT
	KIEC1*	Insertion	17	C C	
	COL443	Deletion	1	GT	6
		Deletion	1		G
		Deletion	1	GCC	G
		Deletion	2	G	GI
		Deletion	2	GA	G
	ULFR1133	Deletion	2	CA	C
		Deletion	4	GC	G
	ADAMITS3	Deletion	5	GI	G
	AP1S1	Deletion	5	AICC	A
	ADCK2	Deletion	Ь	IGIGGA	1
G3	CD163	Deletion	6	CA	С
	BHLHE41	Deletion	6	Deletion of 33 bp	-
	CACNG8*	Deletion	7	GC	G
	MAP4K1	Insertion	7	С	CG
	KLK1B7-ps	Deletion	7	GA	G
	NACAD*	Deletion	11	TG	Т
	LRFN5	Deletion	12	CAGCG	С
	PROX2*	Deletion	12	Deletion of 20 bp	
	RCOR1*	Deletion	12	CG	С
	MAST4*	Insertion	13	Т	TG

Table S5: Genes showing mutations in plck-GAPDH tumors identified by whole exome sequencing other than genes frequently mutated in hAITL.

 Related to Figure 6

	KIFC5B	Insertion	17	Insertion of 25	
				bp	
	KIFC1*	Insertion	17	С	CAA
	LTBP1	Deletion	17	TC	Т
	Gm8369	Deletion	19	CGT	С
	OLFR1323	Deletion	Х	GA	G
	RIK	Deletion	1	ACT	А
	SPTBN5*	Deletion	2	Deletion of 51 bp	
	SPEER4D	Deletion	5	CA	С
	CUX1*	Deletion	5	CCAGGGCTGGT	С
64	CACNG8*	Deletion	7	GC	G
64	Gm4978*	Deletion	9	AG	Α
	Gm6316	Deletion	12	AG	А
	DBPHT22	Deletion	12	GCA	G
	PROX2*	Deletion	12	Deletion of 20 bp	
	TECTB*	Deletion	19	TC	Т
	ANKRD45	Deletion	1	AT	А
	SPTBN5*	Deletion	2	Deletion of 51 bp	
	CADPS2	Deletion	6	GA	G
	HOXA13	Deletion	6	GC	G
	Gm5611	Deletion	9	GTT	G
G5	ACAD11	Insertion	9	С	CA
	KIF1BP*	Deletion	10	GCA	G
	RAB10	Deletion	12	AG	А
	RCOR1*	Deletion	12	CG	С
	Gm5464	Insertion	14	G	GT
	PKHD1L1	Deletion	15	AT	Α
	RIK*	Deletion	2	AC	А
	SPTNB5*	Deletion	2	Deletion of 51 bp	
	TOMM34*	Insertion	2	Insertion of 64	
				bp	
	PLA2G5	Deletion	4	TG	Т
	WDR66	Insertion	5	G	GT
	CUX1*	Insertion	5	G	GA
	TMCC1	Insertion	6	Insertion of 80	
G6				bp	
	CACNG8*	Deletion	7	GC	G
	Gm10720	Deletion	9	TGG	Т
	Gm5611	Deletion	9	GTT	G
	FYCO1*	Deletion	9	ATTC	А
	KIF1BP*	Deletion	10	GCA	G
	NACAD*	Deletion	11	TG	Т
	KIFC1*	Insertion	17	С	CAA
	TECTB*	Deletion	19	TC	Т
*	1				

* : repeated in more than one mouse tumor

Tumor	Gene	Function	Chr	Ref	Obs
Tullion	SP110*		1	т	6
	VMN1877	Nonsynonymous SNP	7	Δ	6
61		Nonsynonymous SND	/	~	6
G1	CTF2A1	Nonsynonymous SNP	10	C	G T
	GIFZAL	Nonsynonymous SNP	12	C	1
	FBXW4*	Nonsynonymous SNP	19	G	A
	SP110*	Nonsynonymous SNP	1	Î	G
	PPIG*	Nonsynonymous SNP	2	C	
	PGD	Nonsynonymous SNP	4	G	
	CDHII	Nonsynonymous SNP	8	C	
	DPPA5A*	Nonsynonymous SNP	9	G	1
G2	SMAD6	Nonsynonymous SNP	9	С	G
	AW822073	Nonsynonymous SNP	10	G	Т
	DUXF3*	Nonsynonymous SNP	10	C	G
	PHF10	Nonsynonymous SNP	17	G	A
	TMEFF2	Nonsynonymous SNP	1	Т	С
	HMCN1	Nonsynonymous SNP	1	С	Т
	PPIG*	Nonsynonymous SNP	2	С	Т
	SRSF6*	Nonsynonymous SNP	2	С	G
	CHRNA1	Nonsynonymous SNP	2	А	С
	PDIA3	Nonsynonymous SNP	2	G	A
	DUSP2	Nonsynonymous SNP	2	G	С
	SPG20	Nonsynonymous SNP	3	G	Т
	RSBN1	Nonsynonymous SNP	3	Т	С
	CNTLN	Nonsynonymous SNP	4	А	G
	ADCK2	Nonsynonymous SNP	6	G	С
	MGAM	Nonsynonymous SNP	6	Т	С
	KCNA6	Nonsynonymous SNP	6	Т	С
63	CLEC2D	Nonsynonymous SNP	6	G	Т
05	HYDIN	Nonsynonymous SNP	8	С	Т
	ING2	Nonsynonymous SNP	8	G	А
	VWA5A	Nonsynonymous SNP	9	Т	А
	TRP53	Nonsynonymous SNP	11	С	Т
	FGF11	Nonsynonymous SNP	11	А	G
	HIST1H1D	Nonsynonymous SNP	13	С	Т
	IGLV3	Nonsynonymous SNP	16	С	Т
	IGLV1*	Nonsynonymous SNP	16	Т	G
	ROBO2	Nonsynonymous SNP	16	С	A
	PIM1	Nonsynonymous SNP	17	Т	А
	SP110*	Nonsynonymous SNP	1	Т	G
	PPIG*	Nonsynonymous SNP	2	С	Т
	FER1L4	Nonsynonymous SNP	2	G	C
	SRSF6*	Nonsynonymous SNP	2	А	Т
	KCTD8	Nonsynonymous SNP	5	А	Т
	LSM3	Nonsynonymous SNP	6	А	G
64	MAZ	Nonsynonymous SNP	7	А	G
04	DPPA5A*	Nonsynonymous SNP	9	G	Т
	PCDH1	Nonsynonymous SNP	18	G	Т
	FBXW4*	Nonsynonymous SNP	19	G	А
	FAT4*	Nonsynonymous SNP	3	С	А
	BRCA2	Nonsynonymous SNP	5	Т	А
	LDHa	Nonsynonymous SNP	7	С	Т
	CCR1L1*	Nonsynonymous SNP	9	Т	G
C.F.	DNAL1	Nonsynonymous SNP	12	А	G
60	DRD1	Nonsynonymous SNP	13	G	А
	LIMA1	Nonsynonymous SNP	15	G	А
	IGLV1*	Nonsynonymous SNP	16	G	A

Table S6: Genes identified by whole exome sequencing of 6 plck-GAPDH mouselymphomasshowing a non-synonymous SNP mutations. Related to Figure 6

	MUC4	Nonsynonymous SNP	16	Т	G
	FAS*	Nonsynonymous SNP	19	А	С
	CD5	Nonsynonymous SNP	19	G	А
	OLFR1143	Nonsynonymous SNP	2	А	С
	OLFR1179	Nonsynonymous SNP	2	G	С
	OLFR1198	Nonsynonymous SNP	2	G	Т
	AQR	Nonsynonymous SNP	2	С	G
	MAPKBP1	Nonsynonymous SNP	2	G	Т
	SPG11	Nonsynonymous SNP	2	Т	G
	CENPB	Nonsynonymous SNP	2	С	G
	EEF1A2	Nonsynonymous SNP	2	Т	С
	MYT1	Stopgain	2	С	Т
	FAT4*	Nonsynonymous SNP	3	Т	С
	NAA15	Nonsynonymous SNP	3	Т	G
	CACNG8	Nonsynonymous SNP	7	Т	G
	CTSC	Nonsynonymous SNP	7	Т	С
	ANKS4B	Nonsynonymous SNP	7	G	А
	MTUS1	Nonsynonymous SNP	8	А	G
G6	DDX60	Nonsynonymous SNP	8	А	G
	TMED6	Nonsynonymous SNP	8	G	Т
	PKD1L2	Nonsynonymous SNP	8	А	С
	ADAMTS7	Stopgain	9	Т	G
	CCR1L1*	Nonsynonymous SNP	9	Т	G
	HELB	Nonsynonymous SNP	10	А	С
	LAMA1	Nonsynonymous SNP	17	С	G
	NRXN1	Nonsynonymous SNP	17	T	c
	ASXI 3	Nonsynonymous SNP	18	т	G
	PCDHB10	Nonsynonymous SNP	18	G	A
	FBN2	Nonsynonymous SNP	18	G	A
	AHNAK	Nonsynonymous SNP	19	С	т
	FAS*	Stopgain	19	Т	G

*repeated in more than 1 plck-GAPDH tumor

ivietitous	
NF-κB1 Forward	CATGGCAGACGATGATCCCTACG
NF-kB1 Reverse	ATTTGAAGGTATGGGCCATCTGTTGA
RelA Forward	TGTGGAGATCATCGAACAGCCG
RelA Reverse	TTCCTGGTCCTGTGTAGCCATTGAT
cRel Forward	GCCGTGTGAACAAGAACTGTGGAA
cRel Reverse	TTGTGAAAAAACACCTCTGGCTTCC
GADD45B Forward	CATCGCCCTGCAAATCCACTTCACGCTCAT
GADD45B Reverse	TTGTTGCCCCGGCTTTCTTCGCAGTAGCTG
E2F1 Forward	CAGTCTCTACTCAGCCTGGAGCAAGAACCG
E2F1 Reverse	GAGGTCCCCAAAGTCACAGTCGAAGAGGTC
NFkBia Forward	CCACTCCATCCTGAAGGCTACCAACTACAA
NFkBia Reverse	CCCCACACTTCAACAGGAGTGACACCAG
NF-KB2 Forward	AGCAGTGTTCAGAGTTGGGAGTGT
NF-ĸB2 Reverse	GGATCATAATCTCCATCATGTTCTTCTT
RelB Forward	GCCGAATCAACAAGGAGAGCG
RelB Reverse	
Ezh2 Forward	GCCAGACTGGGAAGAAATCTG
Ezh2 Reverse	TGTGCTGGAAAATCCAAGTCA
SP1 Forward	GTGTTGCTTGGTGTTTCCAC
SP1 Reverse	GCAGAATCAGCAGTTCCAGA
IRF4 Forward	
IRF4 Reverse	
ENPP2 Forward	
ENPP2 Reverse	
NOD2 Forward	
NOD2 Reverse	GAAGGGGAGAAGCCAATTIC
18s Forward	GTAACCCGTTGAACCCCATT
18s Reverse	CCATCCAATCGGTAGTAGCG
CXCL13 Forward	CTCTCCAGGCCACGGTATTC
CXCL13 Reverse	TGGCACGAGGATTCACACAT
GAPDH Forward	CGAGTTGGATAGGGCCTCT
GAPDH Reverse	CAAGGAGTAAGAAACCCTGGACC
IL-21 Forward	GGACAGTGGCCCATAAATCA
IL-21 Reverse	CAGGGTTTGATGGCTTGAGT
Bcl-6 Forward	GGAAGTTCATCAAGGCCAGT
Bcl-6 Reverse	GACCTCGGTAGGCCATGA
NIK Forward	GGAAATGGCACCTATGGACAA
NIK Reverse	CCAGCCCCACAAAACTCC
RHOA Forward	CTGCCATCAGGAAGAAACTGGTG
RHOA Reverse	CTGCTTCCCATCCACCTCGATATC
TET2 Forward	TGGTGGTCACCCTCAATA
TET2 Reverse	GAAGACTTTCCCTTCTCAGTC
IDH2 Forward	GTTCAAGCTGAAGAAAATGTGG
IDH2 Reverse	CTGGTCGCCATGGGCGTGCC
DNMT3A Forward	GGGTGTTTGGCTTCCCCGTCC
DNMT3A Reverse	CAGTCACAGCAAGAAACAAAAACC
Gamma7 Forward	GTGATACTCACAGAACAGGC
Gamma1 Forward	CCGGCAAAAAGCAAAAAGT
Gamma4 Forward	TGTCCTTGCAACCCCTACCCA
Common Gamma	CTTATGGAGATTTGTTTCAGC
Actin Forward	TTCTACAATGAGCTGCGTGTGGCT
Actin Reverse	TCTCCTTAATGTCACGCACGA
Human Ezh2 Forward	GGACCACAGTGTTACCAGCAT
Human Ezh2 Reverse	GTGGGGTCTTTATCCGCTCAG
Human CDKN1A Forward	ACCCTTGTGCCTCGCTCA
Human CDKN1A Reverse	AATCTGTCATGCTGGTCTGCC

Table S7 : Primers used for quantification of mRNA and for sequencing. Related to Star Methods

Article 2
La reprogrammation métabolique vers la phosphorylation oxidative des lymphocytes T folliculaires helper PD-1^{high} révèle une nouvelle option thérapeutique pour le lymphome T angioimmunoblastique

La reprogrammation métabolique est un processus majeur de la transformation tumorale. Elle implique un détournement des voies métaboliques physiologiquement actives dans une cellule saine, dans le but de permettre à la cellule tumorale de maintenir un statut bioénergétique compatible avec sa survie et sa proliferation. Il a été admis pendant plusieurs décennies que le métabolisme tumoral était fondé sur une utilisation accrue du glucose, une théorie connue comme l'effet Warburg. Cependant, au cours des dernières années, plusieurs études ont montré que les cellules cancéreuses peuvent aussi utiliser vers d'autres voies métaboliques pour faire face aux conditions environnementales stressantes.

Dans cette étude nous avons montré que les cellules tumorales du lymphome T angioimmunoblastique (AITL) utilisent la phosphorylation oxidative (OXPHOS) pour satisfaire leurs besoins énergétiques. En utilisant notre modèle préclinique plck-GAPDH qui récapitule étroitement les caractéristiques pathologiques et immunologiques de la pathologie humaine, nous avons observé que les cellules tumorales T folliculaires helper présentent une reduction du flux glycolytique et de la consommation du glucose associée à une forte dépendance à la respiration mitochondriale. De manière intéressante, nous avons démontré que ces perturbations métaboliques coincident avec une expression élevée du récepteur de coinhibition PD-1. Pour confirmer nos observations, nous avons montré que l'inhibition de la voie OXPHOS à l'aide d'un inhibiteur ciblant le complexe I de la chaîne respiratoire mitochondriale améliore la survie des souris greffées avec les cellules tumorales mAITL et permet de ralentir la croissance tumorale.

L'ensemble de nos résultats suggèrent que le ciblage de cette voie métabolique peut constituer une nouvelle approche thérapeutique efficace pour les patients atteints d'AITL.

Metabolic reprogramming toward oxidative phosphorylation in tumorogenic PD-1^{high} Follicular Helper T cells reveals a new therapeutic target for Angioimmunoblastic T Cell Lymphoma

Rana Mhaidly¹, Marie Tosolini², Johanna Chiche¹, Laura Mondragon¹, Philippe Gaulard³, François Lemonnier³, Jean-Jacques Fournié⁴, Jean-Ehrland Ricci^{1,6} and Els Verhoeyen^{1,5,6}

¹Université Côte d'Azur, INSERM, C3M, 06204 Nice, France

²Pôle Technologique du CRCT-Plateau BioInformatique, INSERM-UMR1037

³Université Paris-Est Créteil; Institut Mondor de Recherche Biomédicale, INSERMU955; Unité hémopathies lymphoïdes, Hôpitaux Universitaires Henri Mondor, Assistance publique des Hôpitaux de Paris, Créteil, France

⁴CRCT ; INSERM U1037-Université Paul Sabatier-CNRS ERL5294, Université de Toulouse ; Laboratoire d'Excellence TOUCAN; Programme Hospitalo-Universitaire en Cancérologie CAPTOR, Toulouse, France; IUCT, 31037 Toulouse, France

⁵CIRI, Université de Lyon; INSERM U1111; ENS de Lyon; University Lyon1; CNRS, UMR5308, 69007 Lyon, France

⁶Co-senior authors

Correspondance : Els Verhoeyen (els.verhoeyen@unice.fr/els.verhoeyen@ens-lyon.fr)

Key words: AITL, metabolism, glycolysis, oxidative phosphorylation, PD-1, GAPDH.

Abstract:

Cancer metabolic reprogramming has been recognized as one of the cancer hallmarks that promote cell proliferation, survival, as well as therapeutic resistance. However, the regulation of metabolism in T cell lymphoma is poorly understood. We demonstrated here that PD-1 co-inhibitory signals co-incided with a change in metabolic phenotype in angioimmunoblastic T cell lymphoma (AITL). Through genomic analysis of clinical human AITL data, we identified that AITL tumor cells use OXPHOS metabolism and enhanced mitochondrial reactions to fulfill their energetic requirement. By using our pre-clinical mouse model plck-GAPDH mimicking closely human AITL features, we confirmed that PD-1^{high} Tfh tumor cells exhibit a reduction of glycolytic flux and glucose uptake *in vivo* associated with a strong enrichment of OXPHOS metabolism using an inhibitor of mitochondrial electron transport chain (ETC) complex improved survival of AITL lymphoma-bearing mice. Taking together, our finding suggests that targeting this metabolic pathway can be a clinically efficient approach to inhibit tumor growth in AITL disease.

Discussions et Perspectives

Discussions et Perspectives

Le lymphome T angioimmunoblastique (AITL) est une hémopathie maligne caractérisée par des polyadénopathies et des symptômes atypiques. C'est un lymphome T périphérique qui, en raison de sa rareté, bénéficie de peu d'études (Leval et al., 2010). Jusqu'à présent, il n'existait que peu de modèles murins qui récapitulaient étroitement toutes les caractéristiques physiopathologiques et immuno-phénotypiques de cette pathologie humaine (Cortes et al., 2018).

Les travaux que nous avons réalisés au cours de cette étude nous ont permis de mettre en place un nouveau modèle préclinique d'AITL (plck-GAPDH) basé sur la surexpression de l'enzyme glycolytique GAPDH (Glycéraldéhyde-3-Phosphate Déshydrogénase), exclusivement dans les LT. Par des approches génétiques et immunologiques, nous avons montré que les souris plck-GAPDH présentaient des changements phénotypiques évidents, notamment : une splénomégalie, des ganglions lymphatiques hypertrophiés et une infiltration lymphocytaire importante au niveau du foie et de la moelle osseuse. De manière similaire à ce qu'il a été décrit chez les patients, nous avons montré chez ces souris une augmentation de la proportion des LT folliculaires helper CD4⁺ PD-1⁺ CXCR5⁺ Bcl-6⁺, oligoclonaux et prolifératifs, qui interagissaient avec les LB du centre germinatif et les cellules dendritiques folliculaires, formant ainsi un microenvironnement tumoral dans notre modèle murin. De manière intéressante, nous avons mis en évidence que la GAPDH induisait une activation de la voie de signalisation NF- κ B non canonique dans les souris plck-GAPDH et que cette voie était également activée chez les patients AITL. Le ciblage de cette voie par un nouvel inhibiteur de la kinase activatrice de NF-κB (NIK) associé à l'immunothérapie anti-PD-1 a montré des résultats satisfaisants se traduisant par une augmentation de la survie des souris traitées, associée à une induction efficace de la réponse immunitaire anticancéreuse (Mondragón et al., 2019).

Cependant, nos résultats soulèvent plusieurs questions importantes dans le but de finaliser la caractérisation de ce modèle murin ainsi que pour élucider de nouveaux mécanismes impliqués dans la pathogenèse de l'AITL et pour tester de nouvelles lignes thérapeutiques.

1. Rôle de la GAPDH dans la pathogenèse de l'AITL

La première partie de mon travail a consisté à caractériser les effets de la surexpression de la GAPDH sur le développement lymphocytaire T. Pour cela, nous avons généré un modèle de souris transgéniques qui surexpriment l'enzyme glycolytique la GAPDH uniquement dans les cellules T. Comme décrit précédemment, la GAPDH est connue pour être une protéine multifonctionnelle qui, en plus de son rôle dans le métabolisme glycolytique, remplit de nombreuses autres fonctions non-glycolytiques (Tableau 1).

Nous savions que le développement thymique nécessite une élimination des LT auto-réactifs ou non-fonctionnels par plusieurs processus de mort cellulaire (Hernandez et al., 2010). Comme décrit dans la partie introductive, il a été démontré que la surexpression de la GAPDH permet aux cellules d'échapper aux processus de la mort cellulaire indépendante des caspases (CICD) (Colell et al., 2007; Jacquin et al., 2013). De plus, la présence de la CICD au cours du développement des LT semble évidente puisque l'inhibition des caspases ou d'autres composants de l'apoptosome ne perturbe pas le développement et la maturation des LT (Bidère and Senik, 2001; Jäättelä and Tschopp, 2003). Ainsi, afin d'étudier le lien entre la GAPDH et la mort cellulaire dans notre modèle plck-GAPDH, nous avons analysé premièrement, les différentes populations de progéniteurs T (DN, DP, CD4⁺SP, CD8⁺SP) au cours des stades précoces de développement. Cependant, nous n'avons identifié aucune modification significative de distribution des LT dans le thymus des souris plck-GAPDH par rapport aux souris contrôles (Mondragón et al., 2019). Nos résultats nous ont permis d'éliminer l'hypothèse de départ qui suggérait un effet direct de la surexpression de la GAPDH sur la sélection et l'éducation des LT.

D'autre part, nous avons souligné un rôle de la GAPDH dans l'activation de la voie NF- κ B canonique. Comme décrit dans des études précédentes, nos résultats ont montré une interaction entre la GAPDH et la protéine activatrice de la voie NF- κ B TRAF2 (Chiche et al., 2015; Gao et al., 2013). Cette interaction induit une stabilisation de TRAF2 et une activation constitutive de la voie de signalisation. Dans notre modèle murin nous avons confirmé dans les thymocytes des souris jeunes, une activation de la voie NF- κ B canonique se traduisant par une surexpression des gènes cibles, y compris les gènes codant pour les cytokines et les chimiokines. Nous suggérons dans notre étude, que cette activation permet de mettre en place un microenvironnement inflammatoire qui est à l'origine du déclenchement de la voie non-canonique, retrouvée fortement activée dans les tumeurs AITL des souris plck-GAPDH. Nous avons ainsi établi un rôle de la GAPDH dans le déclenchement de l'AITL dans le modèle murin

plck-GAPDH, via sa fonction non-glycolytique d'activation de la voie NF-κB (Mondragón et al., 2019).

Pour évaluer l'implication plus tardive de la GAPDH dans le maintien de la pathologie, nous avons analysé l'effet de l'administration d'un inhibiteur de la GAPDH « l'acide koningique KA » sur la progression tumorale (Liberti et al., 2017). De façon surprenante, bien que l'activité de l'enzyme soit inhibée, nous n'avons pas observé de déplétion des cellules tumorales dans les souris traitées par rapport aux souris contrôles. Ces résultats nous ont permis de conclure que l'importance de la GAPDH dans la pathogenèse de l'AITL intervient lors des les stades précoces de déclenchement de la maladie qui, une fois établie, ne peut pas être inversée par une inhibition de la GAPDH.

Ainsi, sur la base de ces résultats nous avons proposé un nouveau modèle de développement de l'AITL dans lequel une surexpression de la GAPDH dans les progéniteurs T induit une activation constitutive de la voie NF-κB canonique et établit un clone précancéreux associé à un environnement inflammatoire favorable à la croissance tumorale. Suite à l'activation de la voie NF-κB non canonique, le développement T se réoriente vers la lignée Tfh qui est à l'origine du développement de l'AITLn (Figure 19). Après la mise en place de la maladie, la seule inhibition de la GAPDH est insuffisante pour restaurer un phénotype sain, montrant la présence d'autres mécanismes moléculaires et épigénétiques qui interviennent au cours de la progression tumorale. Nos résultats confirment que la GAPDH n'est pas un oncogène, mais intervient dans la progression tumorale par une activation des voies de signalisation qui présentent un rôle oncogénique.



Figure 19:Modèle de développement de l'AITL basé sur l'expression de la GAPDH et l'activation de la voie NF-kB

Par ailleurs, il a été démontré que, suite à une inhibition de la voie glycolytique, la GAPDH est libérée de ses fonctions glycolytiques et est liée aux éléments riches en AU au niveau de l'extrémité 3'UTR de l'ARN messager (ARNm) de l'IFN-γ induisant une inhibition de sa traduction (Chang et al., 2013). Dans un premier temps, nous avons observé une corrélation négative entre les niveaux d'expression de la GAPDH et de l'IFN-γ aussi bien dans les souris plck-GAPDH que chez les patients AITL (Figure 20A-B) confirmant ainsi qu'une augmentation de l'expression de la GAPDH est corrélée à une diminution de l'expression de la GAPDH est corrélée à une diminution de l'expression de l'IFN-γ. Nous avons confirmé cette observation par une analyse en cytométrie en flux effectuée sur les cellules CD8⁺ des tumeurs plck-GAPDH qui a montré une diminution de la sécrétion d'IFN-γ dans les cellules CD8⁺GAPDH, ce phénomène se traduisait par une diminution de la cytotoxicité des cellules CD8⁺ et par suite une absence de l'immunité anti-tumorale. Cependant, ces résultats sont préliminaires et devront être suivis par des analyses supplémentaires qui confirmeront la liaison de la GAPDH à l'ARNm de l'IFN-γ.

Nos résultats suggèrent ainsi un second rôle de la GAPDH dans la pathogenèse de l'AITL, et cette fois-ci par l'intermédiaire de sa fonction d'inhibition de la traduction des ARNm de l'IFN- γ qui pourrait être à l'origine de l'inhibition de l'immunité anti-tumorale.



Figure 20:Corrélation entre les niveaux d'expression de la GAPDH et de l'IFNy

- *A.* Corrélation entre les niveaux d'expression de la GAPDH et de l'IFN- γ dans les tumeurs isolées à partir des souris plck-GAPDH.
- *B.* Corrélation entre les niveaux d'expression de la GAPDH et de l'IFN- γ chez les patients *AITL*.
- *C.* Représentation en cytométrie en flux montrant les différences des niveaux de sécrétion de l'IFNγ entre les deux populations GAPDH^{high} et GAPDH^{low} des cellules CD8⁺ de la tumeur isolée à partir des souris plck-GAPDH

L'ensemble de nos résultats souligne un équilibre entre les fonctions glycolytiques et non glycolytiques de la GAPDH. Nous savions que suite à une dépendance cellulaire accrue à la glycolyse, la GAPDH exerce ses fonctions glycolytiques en priorité. Cependant, dans les conditions où la glycolyse est inhibée, la GAPDH se libère de sa fonction principale et exerce d'autres fonctions non-glycolytiques comme la liaison à ARNm de l'IFN- γ (Chang et al., 2013). Dans notre modèle murin basé sur la surexpression de la GAPDH dans la lignée lymphocytaire T, nous suggérons que l'excès de cette enzyme lui permet, en plus de la glycolyse, d'accomplir d'autres fonctions auxiliaires y compris l'activation de la voie NF- κ B comme nous l'avons décrit dans notre étude (Mondragón et al., 2019).

2. Activation et ciblage de la voie de signalisation NF-κB dans les tumeurs AITL

Comme décrit précédemment, nous avons montré dans cette étude une activation de la voie de signalisation NF- κ B non canonique aussi bien dans le modèle murin plck-GAPDH que chez les patients AITL. Nous avons confirmé cette activation par une analyse quantitative des niveaux d'expression de ses gènes cibles qui a révélé une forte augmentation de l'expression d'*Ezh2* et d'*IRF4*.

Pour confirmer que l'activation de cette voie peut être à l'origine du développement de l'AITL, nous avons utilisé des souris plck-IκB^{-/-} qui présentent une activation constitutive de la voie NF-κB canonique dans les LT. Comme pour les souris plck-GAPDH, les souris plck-IκB^{-/-} ont développé un lymphome T périphérique à dominance de cellules Tfh, associé à une prolifération des LB du centre germinatif. De manière intéressante, nous avons montré dans ces souris, une activation de la voie NF-κB non-canonique, avec une expression élevée des gènes cibles Ezh2 et IRF4, montrant une interconnexion entre les deux voies et confirmant ainsi nos résultats obtenus dans le modèle préclinique.

De plus, pour confirmer nos résultats, nous avons généré des souris plck-GAPDH/p65^{-/-} dans lesquelles la surexpression de la GAPDH est associée à une inactivation de la voie NF-κB. Nous avons montré que les cellules spléniques CD4⁺ isolées à partir de ces souris et cultivées dans des conditions cytokiniques spécifiques sont incapables de se différencier en Tfh, contrairement aux cellules isolées à partir des souris plck-GAPDH ou plck-IκB^{-/-} (Mondragón et al., 2019). Nos résultats ont souligné donc l'importance de la GAPDH dans l'induction initiale de la voie NF-κB canonique, suivie par une activation de la voie non-canonique, qui est à l'origine d'un développement accéléré des Tfh (Figure 21).



<u>Figure 21:Schéma récapitulatif de l'implicaton de GAPDH et de la voie NF-κB dans le</u> <u>développement de l'AITL</u>

Pour poursuivre notre étude, nous nous sommes intéressés à la kinase NIK responsable de l'activation de la voie NF-κB non canonique. Nous avons observé dans notre modèle murin des niveaux élevés d'expression de NIK dans les Tfh CD4⁺ et dans les LB du centre germinatif. Par conséquent, nous avons proposé NIK comme une nouvelle cible thérapeutique pour le traitement des AITL. Afin de tester notre hypothèse, nous avons développé, en collaboration avec l'Institut de Chimie de Nice (ICN), un nouvel inhibiteur de NIK, caractérisé par une

activité puissante à faible dose (Brevet PCT/EP2017/067306). L'utilisation de cet inhibiteur *in vivo* sur des souris greffées avec les tumeurs mAITL a révélé une efficacité importante traduite par une augmentation de la survie des souris traitées associée à une déplétion des cellules tumorales. De plus, nous avons validé l'effet anti-prolifératif de cet inhibiteur sur des cellules isolées à partir des biopsies des patients AITL. De manière intéressante, nous avons également confirmé l'efficacité de cet inhibiteur sur des lignées cellulaires de mélanome et de DLBCL présentant une activation majeure de la voie NF-κB non canonique. Ces résultats mettent en évidence l'importance de cet inhibiteur comme nouvelle option thérapeutique dans divers cancers (Mondragón et al., 2019)(Figure 22).

Par ailleurs, il a été décrit précédemment, la présence d'autres inhibiteurs qui ciblent l'activation de la voie NF-κB non canonique tels que les inhibiteurs d'EZH2 (Stazi et al., 2017). Ces composés ciblent l'activité catalytique d'EZH2 et empêchent la méthylation de ses gènes cibles. De manière intéressante, en agissant sur NIK nous avons pu diminuer l'expression d'EZH2 et ainsi inhiber indirectement son activité épigénétique. Dans ce contexte, les inhibiteurs de NIK ciblent à la fois l'activité catalytique et non catalytique d'EZH2.

De plus, il a été démontré qu'EZH2 inhibe la production d'IFN-γ par les cellules immunitaires. L'invalidation de l'expression d'EZH2 contrecarre cette inhibition et favorise la réactivation de l'immunité anti-tumorale. Cependant, les inhibiteurs catalytiques d'EZH2 sont incapables de reproduire cet effet, d'où l'avantage d'utiliser les inhibiteurs de NIK (Wee et al., 2014).



Figure 22:Schéma recapitulatif des deux options thérapeutiques proposées dans notre étude pour le traitement de l'AITL

La combinaison de l'inhibiteur de la voie NF- κB à l'immunothérapie Anti-PD-1 induit une réactivation de l'immunité anti-tumorale des LT CD8⁺ qui se traduit par une augmentation de la production de l'IFN- γ et de la sécrétion des protéines cytotoxiques. Cette activation est à l'origine de la déplétion des cellules tumorales T et B.

3. Révéler de nouvelles options thérapeutiques pour le traitement de l'AITL

Au cours de mon travail de thèse, j'ai validé l'utilité du modèle murin plck-GAPDH comme étant un modèle préclinique valable pour tester de nouvelles stratégies thérapeutiques.

Dans un premier temps, nous avons identifié une activation chronique de la voie NF-kB non canonique dans les tumeurs mAITL et nous avons montré que l'inhibition de cette voie par un nouvel inhibiteur ciblant la kinase NIK pourrait augmenter la survie des souris traitées. De manière intéressante, nous avons confirmé l'efficacité de cet inhibiteur sur des biopsies de patients AITL *in vitro*. Par ailleurs, nous nous sommes aussi intéressés à l'efficacité de l'immunothérapie anti-PD1 sur la croissance des tumeurs mAITL. Nous avons obtenu des résultats satisfaisants montrant une déplétion des cellules tumorales CD4⁺PD-1^{high} associée à une réactivation de la cytotoxicité des cellules immunitaires CD8⁺ et à une induction efficace de la réponse immunitaire anticancéreuse. L'ensemble de nos résultats suggère donc la possibilité de cibler d'autres composants ou acteurs moléculaires de la tumeur AITL dans le but de proposer de nouvelles options thérapeutiques.

Sachant que le maintien de la pathologie dépend principalement de la relation entre les cellules tumorales (Tfh) et les cellules du microenvironnement (LB et FDC), il serait intéressant de proposer de nouveaux traitements induisant une rupture de cette interaction, ou encore cibler des acteurs moléculaires déjà connus comme ayant un rôle majeur dans le développement des différents composants de la tumeur.

En premier lieu, nous suggérons d'utiliser les anticorps monoclonaux anti-ICOS qui permettent de rompre l'interaction entre les cellules tumorales Tfh et les cellules B du centre germinatif et donc nous formulons l'hypothèse qu'ils participent à une réduction de la progression tumorale. De manière intéressante il a été démontré que l'utilisation de ces anticorps participent à l'élimination des Tregs qui interviennent également dans la suppression immunitaire (Burlion et al., 2019)

D'autre part, comme décrit dans la partie introductive, les Tfh sont les cellules à l'origine du développement de l'AITL (Crotty, 2019; Lunning and Vose, 2017). La différenciation des Tfh est un processus complexe qui nécessite plusieurs interactions avec les cellules de l'environnement, en particulier les lymphocytes B du centre germinatif. Parmi les facteurs impliqués dans la génération des Tfh, Bcl-6 est désigné comme étant le facteur de transcription clé (Hatzi et al., 2015; Nurieva et al., 2009). Ainsi, ila été démontré que la différenciation des Tfh est interrompue en absence de Bcl-6 (Yu et al., 2009). De plus, Bcl-6 reprogramme

l'expression de toutes les molécules fonctionnelles caractéristiques de la lignée Tfh (Jogdand et al., 2016).

D'autre part, Bcl-6 est fortement exprimé dans les lymphocytes B du centre germinatif et joue un rôle majeur dans l'adaptation de ces cellules au stress génotoxique induit par les cassures des brins d'ADN et les mécanismes de mutations ponctuelles intervenant au cours de la différenciation plasmocytaire (Basso and Dalla-Favera, 2012). De plus, une expression dérégulée de Bcl-6 a été associée aux mécanismes de lymphomagénèse B, en particulier dans les DLBCL (Polo et al., 2007).

L'ensemble de ces données soulève une question importante est-ce que l'inhibition de Bcl-6 pourrait être une nouvelle option thérapeutique pour le traitement des patients AITL ? Récemment, une nouvelle catégorie d'inhibiteurs de Bcl-6 a été identifié (Kerres et al., 2017). Ces composés interagissent avec le site actif de Bcl-6 qui correspond au domaine BTB et inhibent son interaction avec les complexes répresseurs qui sont à l'origine de son activité oncogénique. Contrairement à d'autres inhibiteurs, ces composés se lient à Bcl-6 et favorisent sa polyubiquitination et sa dégradation, appelés ainsi « Bcl-6 degrader ». L'activité anti-tumorale de ces inhibiteurs a été testé sur des lignées cellulaires de DLBCL. De manière intéressante, les résultats obtenus montrent que ces inhibiteurs ne favorisent pas la mort cellulaire mais induisent une activité anti-proliférative des cellules tumorales et permettent une expression importante des gènes réprimés par Bcl-6 (Kerres et al., 2017).

Dans le modèle plck-GAPDH, nous avons montré une surexpression de Bcl-6 dans les cellules Tfh CD4⁺ ainsi que dans les cellules B du centre germinatif. Ainsi, il serait intéressant de déterminer l'effet de cet inhibiteur sur la croissance tumorale dans ce modèle préclinique mAITL. Nous nous attendons principalement à retrouver une régression du nombre de cellules tumorales Tfh ainsi que les LB du centre germinatif rejoignant ainsi les études effectuées dans le cadre des DLBCL. De plus, nous pourrions également tester l'activité de cet inhibiteur *in vitro* sur les biopsies de patients AITL.

Ensuite, pour garantir une activation de l'immunité anti-tumorale comme nous l'avons montré dans notre étude précédente, nous pourrions également combiner cet inhibiteur à l'immunothérapie anti-PD-1 dans le modèle mAITL. En effet, l'inhibiteur de Bcl-6 semblant seulement anti-prolifératif, une combinaison avec l'immunothérapie anti-PD-1 permettra, en plus de l'inhibition de la prolifération, une restauration de l'activité cytotoxique anti-tumorale des cellules immunitaires CD8⁺, et hypothétiquement une régression tumorale.

4. Métabolisme énergétique des cellules tumorales Tfh et des cellules du microenvironnement

Dans la deuxième partie de mon travail, nous nous sommes intéressées à étudier le métabolisme énergétique des cellules tumorales dans le modèle plck-GAPDH. Bien que la GAPDH soit une enzyme glycolytique, les résultats obtenus dans notre étude ont montré que les cellules tumorales Tfh dans le modèle mAITL dépendent du métabolisme oxydatif pour leur développement (Article 2, Mhaidly et al., en préparation). Dans un premier temps, nous avons observé une surexpression des gènes codant pour les enzymes impliquées dans la respiration mitochondriale, aussi bien dans la cohorte de patients AITL que dans les tumeurs isolées des souris plck-GAPDH. Par la suite, nous avons détecté une diminution de la production de l'ATP glycolytique, accompagnée avec une dépendance plus importante à l'énergie produite par la phosphorylation oxydative. De plus, nous avons pu montrer que le traitement des souris greffées avec les tumeurs mAITL, avec un inhibiteur du complexe I de la chaîne respiratoire mitochondriale « la metformine », augmente leur survie et induit un ralentissement de la croissance tumorale. L'ensemble de nos résultats suggère donc que le métabolisme mitochondrial favorise la croissance et l'envahissement tumoral dans les AITL.

Nos observations corrèlent avec l'ensemble des données publiées récemment suggérant que le métabolisme énergétique des cellules cancéreuses est un processus plus complexe qu'initialement décrit. Contrairement à ce qui avait été suggéré par Otto Warburg qui a décrit que la majorité des tumeurs se limitent seulement à l'utilisation de la glycolyse pour leur progression, il apparaît aujourd'hui que le métabolisme mitochondrial est également impliqué dans les processus de croissance tumorale (Chiche et al., 2019; Sica et al., 2019; Zhang et al., 2019). Une telle hétérogénéité inter-tumorale semble résulter d'une combinaison de facteurs internes (profil génétique de la tumeur) ou externes (microenvironnement tumoral) qui peuvent contrôler la reprogrammation métabolique des cellules cancéreuses.

En effet, comme nous avons pu le voir dans la partie introductive, plusieurs acteurs moléculaires régulant la différenciation des LT en Tfh interviennent dans la régulation du métabolisme énergétique de ces cellules (Chatterjee et al., 2013; Oestreich et al., 2014; Patsoukis et al., 2015). Nous pouvons ainsi émettre deux hypothèses expliquant l'activation du métabolisme oxydatif dans les tumeurs AITL.

Premièrement, il a été bien établi que Bcl-6, le facteur clé du développement des Tfh, inhibe le métabolisme glycolytique en réprimant les gènes codant pour les transporteurs de glucose ou pour les enzymes catalysant la voie glycolytique (Oestreich et al., 2014; Ray et al., 2015). Nous

pouvons donc penser que l'expression élevée de Bcl-6 dans les tumeurs AITL est à l'origine de la reprogrammation métabolique des cellules Tfh vers la phosphorylation oxydative. Nos résultats préliminaires obtenus à partir des analyses transcriptomiques effectuées sur les tumeurs plck-GAPDH montrent en effet une corrélation positive entre le niveau d'expression de Bcl-6 et le métabolisme oxydatif (Figure 23A). Comme attendu, une corrélation négative est observé entre l'expression de Bcl-6 et la glycolyse. Des résultats similaires ont été obtenus à partir d'analyses effectuées sur des datas de transcriptomie d'une cohorte de patients AITL (Figure 23B). Toutefois, ces observations sont préliminaires et nécessitent des analyses supplémentaires pour les confirmer. Nous pourrions par exemple analyser le métabolisme énergétique des cellules Tfh isolées à partir des tumeurs mAITL après un traitement avec les inhibiteurs de Bcl-6 « Bcl-6 degrader » cités précédemment. De plus, nous pourrions invalider génétiquement l'expression de Bcl-6 dans les cellules CD4⁺ Tfh isolées à partir des biopsies de patients et analyser ensuite la production d'ATP glycolytique par ces cellules.



Figure 23:Corrélation entre le niveau d'expression de Bcl-6 et le métabolisme énergétique des cellules tumorales des souris plck-GAPDH (A) ou dans les biopsies de patients AITL (B)

Dans un second temps, il serait intéressant d'analyser le lien entre l'activation de la voie NF- κ B et le métabolisme oxydatif observé dans les cellules Tfh tumorales. Il a été démontré que

l'activation chronique de la voie NF- κ B non canonique dans les DLBCL induit une dérégulation de l'expression de Bcl-6 qui est à l'origine de la progression tumorale (Zhang et al., 2015). Nous suggérons dons que l'activation de la voie NF- κ B dans notre modèle plck-GAPDH soit à l'origine de la dérégulation de l'expression de Bcl-6, ce qui déclenche la reprogrammation métabolique des cellules tumorales Tfh.

D'autre part, il a précédemment été décrit que le récepteur co-inhibiteur PD-1 est un régulateur majeur de la reprogrammation métabolique des LT. Il apparaît que les signaux déclenchés par l'activation de PD-1 limitent la capture du glucose et induisent une diminution du flux glycolytique (Chatterjee et al., 2013; Patsoukis et al., 2015). Dans nos conditions, nous avons observé une diminution de la production de l'ATP glycolytique associée à une faible consommation du glucose dans les cellules tumorales CD4⁺PD-1^{high} isolées à partir des tumeurs mAITL en comparaison aux PD-1⁻. De plus, nous avons observé une corrélation positive entre le niveau d'expression de PD-1 et l'activation de la voie de phosphorylation oxydative chez les patients AITL. Ces observations nous ont permis de poser la question suivante : est-ce que le récepteur PD-1 pourrait être à l'origine de la reprogrammation métabolique observée dans les tumeurs AITL ? Pour répondre à cette question, nous pourrions analyser le métabolisme cellulaire des cellules tumorales suite à un traitement avec l'immunothérapie anti-PD-1.

Par ailleurs, il a été démontré que PD-1 active la lipolyse et la β -oxydation des acides gras par une régulation des deux enzymes clés ATGL et CPT1A (Patsoukis et al., 2015). Il serait ainsi intéressant d'analyser dans notre modèle plck-GAPDH l'activation de cette voie métabolique. Pour cela, il faudra incuber les cellules T CD4⁺PD-1^{high} isolées à partir des tumeurs mAITL avec du palmitate radiomarqué ³H pendant 48h. Ensuite, la β -oxydation du palmitate sera évaluée en mesurant à différents intervalles de temps le taux du ³H₂O , qui correspond au produit final du métabolisme des acides gras.

D'autre part, les cellules cancéreuses orientent leur métabolisme en fonction de leur environnement (Sun et al., 2018). Comme décrit précédemment, dans l'AITL, les cellules tumorales interagissent avec les cellules du microenvironnement pour assurer le maintien de la pathologie. Il serait dons intéressant d'analyser le métabolisme énergétique des cellules B du centre germinatif et des LT CD8⁺ infiltrés au sein de la tumeur. Les résultats préliminaires obtenus montrent que les cellules B isolées à partir des tumeurs des plck-GAPDH produisent majoritairement leur énergie par la voie glycolytique (Figure 24A). Cette observation pourrait expliquer partiellement l'orientation des cellules Tfh vers un métabolisme oxydatif (Sukumar et al., 2015). En effet, les cellules du microenvironnement tumoral agissent de manière compétitive pour assurer leur apport en oxygène et en nutriments. L'augmentation de l'apport glycolytique dans les LB pourrait induire une reprogrammation métabolique des LT vers une autre voie métabolique pour compenser leurs pertes énergétiques.

D'autre part, nous avons observé que les LT CD8⁺ infiltrant la tumeur se présentent à l'état quiescent non stimulé avec une production énergétique semblable à celle des LT CD8⁺ isolés à partir des souris contrôles (Figure 24B). Ces résultats corrèlent avec nos résultats précédents montrant une réduction de l'immunité anti-tumorale et une absence des LT CD8 cytotoxiques dans les tumeurs AITL. Toutefois, ces résultats sont préliminaires et devront être confirmés et reproduits sur un nombre plus élevé de tumeurs.



Figure 24:Mesure de la production de l'ATP glycolytique sur les cellules B CD19+ (A) et les cellules T CD8+ (B) isolées à partir des tumeurs spléniques des souris plck-GAPDH en comparaison à des cellules normales isolées de la rate des souris contrôles

Enfin, une des caractéristiques des cellules tumorales qui favorisent leur expansion et leur résistance aux traitements est leur flexibilité métabolique (Ricci and Chiche, 2018). C'est un phénomène réversible, défini par un passage d'un état métabolique à un autre, mieux adapté pour résister aux conditions stressantes de l'environnement. Ce mécanisme de plasticité a été décrit récemment dans des lignées cellulaire de lymphome du manteau, dans lesquelles les cellules tumorales réorientent leur métabolisme vers la phosphorylation oxydative pour résister au traitement par l'ibrutinib (Zhang et al., 2019). Dans notre modèle, suite au traitement par la metformine, nous avons remarqué la persistance d'une population de LT CD4⁺PD-1^{high} résistant au traitement. Nous avons observé dans cette population cellulaire une augmentation de la production de l'ATP glycolytique contrairement aux cellules isolées à partir de la tumeur d'origine (Figure 25). Ces résultats suggèrent un processus d'adaptation et de flexibilité métabolique dans les tumeurs mAITL suite au traitement avec l'inhibiteur du complexe I de la chaîne mitochondriale. Pour vérifier cette hypothèse, nous devrions tout d'abord reproduire

cette expérience pour confirmer nos observations, et ensuite effectuer une greffe secondaire des cellules résistantes dans des souris NSG receveuses et les traiter avec un inhibiteur de la glycolyse « le 2-déoxyglucose » pour savoir si nous pourrions les éliminer. Nous pourrions également effectuer une analyse métabolomique détaillée de ces cellules résistantes par rapport aux cellules sensibles pour détecter une possible reprogrammation métabolique (glutaminolyse, métabolisme des acides gras ou des acides aminés).



Figure 25 : Pourcentage de l'ATP glycolytique produite par les cellules T CD4+PD-1high isolées à partir de la tumeur plck-GAPDH d'origine ou la tumeur traitée par la metformine

En conclusion, nous pourrions valider l'ensemble de nos résultats par une analyse métabolomique sur des cellules tumorales isolées à partir des tumeurs plck-GAPDH. Cette analyse permettra, par une approche non ciblée, d'identifier les différents métabolites présents dans les cellules tumorales AITL en comparaison à des cellules normales et de détecter d'autres perturbations discrètes du métabolisme cellulaire associées à l'agressivité tumorale.

5. Analyse des modifications épigénétiques dans les tumeurs mAITL

Comme décrit dans la partie introductive, il a bien été établi la présence des perturbations des signalisations épigénétiques dans l'AITL (Couronné et al., 2012; Palomero et al., 2014; Zang et al., 2017). Cependant il est difficile de comprendre la suite des évènements qui contrôlent ces modifications. Elles peuvent intervenir aussi bien dans les phases d'initiation du cancer que lors de sa progression. Les causes des différentes aberrations épigénétiques découlent le plus souvent d'une dérégulation initiale de l'expression des protéines capables d'écrire ou d'effacer ces empreintes. Cette dérégulation entraîne une reprogrammation complète de l'expression des gènes responsables des différents stades de la tumorigénèse, y compris la répression des gènes suppresseurs de tumeur, ou au contraire l'activation des oncogènes.

Dans l'AITL, des mutations inactivatrices des gènes *TET2* et *DNMT3A* sont retrouvées dans 20 à 30% des cas (Couronné et al., 2012; Scourzic et al., 2016). Ces gènes régulent les profils de méthylation des motifs CpG répartis au niveau des séquences d'ADN. Par exemple, il a été décrit que l'invalidation du gène *TET2* induit une hyperméthylation des loci CpG de l'intron 1 de Bcl-6 qui est à l'origine du développement anormal des Tfh (Nishizawa et al., 2017).

Les premières analyses effectuées sur les tumeurs mAITL ont révélé la présence des mutations affectant les régions introniques des principaux régulateurs épigénétiques retrouvés mutés dans la pathologie humaine (TET2, DNMT3A, EZH2...) (Mondragón et al., 2019) (Figure 26). Nous ignorons encore l'influence de ces mutations sur la pathogenèse de l'AITL dans notre modèle murin. Ainsi, il serait intéressant de caractériser les différentes modifications épigénétiques qui peuvent être régulées par ces acteurs moléculaires.

Afin d'examiner les changements ou les marques épigénétiques dans les cellules du lymphome des souris plck-GAPDH, nous effectuerons une immunoprécipitation de l'ADN méthylé, suivie par un séquençage à haut débit. Ainsi les méthylations dans les régions initiatrices de la transcription ou dans les îlots CpG seront évaluées dans les cellules tumorales de notre modèle murin par rapport aux cellules T CD4⁺ contrôles. Ensuite, nous combinerons ces résultats avec les analyses transcriptomiques. Généralement, la méthylation de l'ADN dans la région promotrice est associée à une inactivation de l'expression génique. Ces analyses nous permettront d'identifier de nouveaux gènes ou voies de signalisation participant à la pathogenèse de l'AITL.

EZH2 est la sous-unité catalytique du complexe répressif polycomb PRC2, responsable de la méthylation de l'histone H3K27, qui est associée à une inhibition de la transcription. Il a été démontré qu'EZH2 est surexprimée dans beaucoup de cancers solides dont le mélanome (Bachmann et al., 2006). Ses effets oncogènes sont dus à son rôle épigénétique qui induit une suppression des gènes suppresseurs des tumeurs comme CDKN1C, p27 et BRCA1 (Chi et al., 2010). Nous avons montré dans notre modèle préclinique mAITL une surexpression aberrante d'EZH2, due à une activation chronique de la voie NF-κB. Nous pouvons donc nous demander quel effet pourrait avoir cette surexpression sur la modification épigénétique des histones. Pour tester cette hypothèse, nous effectuerons une immunoprécipitation des histones méthylées (ChIP-seq), suivie par un séquençage à haut débit.

En plus de la méthylation contrôlée par EZH2, les histones peuvent être modifiées par un ajout ou une suppression des groupements acétyl. Dans notre modèle plck-GAPDH, nous avons observé des mutations affectant les gènes codants pour les enzymes régulant ces modifications (HAT1, HDAC2). Ces observations nous incitent à analyser les niveaux d'acétylation des histones et par la suite détecter les défauts de transcription qui peuvent en résulter.

Enfin, il est important de noter que les protéines du bromodomaine (BET) sont des lecteurs épigénétiques qui reconnaissent les modifications des histones et de la chromatine et régulant ainsi l'expression génique. Il a été établi que certains BET (BRD2, BRD4) maintiennent l'expression d'IKK stabilisant l'activité de la voie NF-kB dans des lignées cellulaires de DLBCL (Ceribelli et al., 2014). Il serait ainsi intéressant de vérifier cette connexion dans notre modèle de lymphome mAITL. Ces analyses pourraient aboutir à l'identification de nouvelles cibles thérapeutiques pour le traitement de l'AITL.



Figure 26 : Liste des gènes codant pour les régulateurs épigénétiques qui sont modifiés dans six tumeurs plck-GAPDH différents, détectés par un séquençage du génome entier

Conclusion

Conclusion

Le lymphome T angioimmunoblastique (AITL) est une pathologie clonale des lymphocytes T CD4⁺ qui, à l'exception des autres lymphomes T périphériques, se caractérise par des interactions entre les cellules T tumorales et les autres composants du microenvironnement. Ce dialogue cellulaire a constitué un obstacle pour l'établissement d'un modèle de culture *in vitro* de lymphome T AITL. Ainsi a émergé la nécessité de mettre en place des modèles murins qui permettent de récapituler les caractéristiques pathologiques et immunophénotypiques de la maladie humaine.

Notre travail a permis de mettre en place et de caractériser un nouveau modèle murin de tumeur AITL, basé sur l'expression de l'enzyme glycolytique « la GAPDH » uniquement dans les LT. Ce modèle préclinique a servi, en premier lieu, à identifier une activation chronique de la voie de signalisation NF- κ B comme jouant un rôle central dans la pathogenèse et l'agressivité des tumeurs. En plus, nous avons testé et validé l'efficacité de deux nouvelles approches thérapeutiques qui présentaient des résultats prometteurs pour le traitement des patients atteints d'AITL.

D'autre part, nous avons analysé le métabolisme énergétique des cellules tumorales du lymphome AITL et nous avons identifié une dépendance accrue à la phosphorylation oxydative avec une réduction du flux glycolytique et de la consommation du glucose.

Enfin, l'ensemble de nos résultats mettent en place un nouveau modèle préclinique qui mime la pathologie humaine (AITL) ce qui aboutiera à une meilleure compréhension des mécanismes moléculaires impliqués dans cette pathologie rare et permettra de tester de nouvelles stratégies thérapeutiques.

Bibliographie

А

Abdel-Wahab, O., Mullally, A., Hedvat, C., Garcia-Manero, G., Patel, J., Wadleigh, M., Malinge, S., Yao, J., Kilpivaara, O., Bhat, R., et al. (2009). Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood *114*, 144–147.

Abril-Rodriguez, G., and Ribas, A. (2017). SnapShot: Immune Checkpoint Inhibitors. Cancer Cell 31, 848-848.e1.

Ahn, H., Yang, J.M., Jeon, Y.K., and Paik, J.H. (2018). Clinicopathologic implications of TNFAIP3/A20 deletions in extranodal NK/T-cell lymphoma. Genes Chromosomes Cancer 57, 231–239.

Akagi, T., Motegi, M., Tamura, A., Suzuki, R., Hosokawa, Y., Suzuki, H., Ota, H., Nakamura, S., Morishima, Y., Taniwaki, M., et al. (1999). A novel gene, MALT1 at 18q21, is involved in t(11;18) (q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue. Oncogene *18*, 5785–5794.

Akram, M. (2014). Citric Acid Cycle and Role of its Intermediates in Metabolism. Cell Biochem Biophys 68, 475–478.

Alizadeh, A.A., and Advani, R.H. (2008). Evaluation and management of angioimmunoblastic T-cell lymphoma: a review of current approaches and future strategies. Clin Adv Hematol Oncol *6*, 899–909.

Al-Katib, A., Arnold, A.A., Aboukameel, A., Sosin, A., Smith, P., Mohamed, A.N., Beck, F.W., and Mohammad, R.M. (2010). I-kappa-kinase-2 (IKK-2) inhibition potentiates vincristine cytotoxicity in non-Hodgkin's lymphoma. Mol. Cancer 9, 228.

Almeida, L., Lochner, M., Berod, L., and Sparwasser, T. (2016). Metabolic pathways in T cell activation and lineage differentiation. Semin. Immunol. 28, 514–524.

Altenberg, B., and Greulich, K.O. (2004). Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. Genomics *84*, 1014–1020.

Ansell, S.M., Lesokhin, A.M., Borrello, I., Halwani, A., Scott, E.C., Gutierrez, M., Schuster, S.J., Millenson, M.M., Cattry, D., Freeman, G.J., et al. (2015). PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. N. Engl. J. Med. *372*, 311–319.

Antonia, S.J., Villegas, A., Daniel, D., Vicente, D., Murakami, S., Hui, R., Yokoi, T., Chiappori, A., Lee, K.H., de Wit, M., et al. (2017). Durvalumab after Chemoradiotherapy in Stage III Non–Small-Cell Lung Cancer. New England Journal of Medicine *377*, 1919–1929.

Aronica, M.A., Mora, A.L., Mitchell, D.B., Finn, P.W., Johnson, J.E., Sheller, J.R., and Boothby, M.R. (1999). Preferential role for NF-kappa B/Rel signaling in the type 1 but not type 2 T cell-dependent immune response in vivo. J. Immunol. *163*, 5116–5124.

Attygalle, A., Al-Jehani, R., Diss, T.C., Munson, P., Liu, H., Du, M.-Q., Isaacson, P.G., and Dogan, A. (2002). Neoplastic T cells in angioimmunoblastic T-cell lymphoma express CD10. Blood *99*, 627–633. Audet-Walsh, É., Yee, T., McGuirk, S., Vernier, M., Ouellet, C., St-Pierre, J., and Giguère, V. (2017). Androgen-Dependent Repression of ERRγ Reprograms Metabolism in Prostate Cancer. Cancer Res. *77*, 378–389.

Auguste, T., Travert, M., Tarte, K., Amé-Thomas, P., Artchounin, C., Martin-Garcia, N., de Reynies, A., de Leval, L., Gaulard, P., and Delfau-Larue, M.-H. (2013). ROQUIN/RC3H1 alterations are not

found in angioimmunoblastic T-cell lymphoma. PLoS ONE 8, e64536.

Azuma, T., Yao, S., Zhu, G., Flies, A.S., Flies, S.J., and Chen, L. (2008). B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. Blood *111*, 3635–3643.

В

Bachmann, I.M., Halvorsen, O.J., Collett, K., Stefansson, I.M., Straume, O., Haukaas, S.A., Salvesen, H.B., Otte, A.P., and Akslen, L.A. (2006). EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J. Clin. Oncol. *24*, 268–273.

Bailis, W., Shyer, J.A., Zhao, J., Canaveras, J.C.G., Al Khazal, F.J., Qu, R., Steach, H.R., Bielecki, P., Khan, O., Jackson, R., et al. (2019). Distinct modes of mitochondrial metabolism uncouple T cell differentiation and function. Nature.

Balasubramani, A., Shibata, Y., Crawford, G.E., Baldwin, A.S., Hatton, R.D., and Weaver, C.T. (2010). Modular Utilization of Distal cis-Regulatory Elements Controls Ifng Gene Expression in T Cells Activated by Distinct Stimuli. Immunity *33*, 35–47.

Balmer, M.L., Ma, E.H., Bantug, G.R., Grählert, J., Pfister, S., Glatter, T., Jauch, A., Dimeloe, S., Slack, E., Dehio, P., et al. (2016). Memory CD8+ T Cells Require Increased Concentrations of Acetate Induced by Stress for Optimal Function. Immunity *44*, 1312–1324.

Baseggio, L., Berger, F., Morel, D., Delfau-Larue, M. -h, Goedert, G., Salles, G., Magaud, J. -p, and Felman, P. (2006). Identification of circulating CD10 positive T cells in angioimmunoblastic T-cell lymphoma. Leukemia *20*, 296.

Basso, K., and Dalla-Favera, R. (2012). Roles of BCL6 in normal and transformed germinal center B cells. Immunol. Rev. 247, 172–183.

Baumgartner, B., Weber, M., Quirling, M., Fischer, C., Page, S., Adam, M., Von Schilling, C., Waterhouse, C., Schmid, C., Neumeier, D., et al. (2002). Increased IkappaB kinase activity is associated with activated NF-kappaB in acute myeloid blasts. Leukemia *16*, 2062–2071.

Beier, U.H., Angelin, A., Akimova, T., Wang, L., Liu, Y., Xiao, H., Koike, M.A., Hancock, S.A., Bhatti, T.R., Han, R., et al. (2015). Essential role of mitochondrial energy metabolism in Foxp3+ T-regulatory cell function and allograft survival. FASEB J *29*, 2315–2326.

Bennaceur-Griscelli, A., Bosq, J., Koscielny, S., Lefrère, F., Turhan, A., Brousse, N., Hermine, O., and Ribrag, V. (2004). High level of glutathione-S-transferase pi expression in mantle cell lymphomas. Clin. Cancer Res. *10*, 3029–3034.

Berg, J.M., Tymoczko, J.L., and Stryer, L. (2002). The Regulation of Cellular Respiration Is Governed Primarily by the Need for ATP. Biochemistry. 5th Edition.

Bettonville, M., d'Aria, S., Weatherly, K., Porporato, P.E., Zhang, J., Bousbata, S., Sonveaux, P., and Braun, M.Y. (2018). Long-term antigen exposure irreversibly modifies metabolic requirements for T cell function. Elife 7.

Bidère, N., and Senik, A. (2001). Caspase-independent apoptotic pathways in T lymphocytes: A minireview. Apoptosis 6, 371–375.

Biswas, D.K., Shi, Q., Baily, S., Strickland, I., Ghosh, S., Pardee, A.B., and Iglehart, J.D. (2004). NF-

kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. Proc. Natl. Acad. Sci. U.S.A. 101, 10137–10142.

Boolbol, S.K., Dannenberg, A.J., Chadburn, A., Martucci, C., Guo, X.J., Ramonetti, J.T., Abreu-Goris, M., Newmark, H.L., Lipkin, M.L., DeCosse, J.J., et al. (1996). Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. Cancer Res. *56*, 2556–2560.

Botros, N., Cerroni, L., Shawwa, A., Green, P.J., Greer, W., Pasternak, S., and Walsh, N.M. (2015). Cutaneous manifestations of angioimmunoblastic T-cell lymphoma: clinical and pathological characteristics. Am J Dermatopathol *37*, 274–283.

Brahmer, J., Reckamp, K.L., Baas, P., Crinò, L., Eberhardt, W.E.E., Poddubskaya, E., Antonia, S., Pluzanski, A., Vokes, E.E., Holgado, E., et al. (2015). Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. N. Engl. J. Med. *373*, 123–135.

Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M., and Förster, R. (2000). Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J. Exp. Med. *192*, 1545–1552.

Brightbill, H.D., Suto, E., Blaquiere, N., Ramamoorthi, N., Sujatha-Bhaskar, S., Gogol, E.B., Castanedo, G.M., Jackson, B.T., Kwon, Y.C., Haller, S., et al. (2018). NF- κ B inducing kinase is a therapeutic target for systemic lupus erythematosus. Nat Commun *9*, 179.

Burlion, A., Ramos, R.N., KC, P., Sendeyo, K., Corneau, A., Ménétrier-Caux, C., Piaggio, E., Olive, D., Caux, C., and Marodon, G. (2019). A novel combination of chemotherapy and immunotherapy controls tumor growth in mice with a human immune system. Oncoimmunology *8*.

С

Cairns, R.A., Iqbal, J., Lemonnier, F., Kucuk, C., Leval, L. de, Jais, J.-P., Parrens, M., Martin, A., Xerri, L., Brousset, P., et al. (2012). IDH2 mutations are frequent in angioimmunoblastic T-cell lymphoma. Blood *119*, 1901–1903.

Carrà, G., Torti, D., Crivellaro, S., Panuzzo, C., Taulli, R., Cilloni, D., Guerrasio, A., Saglio, G., and Morotti, A. (2016). The BCR-ABL/NF-κB signal transduction network: a long lasting relationship in Philadelphia positive Leukemias. Oncotarget *7*, 66287–66298.

Carracedo, A., Cantley, L.C., and Pandolfi, P.P. (2013). Cancer metabolism: fatty acid oxidation in the limelight. Nature Reviews Cancer 13, 227–232.

Ceribelli, M., Kelly, P.N., Shaffer, A.L., Wright, G.W., Xiao, W., Yang, Y., Mathews Griner, L.A., Guha, R., Shinn, P., Keller, J.M., et al. (2014). Blockade of oncogenic IkB kinase activity in diffuse large B-cell lymphoma by bromodomain and extraterminal domain protein inhibitors. Proc. Natl. Acad. Sci. U.S.A. *111*, 11365–11370.

Chang, C., Su, H., Zhang, D., Wang, Y., Shen, Q., Liu, B., Huang, R., Zhou, T., Peng, C., Wong, C.C.L., et al. (2015). AMPK-Dependent Phosphorylation of GAPDH Triggers Sirt1 Activation and Is Necessary for Autophagy upon Glucose Starvation. Mol. Cell *60*, 930–940.

Chang, C.-H., Curtis, J.D., Maggi, L.B., Faubert, B., Villarino, A.V., O'Sullivan, D., Huang, S.C.-C., van der Windt, G.J.W., Blagih, J., Qiu, J., et al. (2013). Posttranscriptional control of T cell effector function by aerobic glycolysis. Cell *153*, 1239–1251.

Chang, J.-H., Xiao, Y., Hu, H., Jin, J., Yu, J., Zhou, X., Wu, X., Johnson, H.M., Akira, S., Pasparakis, M., et al. (2012). Ubc13 maintains the suppressive function of regulatory T cells and prevents their conversion into effector-like T cells. Nat. Immunol. *13*, 481–490.

Chatterjee, P., Sari, D., Petkova, V., Li, L., and Boussiotis, V.A. (2013). PD-1 Induces Metabolic Reprogramming Of Activated T Cells From Glycolysis To Lipid Oxidation. Blood *122*, 187–187.

Chen, D., Frezza, M., Schmitt, S., Kanwar, J., and Dou, Q.P. (2011). Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. Curr Cancer Drug Targets *11*, 239–253. Chen, G., Hardy, K., Bunting, K., Daley, S., Ma, L., and Shannon, M.F. (2010). Regulation of the IL-21 gene by the NF-κB transcription factor c-Rel. J. Immunol. *185*, 2350–2359.

Chen, X., Willette-Brown, J., Wu, X., Hu, Y., Howard, O.M.Z., Hu, Y., and Oppenheim, J.J. (2015). IKKα is required for the homeostasis of regulatory T cells and for the expansion of both regulatory and effector CD4 T cells. FASEB J. 29, 443–454.

Chi, P., Allis, C.D., and Wang, G.G. (2010). Covalent histone modifications: miswritten, misinterpreted, and miserased in human cancers. Nat Rev Cancer 10, 457–469.

Chiche, J., Pommier, S., Beneteau, M., Mondragón, L., Meynet, O., Zunino, B., Mouchotte, A., Verhoeyen, E., Guyot, M., Pagès, G., et al. (2015). GAPDH enhances the aggressiveness and the vascularization of non-Hodgkin's B lymphomas via NF- κ B-dependent induction of HIF-1 α . Leukemia 29, 1163–1176.

Chiche, J., Reverso-Meinietti, J., Mouchotte, A., Rubio-Patiño, C., Mhaidly, R., Villa, E., Bossowski, J.P., Proics, E., Grima-Reyes, M., Paquet, A., et al. (2019). GAPDH Expression Predicts the Response to R-CHOP, the Tumor Metabolic Status, and the Response of DLBCL Patients to Metabolic Inhibitors. Cell Metab. *29*, 1243-1257.e10.

Choi, S.-C., Titov, A.A., Abboud, G., Seay, H.R., Brusko, T.M., Roopenian, D.C., Salek-Ardakani, S., and Morel, L. (2018). Inhibition of glucose metabolism selectively targets autoreactive follicular helper T cells. Nat Commun *9*, 4369.

Cilloni, D., Messa, F., Arruga, F., Defilippi, I., Morotti, A., Messa, E., Carturan, S., Giugliano, E., Pautasso, M., Bracco, E., et al. (2006). The NF-kappaB pathway blockade by the IKK inhibitor PS1145 can overcome imatinib resistance. Leukemia 20, 61–67.

Colell, A., Ricci, J.-E., Tait, S., Milasta, S., Maurer, U., Bouchier-Hayes, L., Fitzgerald, P., Guio-Carrion, A., Waterhouse, N.J., Li, C.W., et al. (2007). GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. Cell *129*, 983–997.

Colell, A., Green, D.R., and Ricci, J.-E. (2009). Novel roles for GAPDH in cell death and carcinogenesis. Cell Death Differ 16, 1573–1581.

Corn, R.A., Hunter, C., Liou, H.-C., Siebenlist, U., and Boothby, M.R. (2005). Opposing Roles for RelB and Bcl-3 in Regulation of T-Box Expressed in T Cells, GATA-3, and Th Effector Differentiation. The Journal of Immunology *175*, 2102–2110.

Cortes, J.R., Ambesi-Impiombato, A., Couronné, L., Quinn, S.A., Kim, C.S., da Silva Almeida, A.C., West, Z., Belver, L., Martin, M.S., Scourzic, L., et al. (2018). RHOA G17V Induces T Follicular Helper Cell Specification and Promotes Lymphomagenesis. Cancer Cell *33*, 259-273.e7.

Couronné, L., Bastard, C., and Bernard, O.A. (2012). TET2 and DNMT3A mutations in human T-cell lymphoma. N. Engl. J. Med. *366*, 95–96.

Cretenet, G., Clerc, I., Matias, M., Loisel, S., Craveiro, M., Oburoglu, L., Kinet, S., Mongellaz, C., Dardalhon, V., and Taylor, N. (2016). Cell surface Glut1 levels distinguish human CD4 and CD8 T lymphocyte subsets with distinct effector functions. Sci Rep *6*, 24129.

Crotty, S. (2011). Follicular helper CD4 T cells (TFH). Annu. Rev. Immunol. 29, 621–663.

Crotty, S. (2019). T Follicular Helper Cell Biology: A Decade of Discovery and Diseases. Immunity 50, 1132–1148.

\mathcal{D}

Dastoor, Z., and Dreyer, J.L. (2001). Potential role of nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase in apoptosis and oxidative stress. J. Cell. Sci. 114, 1643–1653.

Davis, R.E., Brown, K.D., Siebenlist, U., and Staudt, L.M. (2001). Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J. Exp. Med. *194*, 1861–1874.

De Donatis, G.M., Le Pape, E., Pierron, A., Cheli, Y., Hofman, V., Hofman, P., Allegra, M., Zahaf, K., Bahadoran, P., Rocchi, S., et al. (2016). NF-kB2 induces senescence bypass in melanoma via a direct transcriptional activation of EZH2. Oncogene *35*, 2735–2745.

Dejardin, E., Droin, N.M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z.-W., Karin, M., Ware, C.F., and Green, D.R. (2002). The Lymphotoxin- β Receptor Induces Different Patterns of Gene Expression via Two NF- κ B Pathways. Immunity 17, 525–535.

Delarue, R., Dupuis, J., Sujobert, P., Barbieux, S., Marçais, A., Tournilhac, O., Lachenal, F., Cheminant, M., Sarkozy, C., Fataccioli, V., et al. (2016). Treatment with Hypomethylating Agent 5-Azacytidine Induces Sustained Response in Angioimmunoblastic T Cell Lymphomas. Blood *128*, 4164–4164.

Delfau-Larue, M.-H., de Leval, L., Joly, B., Plonquet, A., Challine, D., Parrens, M., Delmer, A., Salles, G., Morschhauser, F., Delarue, R., et al. (2012). Targeting intratumoral B cells with rituximab in addition to CHOP in angioimmunoblastic T-cell lymphoma. A clinicobiological study of the GELA. Haematologica *97*, 1594–1602.

Demchenko, Y.N., Glebov, O.K., Zingone, A., Keats, J.J., Bergsagel, P.L., and Kuehl, W.M. (2010). Classical and/or alternative NF-κB pathway activation in multiple myeloma. Blood *115*, 3541–3552.

Demchenko, Y.N., Brents, L.A., Li, Z., Bergsagel, L.P., McGee, L.R., and Kuehl, M.W. (2014). Novel inhibitors are cytotoxic for myeloma cells with NFkB inducing kinase-dependent activation of NFkB. Oncotarget *5*, 4554–4566.

DeNardo, D.G., Barreto, J.B., Andreu, P., Vasquez, L., Tawfik, D., Kolhatkar, N., and Coussens, L.M. (2009). CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. Cancer Cell *16*, 91–102.

Döbbeling, U. (2007). Transcription factor profiling shows new ways towards new treatment options of cutaneous T cell lymphomas. Curr Drug Discov Technol *4*, 24–30.

Dogan, A., Attygalle, A.D., and Kyriakou, C. (2003). Angioimmunoblastic T-cell lymphoma. British Journal of Haematology *121*, 681–691.

Dong, C., Juedes, A.E., Temann, U.-A., Shresta, S., Allison, J.P., Ruddle, N.H., and Flavell, R.A. (2001). ICOS co-stimulatory receptor is essential for T-cell activation and function. Nature *409*, 97.

Dong, H., Strome, S.E., Salomao, D.R., Tamura, H., Hirano, F., Flies, D.B., Roche, P.C., Lu, J., Zhu, G., Tamada, K., et al. (2002). Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat. Med. *8*, 793–800.

Donnelly, R.P., and Finlay, D.K. (2015). Glucose, glycolysis and lymphocyte responses. Molecular Immunology 68, 513–519.

Du, M.-Q. (2016). MALT lymphoma: A paradigm of NF-κB dysregulation. Semin. Cancer Biol. 39, 49-60.

Du, J.-W., Xu, K.-Y., Fang, L.-Y., and Qi, X.-L. (2012). Interleukin-17, produced by lymphocytes, promotes tumor growth and angiogenesis in a mouse model of breast cancer. Mol Med Rep *6*, 1099–1102.

Du, Z.-X., Wang, H.-Q., Zhang, H.-Y., and Gao, D.-X. (2007). Involvement of Glyceraldehyde-3-Phosphate Dehydrogenase in Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Mediated Death of Thyroid Cancer Cells. Endocrinology *148*, 4352–4361.

Dunn, G.P., Old, L.J., and Schreiber, R.D. (2004). The immunobiology of cancer immunosurveillance and immunoediting. Immunity 21, 137–148.

Dwyer, K.M., Deaglio, S., Gao, W., Friedman, D., Strom, T.B., and Robson, S.C. (2007). CD39 and control of cellular immune responses. Purinergic Signal. *3*, 171–180.

E

Ellyard, J.I., Chia, T., Rodriguez-Pinilla, S.-M., Martin, J.L., Hu, X., Navarro-Gonzalez, M., Garcia, J.F., Delfau-Larue, M.-H., Montes-Moreno, S., Gaulard, P., et al. (2012). Heterozygosity for Roquinsan leads to angioimmunoblastic T-cell lymphoma-like tumors in mice. Blood *120*, 812–821.

Epner, D.E., and Coffey, D.S. (1996). There are multiple forms of glyceraldehyde-3-phosphate dehydrogenase in prostate cancer cells and normal prostate tissue. The Prostate 28, 372–378.

F

Ferris, R.L., Blumenschein, G., Fayette, J., Guigay, J., Colevas, A.D., Licitra, L., Harrington, K., Kasper, S., Vokes, E.E., Even, C., et al. (2016). Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. N. Engl. J. Med. *375*, 1856–1867.

Foss, H.D., Anagnostopoulos, I., Herbst, H., Grebe, M., Ziemann, K., Hummel, M., and Stein, H. (1995). Patterns of cytokine gene expression in peripheral T-cell lymphoma of angioimmunoblastic lymphadenopathy type. Blood *85*, 2862–2869.

Foy, T.M., Laman, J.D., Ledbetter, J.A., Aruffo, A., Claassen, E., and Noelle, R.J. (1994). gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. J. Exp. Med. *180*, 157–163.

Frauwirth, K.A., Riley, J.L., Harris, M.H., Parry, R.V., Rathmell, J.C., Plas, D.R., Elstrom, R.L., June, C.H., and Thompson, C.B. (2002). The CD28 Signaling Pathway Regulates Glucose Metabolism. Immunity *16*, 769–777.

Fujisawa, M., Chiba, S., and Sakata-Yanagimoto, M. (2017). Recent Progress in the Understanding of Angioimmunoblastic T-cell Lymphoma. Journal of Clinical and Experimental Hematopathology : JCEH

57, 109–119.

Furuta, H., Osawa, K., Shin, M., Ishikawa, A., Matsuo, K., Khan, M., Aoki, K., Ohya, K., Okamoto, M., Tominaga, K., et al. (2012). Selective inhibition of NF-κB suppresses bone invasion by oral squamous cell carcinoma in vivo. Int. J. Cancer *131*, E625-635.

G

Galgani, M., Rosa, V.D., Cava, A.L., and Matarese, G. (2016). Role of Metabolism in the Immunobiology of Regulatory T Cells. The Journal of Immunology 197, 2567–2575.

Gallamini, A., Zaja, F., Patti, C., Billio, A., Specchia, M.R., Tucci, A., Levis, A., Manna, A., Secondo, V., Rigacci, L., et al. (2007). Alemtuzumab (Campath-1H) and CHOP chemotherapy as first-line treatment of peripheral T-cell lymphoma: results of a GITIL (Gruppo Italiano Terapie Innovative nei Linfomi) prospective multicenter trial. Blood *110*, 2316–2323.

Gandolfi, S., Laubach, J.P., Hideshima, T., Chauhan, D., Anderson, K.C., and Richardson, P.G. (2017). The proteasome and proteasome inhibitors in multiple myeloma. Cancer Metastasis Rev. *36*, 561–584. Ganjoo, K., Hong, F., Horning, S.J., Gascoyne, R.D., Natkunam, Y., Swinnen, L.J., Habermann, T.M., Kahl, B.S., and Advani, R.H. (2014). Bevacizumab and cyclosphosphamide, doxorubicin, vincristine and prednisone in combination for patients with peripheral T-cell or natural killer cell neoplasms: an Eastern Cooperative Oncology Group study (E2404). Leuk. Lymphoma *55*, 768–772.

Gao, X., Wang, X., Pham, T.H., Feuerbacher, L.A., Lubos, M.-L., Huang, M., Olsen, R., Mushegian, A., Slawson, C., and Hardwidge, P.R. (2013). NleB, a bacterial effector with glycosyltransferase activity, targets GAPDH function to inhibit NF-κB activation. Cell Host Microbe *13*, 87–99.

Genta, S., Pirosa, M.C., and Stathis, A. (2019). BET and EZH2 Inhibitors: Novel Approaches for Targeting Cancer. Curr Oncol Rep 21, 13.

Gerondakis, S., and Siebenlist, U. (2010). Roles of the NF-kB Pathway in Lymphocyte Development and Function. Cold Spring Harb Perspect Biol 2.

Gerondakis, S., Fulford, T.S., Messina, N.L., and Grumont, R.J. (2014). NF-κB control of T cell development. Nat. Immunol. 15, 15–25.

Ghani, A.M., and Krause, J.R. (1985). Bone marrow biopsy findings in angioimmunoblastic lymphadenopathy. British Journal of Haematology *61*, 203–213.

Ghione, P., Moskowitz, A.J., De Paola, N.E.K., Horwitz, S.M., and Ruella, M. (2018). Novel Immunotherapies for T Cell Lymphoma and Leukemia. Curr Hematol Malig Rep *13*, 494–506.

Ghosh, S., and Karin, M. (2002). Missing Pieces in the NF-kB Puzzle. Cell 109, S81–S96.

Gilmore, T.D. (2006). Introduction to NF-kappaB: players, pathways, perspectives. Oncogene 25, 6680-6684.

Glasmacher, E., Hoefig, K.P., Vogel, K.U., Rath, N., Du, L., Wolf, C., Kremmer, E., Wang, X., and Heissmeyer, V. (2010). Roquin binds inducible costimulator mRNA and effectors of mRNA decay to induce microRNA-independent post-transcriptional repression. Nat. Immunol. *11*, 725–733.

Good-Jacobson, K.L., Szumilas, C.G., Chen, L., Sharpe, A.H., Tomayko, M.M., and Shlomchik, M.J. (2010). PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. Nature Immunology *11*, 535–542.

Goudeau, B., Huetz, F., Samson, S., Di Santo, J.P., Cumano, A., Beg, A., Israël, A., and Mémet, S. (2003). IkappaBalpha/IkappaBepsilon deficiency reveals that a critical NF-kappaB dosage is required for lymphocyte survival. Proc. Natl. Acad. Sci. U.S.A. *100*, 15800–15805.

Grant, C.R., Liberal, R., Mieli-Vergani, G., Vergani, D., and Longhi, M.S. (2015). Regulatory T-cells in autoimmune diseases: challenges, controversies and--yet--unanswered questions. Autoimmun Rev *14*, 105–116.

Grinberg-Bleyer, Y., Caron, R., Seeley, J.J., De Silva, N.S., Schindler, C.W., Hayden, M.S., Klein, U., and Ghosh, S. (2018a). The Alternative NF- κ B Pathway in Regulatory T Cell Homeostasis and Suppressive Function. J. Immunol. *200*, 2362–2371.

Grinberg-Bleyer, Y., Caron, R., Seeley, J.J., De Silva, N.S., Schindler, C.W., Hayden, M.S., Klein, U., and Ghosh, S. (2018b). The alternative NF-κB pathway in regulatory T cell homeostasis and suppressive function. J Immunol *200*, 2362–2371.

Gringhuis, S.I., den Dunnen, J., Litjens, M., van der Vlist, M., Wevers, B., Bruijns, S.C.M., and Geijtenbeek, T.B.H. (2009). Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. Nat. Immunol. *10*, 203–213.

Grosjean-Raillard, J., Adès, L., Boehrer, S., Tailler, M., Fabre, C., Braun, T., De Botton, S., Israel, A., Fenaux, P., and Kroemer, G. (2008). Flt3 receptor inhibition reduces constitutive NFkappaB activation in high-risk myelodysplastic syndrome and acute myeloid leukemia. Apoptosis *13*, 1148–1161.

Guzman, M.L., Neering, S.J., Upchurch, D., Grimes, B., Howard, D.S., Rizzieri, D.A., Luger, S.M., and Jordan, C.T. (2001). Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. Blood *98*, 2301–2307.

Н

Habineza Ndikuyeze, G., Gaurnier-Hausser, A., Patel, R., Baldwin, A.S., May, M.J., Flood, P., Krick, E., Propert, K.J., and Mason, N.J. (2014). A phase I clinical trial of systemically delivered NEMO binding domain peptide in dogs with spontaneous activated B-cell like diffuse large B-cell lymphoma. PLoS ONE *9*, e95404.

Hara, M.R., Agrawal, N., Kim, S.F., Cascio, M.B., Fujimuro, M., Ozeki, Y., Takahashi, M., Cheah, J.H., Tankou, S.K., Hester, L.D., et al. (2005). S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. Nat. Cell Biol. 7, 665–674.

Harb, W., Abramson, J., Lunning, M., Goy, A., Maddocks, K., Lebedinsky, C., Senderowicz, A., Trojer, P., Bradley, W.D., and Flinn, I. (2018). 42OA phase 1 study of CPI-1205, a small molecule inhibitor of EZH2, preliminary safety in patients with B-cell lymphomas. Ann Oncol *29*.

Hatzi, K., Nance, J.P., Kroenke, M.A., Bothwell, M., Haddad, E.K., Melnick, A., and Crotty, S. (2015). BCL6 orchestrates Tfh cell differentiation via multiple distinct mechanisms. J Exp Med *212*, 539–553. Hayden, M.S., and Ghosh, S. (2012). NF-κB, the first quarter-century: remarkable progress and outstanding questions. Genes Dev *26*, 203–234.

Haynes, N.M., Allen, C.D.C., Lesley, R., Ansel, K.M., Killeen, N., and Cyster, J.G. (2007). Role of CXCR5 and CCR7 in Follicular Th Cell Positioning and Appearance of a Programmed Cell Death Gene-1High Germinal Center-Associated Subpopulation. The Journal of Immunology *179*, 5099–5108.

Henning, S.W., Galandrini, R., Hall, A., and Cantrell, D.A. (1997). The GTPase Rho has a critical
regulatory role in thymus development. EMBO J. 16, 2397-2407.

Hernandez, J.B., Newton, R.H., and Walsh, C.M. (2010). Life and death in the thymus – cell death signaling during T cell development. Curr Opin Cell Biol 22, 865–871.

Hettmann, T., DiDonato, J., Karin, M., and Leiden, J.M. (1999). An Essential Role for Nuclear Factor κB in Promoting Double Positive Thymocyte Apoptosis. J Exp Med *189*, 145–158.

Hideshima, T., Chauhan, D., Richardson, P., Mitsiades, C., Mitsiades, N., Hayashi, T., Munshi, N., Dang, L., Castro, A., Palombella, V., et al. (2002). NF-κB as a Therapeutic Target in Multiple Myeloma. J. Biol. Chem. *277*, 16639–16647.

Hodi, F.S., O'Day, S.J., McDermott, D.F., Weber, R.W., Sosman, J.A., Haanen, J.B., Gonzalez, R., Robert, C., Schadendorf, D., Hassel, J.C., et al. (2010). Improved survival with ipilimumab in patients with metastatic melanoma. N. Engl. J. Med. *363*, 711–723.

Honma, K., Tsuzuki, S., Nakagawa, M., Tagawa, H., Nakamura, S., Morishima, Y., and Seto, M. (2009). TNFAIP3/A20 functions as a novel tumor suppressor gene in several subtypes of non-Hodgkin lymphomas. Blood *114*, 2467–2475.

Horwitz, S.M., Advani, R.H., Bartlett, N.L., Jacobsen, E.D., Sharman, J.P., O'Connor, O.A., Siddiqi, T., Kennedy, D.A., and Oki, Y. (2014). Objective responses in relapsed T-cell lymphomas with singleagent brentuximab vedotin. Blood *123*, 3095–3100.

Hu, H., Wu, X., Jin, W., Chang, M., Cheng, X., and Sun, S.-C. (2011). Noncanonical NF-kappaB regulates inducible costimulator (ICOS) ligand expression and T follicular helper cell development. Proc. Natl. Acad. Sci. U.S.A. *108*, 12827–12832.

Huang, J., Zhang, P.-H., Gao, Y.-H., and Qiu, L.-G. (2012). Sequential development of diffuse large B-cell lymphoma in a patient with angioimmunoblastic T-cell lymphoma. Diagn. Cytopathol. *40*, 346–351.

Huitorel, P., and Pantaloni, D. (1985). European Journal of Biochemistry 150, 265-269.

Hulleman, E., Kazemier, K.M., Holleman, A., VanderWeele, D.J., Rudin, C.M., Broekhuis, M.J.C., Evans, W.E., Pieters, R., and Boer, M.L.D. (2009). Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. Blood *113*, 2014–2021.

I

Iqbal, J., Wright, G., Wang, C., Rosenwald, A., Gascoyne, R.D., Weisenburger, D.D., Greiner, T.C., Smith, L., Guo, S., Wilcox, R.A., et al. (2014). Gene expression signatures delineate biological and prognostic subgroups in peripheral T-cell lymphoma. Blood *123*, 2915–2923.

Ishitani, R., Tanaka, M., Sunaga, K., Katsube, N., and Chuang, D.M. (1998). Nuclear localization of overexpressed glyceraldehyde-3-phosphate dehydrogenase in cultured cerebellar neurons undergoing apoptosis. Mol. Pharmacol. *53*, 701–707.

Isomura, I., Palmer, S., Grumont, R.J., Bunting, K., Hoyne, G., Wilkinson, N., Banerjee, A., Proietto, A., Gugasyan, R., Wu, L., et al. (2009). c-Rel is required for the development of thymic Foxp3+ CD4 regulatory T cells. J Exp Med *206*, 3001–3014.

Italiano, A., Soria, J.-C., Toulmonde, M., Michot, J.-M., Lucchesi, C., Varga, A., Coindre, J.-M., Blakemore, S.J., Clawson, A., Suttle, B., et al. (2018). Tazemetostat, an EZH2 inhibitor, in relapsed or

refractory B-cell non-Hodgkin lymphoma and advanced solid tumours: a first-in-human, open-label, phase 1 study. Lancet Oncol. 19, 649-659.

Izban, K.F., Ergin, M., Qin, J.Z., Martinez, R.L., Pooley RJ, J.R., Saeed, S., and Alkan, S. (2000). Constitutive expression of NF-kappa B is a characteristic feature of mycosis fungoides: implications for apoptosis resistance and pathogenesis. Hum. Pathol. *31*, 1482–1490.

J

Jäättelä, M., and Tschopp, J. (2003). Caspase-independent cell death in T lymphocytes. Nat Immunol 4, 416–423.

Jacque, E., Tchenio, T., Piton, G., Romeo, P.-H., and Baud, V. (2005). RelA repression of RelB activity induces selective gene activation downstream of TNF receptors. Proc Natl Acad Sci U S A *102*, 14635–14640.

Jacquin, M.A., Chiche, J., Zunino, B., Bénéteau, M., Meynet, O., Pradelli, L.A., Marchetti, S., Cornille, A., Carles, M., and Ricci, J.-E. (2013). GAPDH binds to active Akt, leading to Bcl-xL increase and escape from caspase-independent cell death. Cell Death Differ. 20, 1043–1054.

Jain, S., Chen, J., Nicolae, A., Wang, H., Shin, D.-M., Adkins, E.B., Sproule, T.J., Leeth, C.M., Sakai, T., Kovalchuk, A.L., et al. (2015). IL-21-driven neoplasms in SJL mice mimic some key features of human angioimmunoblastic T-cell lymphoma. Am. J. Pathol. *185*, 3102–3114.

Jimi, E., Strickland, I., Voll, R.E., Long, M., and Ghosh, S. (2008). Differential role of the transcription factor NF-kappaB in selection and survival of CD4+ and CD8+ thymocytes. Immunity *29*, 523–537.

Jin, H.-T., Ahmed, R., and Okazaki, T. (2011). Role of PD-1 in regulating T-cell immunity. Curr. Top. Microbiol. Immunol. *350*, 17–37.

Jogdand, G.M., Mohanty, S., and Devadas, S. (2016). Regulators of Tfh Cell Differentiation. Front Immunol 7.

Johnston, P.B., Cashen, A.F., Nikolinakos, P.G., Beaven, A.W., Barta, S.K., Bhat, G., Song, T., Choi, M.R., Allen, L.F., Vos, S. de, et al. (2015). Safe and Effective Treatment of Patients with Peripheral T-Cell Lymphoma (PTCL) with the Novel HDAC Inhibitor, Belinostat, in Combination with CHOP: Results of the Bel-CHOP Phase 1 Trial. Blood *126*, 253–253.

Jose, C., Bellance, N., and Rossignol, R. (2011). Choosing between glycolysis and oxidative phosphorylation: A tumor's dilemma? Biochimica et Biophysica Acta (BBA) - Bioenergetics 1807, 552–561.

К

Kaufman, H.L., Russell, J., Hamid, O., Bhatia, S., Terheyden, P., D'Angelo, S.P., Shih, K.C., Lebbé, C., Linette, G.P., Milella, M., et al. (2016). Avelumab in patients with chemotherapy-refractory metastatic Merkel cell carcinoma: a multicentre, single-group, open-label, phase 2 trial. Lancet Oncol. *17*, 1374–1385.

Kawano, R., Ohshima, K., Wakamatsu, S., Suzumiya, J., Kikuchi, M., and Tamura, K. (2005). Epstein-Barr virus genome level, T-cell clonality and the prognosis of angioimmunoblastic T-cell lymphoma. Haematologica *90*, 1192–1196.

Kerres, N., Steurer, S., Schlager, S., Bader, G., Berger, H., Caligiuri, M., Dank, C., Engen, J.R.,

Ettmayer, P., Fischerauer, B., et al. (2017). Chemically Induced Degradation of the Oncogenic Transcription Factor BCL6. Cell Rep 20, 2860–2875.

Khan, N., Ozkaya, N., Moskowitz, A., Dogan, A., and Horwitz, S. (2018). Peripheral T-cell lymphoma - are we making progress? Best Pract Res Clin Haematol *31*, 306–314.

Kim, S.J., Yoon, D.H., Kang, H.J., Kim, J.S., Park, S.K., Kim, H.J., Lee, J., Ryoo, B.-Y., Ko, Y.H., Huh, J., et al. (2012). Bortezomib in combination with CHOP as first-line treatment for patients with stage III/IV peripheral T-cell lymphomas: a multicentre, single-arm, phase 2 trial. Eur. J. Cancer *48*, 3223–3231.

Kirkwood, J.M., Strawderman, M.H., Ernstoff, M.S., Smith, T.J., Borden, E.C., and Blum, R.H. (1996). Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. J. Clin. Oncol. *14*, 7–17.

Konstantinou, K., Yamamoto, K., Ishibashi, F., Mizoguchi, Y., Kurata, M., Nakagawa, Y., Suzuki, K., Sawabe, M., Ohta, M., Miyakoshi, S., et al. (2009). Angiogenic mediators of the angiopoietin system are highly expressed by CD10-positive lymphoma cells in angioimmunoblastic T-cell lymphoma. Br. J. Haematol. *144*, 696–704.

Krynetski, E.Y., Krynetskaia, N.F., Gallo, A.E., Murti, K.G., and Evans, W.E. (2001). Mol Pharmacol *59*, 367–374.

Ĺ

Lavallard, V.J., Pradelli, L.A., Paul, A., Bénéteau, M., Jacquel, A., Auberger, P., and Ricci, J.-E. (2009). Modulation of caspase-independent cell death leads to resensitization of imatinib mesylate-resistant cells. Cancer Res. *69*, 3013–3020.

Le Gouill, S., Milpied, N., Buzyn, A., De Latour, R.P., Vernant, J.-P., Mohty, M., Moles, M.-P., Bouabdallah, K., Bulabois, C.-E., Dupuis, J., et al. (2008). Graft-versus-lymphoma effect for aggressive T-cell lymphomas in adults: a study by the Société Francaise de Greffe de Moëlle et de Thérapie Cellulaire. J. Clin. Oncol. *26*, 2264–2271.

Lee, K., Min, H.J., Jang, E.J., Hong, J.-H., and Hwang, E.S. (2010). In vivo tumor suppression activity by T cell-specific T-bet restoration. Int. J. Cancer *127*, 2129–2137.

Lee, M.N., Ha, S.H., Kim, J., Koh, A., Lee, C.S., Kim, J.H., Jeon, H., Kim, D.-H., Suh, P.-G., and Ryu, S.H. (2009). Glycolytic Flux Signals to mTOR through Glyceraldehyde-3-Phosphate Dehydrogenase-Mediated Regulation of Rheb. Mol Cell Biol *29*, 3991–4001.

Lee, S.H., Kim, J.S., Kim, J., Kim, S.J., Kim, W.S., Lee, S., Ko, Y.H., and Yoo, H.Y. (2015). A highly recurrent novel missense mutation in CD28 among angioimmunoblastic T-cell lymphoma patients. Haematologica *100*, e505-507.

Lemonnier, F., Couronné, L., Parrens, M., Jaïs, J.-P., Travert, M., Lamant, L., Tournillac, O., Rousset, T., Fabiani, B., Cairns, R.A., et al. (2012). Recurrent TET2 mutations in peripheral T-cell lymphomas correlate with TFH-like features and adverse clinical parameters. Blood *120*, 1466–1469.

Lesokhin, A.M., Ansell, S.M., Armand, P., Scott, E.C., Halwani, A., Gutierrez, M., Millenson, M.M., Cohen, A.D., Schuster, S.J., Lebovic, D., et al. (2016). Nivolumab in Patients With Relapsed or Refractory Hematologic Malignancy: Preliminary Results of a Phase Ib Study. J. Clin. Oncol. *34*, 2698–2704.

Leval, L.D., Gisselbrecht, C., and Gaulard, P. (2010). Advances in the understanding and management of angioimmunoblastic T-cell lymphoma. British Journal of Haematology *148*, 673–689.

Liberti, M.V., Dai, Z., Wardell, S.E., Baccile, J.A., Liu, X., Gao, X., Baldi, R., Mehrmohamadi, M., Johnson, M.O., Madhukar, N.S., et al. (2017). A Predictive Model for Selective Targeting of the Warburg Effect through GAPDH Inhibition with a Natural Product. Cell Metab. *26*, 648-659.e8.

Linterman, M.A., Beaton, L., Yu, D., Ramiscal, R.R., Srivastava, M., Hogan, J.J., Verma, N.K., Smyth, M.J., Rigby, R.J., and Vinuesa, C.G. (2010). IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. J. Exp. Med. 207, 353–363.

Liu, X., Yu, H., Yang, W., Zhou, X., Lu, H., and Shi, D. (2010). Mutations of NFKBIA in biopsy specimens from Hodgkin lymphoma. Cancer Genet. Cytogenet. 197, 152–157.

Li-Weber, M., Giaisi, M., Baumann, S., Pálfi, K., and Krammer, P.H. (2004). NF-kappa B synergizes with NF-AT and NF-IL6 in activation of the IL-4 gene in T cells. Eur. J. Immunol. *34*, 1111–1118.

Lu, H., Zhang, Y., Roberts, D.D., Osborne, C.K., and Templeton, N.S. (2002). Enhanced Gene Expression in Breast Cancer Cells in Vitro and Tumors in Vivo. Molecular Therapy *6*, 783–792.

Lu, Y.-S., Yeh, P.-Y., Chuang, S.-E., Gao, M., Kuo, M.-L., and Cheng, A.-L. (2006). Glucocorticoids enhance cytotoxicity of cisplatin via suppression of NF-{kappa}B activation in the glucocorticoid receptor-rich human cervical carcinoma cell line SiHa. J. Endocrinol. *188*, 311–319.

Lunning, M.A., and Vose, J.M. (2017). Angioimmunoblastic T-cell lymphoma: the many-faced lymphoma. Blood *129*, 1095–1102.

Lunt, S.Y., and Vander Heiden, M.G. (2011). Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation. Annu. Rev. Cell Dev. Biol. 27, 441–464.

 \mathcal{M}

Macintyre, A.N., Gerriets, V.A., Nichols, A.G., Michalek, R.D., Rudolph, M.C., Deoliveira, D., Anderson, S.M., Abel, E.D., Chen, B.J., Hale, L.P., et al. (2014). The Glucose Transporter Glut1 Is Selectively Essential for CD4 T Cell Activation and Effector Function. Cell Metabolism *20*, 61–72.

Mandal, M., Borowski, C., Palomero, T., Ferrando, A.A., Oberdoerffer, P., Meng, F., Ruiz-Vela, A., Ciofani, M., Zuniga-Pflucker, J.-C., Screpanti, I., et al. (2005). The BCL2A1 gene as a pre-T cell receptor-induced regulator of thymocyte survival. J. Exp. Med. 201, 603–614.

Mansouri, L., Sutton, L.-A., Ljungström, V., Bondza, S., Arngården, L., Bhoi, S., Larsson, J., Cortese, D., Kalushkova, A., Plevova, K., et al. (2015). Functional loss of IκBε leads to NF-κB deregulation in aggressive chronic lymphocytic leukemia. J. Exp. Med. *212*, 833–843.

Mansouri, L., Papakonstantinou, N., Ntoufa, S., Stamatopoulos, K., and Rosenquist, R. (2016). NF- κ B activation in chronic lymphocytic leukemia: A point of convergence of external triggers and intrinsic lesions. Seminars in Cancer Biology *39*, 40–48.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends in Immunology 23, 549–555.

Mao, Z.J., Surowiecka, M., Linden, M.A., and Singleton, T.P. (2015). Abnormal immunophenotype of the T-cell-receptor beta Chain in follicular-helper T cells of angioimmunoblastic T-cell lymphoma.

Cytometry B Clin Cytom 88, 190–193.

Martínez-Delgado, B., Cuadros, M., Honrado, E., Ruiz de la Parte, A., Roncador, G., Alves, J., Castrillo, J.M., Rivas, C., and Benítez, J. (2005). Differential expression of NF-kappaB pathway genes among peripheral T-cell lymphomas. Leukemia *19*, 2254–2263.

Martin-Orozco, N., Muranski, P., Chung, Y., Yang, X.O., Yamazaki, T., Lu, S., Hwu, P., Restifo, N.P., Overwijk, W.W., and Dong, C. (2009). T helper 17 cells promote cytotoxic T cell activation in tumor immunity. Immunity *31*, 787–798.

Maruyama, D., Tobinai, K., Makita, S., Ishida, T., Kusumoto, S., Ishitsuka, K., Yoshimitsu, M., Imaizumi, Y., Sawayama, Y., Takeuchi, S., et al. (2017). First-in-Human Study of the EZH1/2 Dual Inhibitor DS-3201b in Patients with Relapsed or Refractory Non-Hodgkin Lymphomas — Preliminary Results. Blood *130*, 4070–4070.

Maruyama, W., Akao, Y., Youdim, M.B.H., Davis, B.A., and Naoi, M. (2001). Transfection-enforced Bcl-2 overexpression and an anti-Parkinson drug, rasagiline, prevent nuclear accumulation of glyceraldehyde-3-phosphate dehydrogenase induced by an endogenous dopaminergic neurotoxin, N-methyl(R)salsolinol. Journal of Neurochemistry *78*, 727–735.

Maubach, G., Feige, M.H., Lim, M.C.C., and Naumann, M. (2019). NF-kappaB-inducing kinase in cancer. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer 1871, 40–49.

Messina, N., Fulford, T., O'Reilly, L., Loh, W.X., Motyer, J.M., Ellis, D., McLean, C., Naeem, H., Lin, A., Gugasyan, R., et al. (2016). The NF-κB transcription factor RelA is required for the tolerogenic function of Foxp3(+) regulatory T cells. J. Autoimmun. 70, 52–62.

Mhaidly, R., and Verhoeyen, E. (2019). The Future: In Vivo CAR T Cell Gene Therapy. Mol. Ther. 27, 707–709.

Miranda, T.B., Cortez, C.C., Yoo, C.B., Liang, G., Abe, M., Kelly, T.K., Marquez, V.E., and Jones, P.A. (2009). DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. Mol. Cancer Ther. *8*, 1579–1588.

Mitsuzawa, H., Kimura, M., Kanda, E., and Ishihama, A. (2005). Glyceraldehyde-3-phosphate dehydrogenase and actin associate with RNA polymerase II and interact with its Rpb7 subunit. FEBS Letters *579*, 48–52.

Mondragón, L., Mhaidly, R., De Donatis, G.M., Tosolini, M., Dao, P., Martin, A.R., Pons, C., Chiche, J., Jacquin, M., Imbert, V., et al. (2019). GAPDH Overexpression in the T Cell Lineage Promotes Angioimmunoblastic T Cell Lymphoma through an NF-κB-Dependent Mechanism. Cancer Cell.

Mookherjee, N., Lippert, D.N.D., Hamill, P., Falsafi, R., Nijnik, A., Kindrachuk, J., Pistolic, J., Gardy, J., Miri, P., Naseer, M., et al. (2009). Intracellular receptor for human host defense peptide LL-37 in monocytes. J. Immunol. *183*, 2688–2696.

Morgan, J.A., Yin, Y., Borowsky, A.D., Kuo, F., Nourmand, N., Koontz, J.I., Reynolds, C., Soreng, L., Griffin, C.A., Graeme-Cook, F., et al. (1999). Breakpoints of the t(11;18)(q21;q21) in mucosa-associated lymphoid tissue (MALT) lymphoma lie within or near the previously undescribed gene MALT1 in chromosome 18. Cancer Res. *59*, 6205–6213.

Motzer, R.J., Escudier, B., McDermott, D.F., George, S., Hammers, H.J., Srinivas, S., Tykodi, S.S., Sosman, J.A., Procopio, G., Plimack, E.R., et al. (2015). Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. N. Engl. J. Med. *373*, 1803–1813.

Murray, S.E., Polesso, F., Rowe, A.M., Basak, S., Koguchi, Y., Toren, K.G., Hoffmann, A., and Parker, D.C. (2011). NF- κ B–inducing kinase plays an essential T cell–intrinsic role in graft-versus-host disease and lethal autoimmunity in mice. J. Clin. Invest. *121*, 4775–4786.

Muto, G., Kotani, H., Kondo, T., Morita, R., Tsuruta, S., Kobayashi, T., Luche, H., Fehling, H.J., Walsh, M., Choi, Y., et al. (2013). TRAF6 Is Essential for Maintenance of Regulatory T Cells That Suppress Th2 Type Autoimmunity. PLoS One 8.

Muto, H., Sakata-Yanagimoto, M., Nagae, G., Shiozawa, Y., Miyake, Y., Yoshida, K., Enami, T., Kamada, Y., Kato, T., Uchida, K., et al. (2014). Reduced TET2 function leads to T-cell lymphoma with follicular helper T-cell-like features in mice. Blood Cancer J *4*, e264.

N

Nagy, E., Henics, T., Eckert, M., Miseta, A., Lightowlers, R.N., and Kellermayer, M. (2000). Identification of the NAD+-Binding Fold of Glyceraldehyde-3-Phosphate Dehydrogenase as a Novel RNA-Binding Domain. Biochemical and Biophysical Research Communications *275*, 253–260.

Najafi, S., and Mirshafiey, A. (2019). The role of T helper 17 and regulatory T cells in tumor microenvironment. Immunopharmacology and Immunotoxicology 41, 16–24.

Nakagawa, T., Hirano, Y., Inomata, A., Yokota, S., Miyachi, K., Kaneda, M., Umeda, M., Furukawa, K., Omata, S., and Horigome, T. (2003). J. Biol. Chem. 278, 20395–20404.

Neri, A., Chang, C.C., Lombardi, L., Salina, M., Corradini, P., Maiolo, A.T., Chaganti, R.S., and Dalla-Favera, R. (1991). B cell lymphoma-associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-kappa B p50. Cell *67*, 1075–1087.

Ng, S.Y., Brown, L., Stevenson, K., deSouza, T., Aster, J.C., Louissaint, A., and Weinstock, D.M. (2018). RhoA G17V is sufficient to induce autoimmunity and promotes T-cell lymphomagenesis in mice. Blood *132*, 935–947.

Nishizawa, S., Sakata-Yanagimoto, M., Hattori, K., Muto, H., Nguyen, T., Izutsu, K., Yoshida, K., Ogawa, S., Nakamura, N., and Chiba, S. (2017). BCL6 locus is hypermethylated in angioimmunoblastic T-cell lymphoma. Int. J. Hematol. *105*, 465–469.

Nomoto, J., Hiramoto, N., Kato, M., Sanada, M., Maeshima, A.M., Taniguchi, H., Hosoda, F., Asakura, Y., Munakata, W., Sekiguchi, N., et al. (2012). Deletion of the TNFAIP3/A20 gene detected by FICTION analysis in classical Hodgkin lymphoma. BMC Cancer *12*, 457.

Numasaki, M., Fukushi, J., Ono, M., Narula, S.K., Zavodny, P.J., Kudo, T., Robbins, P.D., Tahara, H., and Lotze, M.T. (2003). Interleukin-17 promotes angiogenesis and tumor growth. Blood *101*, 2620–2627.

Nurieva, R.I., Chung, Y., Martinez, G.J., Yang, X.O., Tanaka, S., Matskevitch, T.D., Wang, Y.-H., and Dong, C. (2009). Bcl6 mediates the development of T follicular helper cells. Science *325*, 1001–1005. Odqvist, L., Sánchez-Beato, M., Montes-Moreno, S., Martín-Sánchez, E., Pajares, R., Sánchez-Verde, L., Ortiz-Romero, P.L., Rodriguez, J., Rodríguez-Pinilla, S.M., Iniesta-Martínez, F., et al. (2013). NIK controls classical and alternative NF-κB activation and is necessary for the survival of human T-cell lymphoma cells. Clin. Cancer Res. *19*, 2319–2330.

Oeckinghaus, A., and Ghosh, S. (2009). The NF-κB Family of Transcription Factors and Its Regulation. Cold Spring Harb Perspect Biol 1.

Oestreich, K.J., Read, K.A., Gilbertson, S.E., Hough, K.P., McDonald, P.W., Krishnamoorthy, V., and Weinmann, A.S. (2014). Bcl-6 directly represses the gene program of the glycolysis pathway. Nat. Immunol. *15*, 957–964.

Oh, H., Grinberg-Bleyer, Y., Liao, W., Maloney, D., Wang, P., Wu, Z., Wang, J., Bhatt, D.M., Heise, N., Schmid, R.M., et al. (2017). An NF-κB transcription factor-dependent, lineage specific transcriptional program promotes regulatory T cell identity and function. Immunity *47*, 450-465.e5.

Ohshima KHaraoka, S., Suzumiya, J., Kawasaki, C., Kanda, M., and Kikuchi, M. (2000). Cytoplasmic cytokines in lymphoproliferative disorders: multiple cytokine production in angioimmunoblastic lymphadenopathy with dysproteinemia. Leuk. Lymphoma *38*, 541–545.

Olver, S., Apte, S.H., Baz, A., Kelso, A., and Kienzle, N. (2013). Interleukin-4-induced loss of CD8 expression and cytolytic function in effector CD8 T cells persists long term in vivo. Immunology *139*, 187–196.

O'Sullivan, D., van der Windt, G.J.W., Huang, S.C.-C., Curtis, J.D., Chang, C.-H., Buck, M.D., Qiu, J., Smith, A.M., Lam, W.Y., DiPlato, L.M., et al. (2014). Memory CD8(+) T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development. Immunity *41*, 75–88.

Otto, C., Giefing, M., Massow, A., Vater, I., Gesk, S., Schlesner, M., Richter, J., Klapper, W., Hansmann, M.-L., Siebert, R., et al. (2012). Genetic lesions of the TRAF3 and MAP3K14 genes in classical Hodgkin lymphoma. Br. J. Haematol. *157*, 702–708.

Ozaki, K., Spolski, R., Ettinger, R., Kim, H.-P., Wang, G., Qi, C.-F., Hwu, P., Shaffer, D.J., Akilesh, S., Roopenian, D.C., et al. (2004). Regulation of B Cell Differentiation and Plasma Cell Generation by IL-21, a Novel Inducer of Blimp-1 and Bcl-6. The Journal of Immunology *173*, 5361–5371.

Р

Pahl, H.L. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18, 6853–6866.

Palayoor, S.T., Youmell, M.Y., Calderwood, S.K., Coleman, C.N., and Price, B.D. (1999). Constitutive activation of IkappaB kinase alpha and NF-kappaB in prostate cancer cells is inhibited by ibuprofen. Oncogene *18*, 7389–7394.

Palomero, T., Couronné, L., Khiabanian, H., Kim, M.-Y., Ambesi-Impiombato, A., Perez-Garcia, A., Carpenter, Z., Abate, F., Allegretta, M., Haydu, J.E., et al. (2014). Recurrent mutations in epigenetic regulators, RHOA and FYN kinase in peripheral T cell lymphomas. Nat. Genet. *46*, 166–170.

Pardoll, D.M. (2012). The blockade of immune checkpoints in cancer immunotherapy. Nature Reviews Cancer 12, 252–264.

Patel, M.R., Ellerton, J., Infante, J.R., Agrawal, M., Gordon, M., Aljumaily, R., Britten, C.D., Dirix, L., Lee, K.-W., Taylor, M., et al. (2018). Avelumab in metastatic urothelial carcinoma after platinum failure (JAVELIN Solid Tumor): pooled results from two expansion cohorts of an open-label, phase 1 trial. Lancet Oncol. *19*, 51–64.

Patsoukis, N., Bardhan, K., Chatterjee, P., Sari, D., Liu, B., Bell, L.N., Karoly, E.D., Freeman, G.J., Petkova, V., Seth, P., et al. (2015). PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis

and promoting lipolysis and fatty acid oxidation. Nat Commun 6, 6692.

Patsoukis, N., Weaver, J.D., Strauss, L., Herbel, C., Seth, P., and Boussiotis, V.A. (2017). Immunometabolic Regulations Mediated by Coinhibitory Receptors and Their Impact on T Cell Immune Responses. Front Immunol *8*, 330.

Pavan, A., Spina, M., Canzonieri, V., Sansonno, S., Toffoli, G., and De Re, V. (2008). Recent prognostic factors in diffuse large B-cell lymphoma indicate NF-kappaB pathway as a target for new therapeutic strategies. Leuk. Lymphoma 49, 2048–2058.

Pearce, E.L., and Pearce, E.J. (2013). Metabolic Pathways in Immune Cell Activation and Quiescence. Immunity *38*, 633–643.

Pellom, S.T., Dudimah, D.F., Thounaojam, M.C., Uzhachenko, R.V., Singhal, A., Richmond, A., and Shanker, A. (2017). Bortezomib augments lymphocyte stimulatory cytokine signaling in the tumor microenvironment to sustain CD8+T cell antitumor function. Oncotarget *8*, 8604–8621.

Pernis, A.B. (2009). Rho GTPase-mediated pathways in mature CD4+ T cells. Autoimmunity Reviews *8*, 199–203.

Polo, J.M., Juszczynski, P., Monti, S., Cerchietti, L., Ye, K., Greally, J.M., Shipp, M., and Melnick, A. (2007). Transcriptional signature with differential expression of BCL6 target genes accurately identifies BCL6-dependent diffuse large B cell lymphomas. PNAS *104*, 3207–3212.

Powles, T., Durán, I., van der Heijden, M.S., Loriot, Y., Vogelzang, N.J., De Giorgi, U., Oudard, S., Retz, M.M., Castellano, D., Bamias, A., et al. (2018). Atezolizumab versus chemotherapy in patients with platinum-treated locally advanced or metastatic urothelial carcinoma (IMvigor211): a multicentre, open-label, phase 3 randomised controlled trial. Lancet *391*, 748–757.

Pro, B., Horwitz, S.M., Prince, H.M., Foss, F.M., Sokol, L., Greenwood, M., Caballero, D., Morschhauser, F., Wilhelm, M., Iyer, S.P., et al. (2017). Romidepsin induces durable responses in patients with relapsed or refractory angioimmunoblastic T-cell lymphoma. Hematol Oncol *35*, 914–917. Puente, X.S., Pinyol, M., Quesada, V., Conde, L., Ordóñez, G.R., Villamor, N., Escaramis, G., Jares, P., Beà, S., González-Díaz, M., et al. (2011). Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature *475*, 101–105.

Q

Qorraj, M., Bruns, H., Böttcher, M., Weigand, L., Saul, D., Mackensen, A., Jitschin, R., and Mougiakakos, D. (2017). The PD-1/PD-L1 axis contributes to immune metabolic dysfunctions of monocytes in chronic lymphocytic leukemia. Leukemia *31*, 470–478.

 \mathcal{R}

Rasmussen, K.D., Jia, G., Johansen, J.V., Pedersen, M.T., Rapin, N., Bagger, F.O., Porse, B.T., Bernard, O.A., Christensen, J., and Helin, K. (2015). Loss of TET2 in hematopoietic cells leads to DNA hypermethylation of active enhancers and induction of leukemogenesis. Genes Dev. *29*, 910–922.

Ratner, L., Waldmann, T.A., Janakiram, M., and Brammer, J.E. (2018). Rapid Progression of Adult T-Cell Leukemia–Lymphoma after PD-1 Inhibitor Therapy. New England Journal of Medicine *378*, 1947–1948.

Ray, J.P., Staron, M.M., Shyer, J.A., Ho, P.-C., Marshall, H.D., Gray, S.M., Laidlaw, B.J., Araki, K., Ahmed, R., Kaech, S.M., et al. (2015). The Interleukin-2-mTORc1 Kinase Axis Defines the Signaling,

Differentiation, and Metabolism of T Helper 1 and Follicular B Helper T Cells. Immunity 43, 690–702. Reck, M., Bondarenko, I., Luft, A., Serwatowski, P., Barlesi, F., Chacko, R., Sebastian, M., Lu, H., Cuillerot, J.-M., and Lynch, T.J. (2013). Ipilimumab in combination with paclitaxel and carboplatin as first-line therapy in extensive-disease-small-cell lung cancer: results from a randomized, double-blind, multicenter phase 2 trial. Ann. Oncol. 24, 75–83.

Reddy, B.S., Rao, C.V., Rivenson, A., and Kelloff, G. (1993). Inhibitory effect of aspirin on azoxymethane-induced colon carcinogenesis in F344 rats. Carcinogenesis 14, 1493–1497.

Ren, X., Li, X., Jia, L., Chen, D., Hou, H., Rui, L., Zhao, Y., and Chen, Z. (2016). A small-molecule inhibitor of NF-κB-inducing kinase (NIK) protects liver from toxin-induced inflammation, oxidative stress, and injury. The FASEB Journal *31*, 711–718.

Ricci, J.-E., and Chiche, J. (2018). Metabolic Reprogramming of Non-Hodgkin's B-Cell Lymphomas and Potential Therapeutic Strategies. Front Oncol *8*, 556.

Robb, R.J., Lineburg, K.E., Kuns, R.D., Wilson, Y.A., Raffelt, N.C., Olver, S.D., Varelias, A., Alexander, K.A., Teal, B.E., Sparwasser, T., et al. (2012). Identification and expansion of highly suppressive CD8+FoxP3+ regulatory T cells after experimental allogeneic bone marrow transplantation. Blood *119*, 5898–5908.

Robbins, A.R., Ward, R.D., and Oliver, C. (1995). A mutation in glyceraldehyde 3-phosphate dehydrogenase alters endocytosis in CHO cells. The Journal of Cell Biology *130*, 1093–1104.

Robbins, G.R., Truax, A.D., Davis, B.K., Zhang, L., Brickey, W.J., and Ting, J.P.-Y. (2012). Regulation of class I major histocompatibility complex (MHC) by nucleotide-binding domain, leucine-rich repeat-containing (NLR) proteins. J. Biol. Chem. 287, 24294–24303.

Robert, C., Thomas, L., Bondarenko, I., O'Day, S., Weber, J., Garbe, C., Lebbe, C., Baurain, J.-F., Testori, A., Grob, J.-J., et al. (2011). Ipilimumab plus Dacarbazine for Previously Untreated Metastatic Melanoma. New England Journal of Medicine *364*, 2517–2526.

Rodríguez-Pascual, F., Redondo-Horcajo, M., Magán-Marchal, N., Lagares, D., Martínez-Ruiz, A., Kleinert, H., and Lamas, S. (2008). Glyceraldehyde-3-Phosphate Dehydrogenase Regulates Endothelin-1 Expression by a Novel, Redox-Sensitive Mechanism Involving mRNA Stability. Molecular and Cellular Biology *28*, 7139–7155.

Rohr, J., Guo, S., Huo, J., Bouska, A., Lachel, C., Li, Y., Simone, P.D., Zhang, W., Gong, Q., Wang, C., et al. (2016). Recurrent activating mutations of CD28 in peripheral T-cell lymphomas. Leukemia *30*, 1062.

Rosenberg, S.A., Yang, J.C., White, D.E., and Steinberg, S.M. (1998). Durability of complete responses in patients with metastatic cancer treated with high-dose interleukin-2: identification of the antigens mediating response. Ann. Surg. 228, 307–319.

Roth, R.B., Hevezi, P., Lee, J., Willhite, D., Lechner, S.M., Foster, A.C., and Zlotnik, A. (2006). Gene expression analyses reveal molecular relationships among 20 regions of the human CNS. Neurogenetics 7, 67–80.

Ruan, Q., Kameswaran, V., Tone, Y., Li, L., Liou, H.-C., Greene, M.I., Tone, M., and Chen, Y.H. (2009). Development of Foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome. Immunity *31*, 932–940.

Sabattini, E., Pizzi, M., Tabanelli, V., Baldin, P., Sacchetti, C.S., Agostinelli, C., Zinzani, P.L., and Pileri, S.A. (2013). CD30 expression in peripheral T-cell lymphomas. Haematologica *98*, e81–e82.

Saito, T., Nishikawa, H., Wada, H., Nagano, Y., Sugiyama, D., Atarashi, K., Maeda, Y., Hamaguchi, M., Ohkura, N., Sato, E., et al. (2016). Two FOXP3⁺CD4⁺ T cell subpopulations distinctly control the prognosis of colorectal cancers. Nature Medicine *22*, 679–684.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J. Immunol. *155*, 1151–1164.

Sakata-Yanagimoto, M., Enami, T., Yoshida, K., Shiraishi, Y., Ishii, R., Miyake, Y., Muto, H., Tsuyama, N., Sato-Otsubo, A., Okuno, Y., et al. (2014). Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. Nat. Genet. *46*, 171–175.

Sato, F., Ishida, T., Ito, A., Mori, F., Masaki, A., Takino, H., Narita, T., Ri, M., Kusumoto, S., Suzuki, S., et al. (2013a). Angioimmunoblastic T-cell lymphoma mice model. Leukemia Research *37*, 21–27.

Sato, T., Kaneda, A., Tsuji, S., Isagawa, T., Yamamoto, S., Fujita, T., Yamanaka, R., Tanaka, Y., Nukiwa, T., Marquez, V.E., et al. (2013b). PRC2 overexpression and PRC2-target gene repression relating to poorer prognosis in small cell lung cancer. Sci Rep *3*.

Saunders, P.A., Chen, R.-W., and Chuang, D.-M. (1999). Nuclear Translocation of Glyceraldehyde-3-Phosphate Dehydrogenase Isoforms During Neuronal Apoptosis. Journal of Neurochemistry 72, 925–932.

Scheidereit, C. (2006). IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. Oncogene 25, 6685–6705.

Scheinman, R.I., Cogswell, P.C., Lofquist, A.K., and Baldwin, A.S. (1995). Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. Science 270, 283–286.

Schmidt-Supprian, M., Courtois, G., Tian, J., Coyle, A.J., Israël, A., Rajewsky, K., and Pasparakis, M. (2003). Mature T cells depend on signaling through the IKK complex. Immunity *19*, 377–389.

Schmitz, N., Trümper, L., Ziepert, M., Nickelsen, M., Ho, A.D., Metzner, B., Peter, N., Loeffler, M., Rosenwald, A., and Pfreundschuh, M. (2010). Treatment and prognosis of mature T-cell and NK-cell lymphoma: an analysis of patients with T-cell lymphoma treated in studies of the German High-Grade Non-Hodgkin Lymphoma Study Group. Blood *116*, 3418–3425.

Schuster, M., Plaza-Sirvent, C., Matthies, A.-M., Heise, U., Jeron, A., Bruder, D., Visekruna, A., Huehn, J., and Schmitz, I. (2017). c-REL and IkBNS Govern Common and Independent Steps of Regulatory T Cell Development from Novel CD122-Expressing Pre-Precursors. J. Immunol. *199*, 920–930.

Scourzic, L., Couronné, L., Pedersen, M.T., Della Valle, V., Diop, M., Mylonas, E., Calvo, J., Mouly, E., Lopez, C.K., Martin, N., et al. (2016). DNMT3A(R882H) mutant and Tet2 inactivation cooperate in the deregulation of DNA methylation control to induce lymphoid malignancies in mice. Leukemia *30*, 1388–1398.

Sen, J., Venkataraman, L., Shinkai, Y., Pierce, J.W., Alt, F.W., Burakoff, S.J., and Sen, R. (1995). Expression and induction of nuclear factor-kappa B-related proteins in thymocytes. J. Immunol. *154*, 3213–3221.

Sena, L.A., Li, S., Jairaman, A., Prakriya, M., Ezponda, T., Hildeman, D.A., Wang, C.-R., Schumacker, P.T., Licht, J.D., Perlman, H., et al. (2013). Mitochondria Are Required for Antigen-Specific T Cell Activation through Reactive Oxygen Species Signaling. Immunity *38*, 225–236.

Serre, K., Mohr, E., Bénézech, C., Bird, R., Khan, M., Caamaño, J.H., Cunningham, A.F., and Maclennan, I.C.M. (2011). Selective effects of NF-κB1 deficiency in CD4⁺ T cells on Th2 and TFh induction by alum-precipitated protein vaccines. Eur. J. Immunol. *41*, 1573–1582.

Shang, B., Liu, Y., Jiang, S., and Liu, Y. (2015). Prognostic value of tumor-infiltrating FoxP3⁺ regulatory T cells in cancers: a systematic review and meta-analysis. Scientific Reports 5, 15179.

Shankaran, V., Ikeda, H., Bruce, A.T., White, J.M., Swanson, P.E., Old, L.J., and Schreiber, R.D. (2001). IFN γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 410, 1107.

Shimizu, J., Yamazaki, S., and Sakaguchi, S. (1999). Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J. Immunol. *163*, 5211–5218.

Sica, V., Bravo-San Pedro, J.M., Stoll, G., and Kroemer, G. (2019). Oxidative phosphorylation as a potential therapeutic target for cancer therapy. Int. J. Cancer.

Singh, R., and Green, M.R. (1993). Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase. Science 259, 365–368.

Slack, M., Wang, T., and Wang, R. (2015). T cell metabolic reprogramming and plasticity. Mol. Immunol. 68, 507–512.

Sors, A., Jean-Louis, F., Bégué, E., Parmentier, L., Dubertret, L., Dreano, M., Courtois, G., Bachelez, H., and Michel, L. (2008). Inhibition of IkappaB kinase subunit 2 in cutaneous T-cell lymphoma down-regulates nuclear factor-kappaB constitutive activation, induces cell death, and potentiates the apoptotic response to antineoplastic chemotherapeutic agents. Clin. Cancer Res. *14*, 901–911.

Spina, V., and Rossi, D. (2016). NF-κB deregulation in splenic marginal zone lymphoma. Semin. Cancer Biol. *39*, 61–67.

Stazi, G., Zwergel, C., Mai, A., and Valente, S. (2017). EZH2 inhibitors: a patent review (2014-2016). Expert Opinion on Therapeutic Patents 27, 797–813.

Streubel, B., Lamprecht, A., Dierlamm, J., Cerroni, L., Stolte, M., Ott, G., Raderer, M., and Chott, A. (2003). T(14;18)(q32;q21) involving IGH and MALT1 is a frequent chromosomal aberration in MALT lymphoma. Blood *101*, 2335–2339.

Su, I.-H., Basavaraj, A., Krutchinsky, A.N., Hobert, O., Ullrich, A., Chait, B.T., and Tarakhovsky, A. (2003). Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. Nat. Immunol. *4*, 124–131.

Sukumar, M., Roychoudhuri, R., and Restifo, N.P. (2015). Nutrient Competition: A New Axis of Tumor Immunosuppression. Cell *162*, 1206–1208.

Sun, S.-C. (2011). Non-canonical NF-kB signaling pathway. Cell Res. 21, 71-85.

Sun, S.-C. (2012). The noncanonical NF-κB pathway. Immunol Rev 246, 125–140. Sun, L., Suo, C., Li, S.-T., Zhang, H., and Gao, P. (2018). Metabolic reprogramming for cancer cells and their microenvironment: Beyond the Warburg Effect. Biochim Biophys Acta Rev Cancer 1870, 51– 66.

Sundararaj, K.P., Wood, R.E., Ponnusamy, S., Salas, A.M., Szulc, Z., Bielawska, A., Obeid, L.M., Hannun, Y.A., and Ogretmen, B. (2004). Rapid shortening of telomere length in response to ceramide involves the inhibition of telomere binding activity of nuclear glyceraldehyde-3-phosphate dehydrogenase. J. Biol. Chem. *279*, 6152–6162.

 \mathcal{T}

Takahashi, S., Harigae, H., Ishii, K.K., Inomata, M., Fujiwara, T., Yokoyama, H., Ishizawa, K., Kameoka, J., Licht, J.D., Sasaki, T., et al. (2005). Over-expression of Flt3 induces NF-kappaB pathway and increases the expression of IL-6. Leuk. Res. *29*, 893–899.

Takeuchi, Y., and Nishikawa, H. (2016). Roles of regulatory T cells in cancer immunity. Int. Immunol. 28, 401–409.

Tan, B.T., Warnke, R.A., and Arber, D.A. (2006). The frequency of B- and T-cell gene rearrangements and epstein-barr virus in T-cell lymphomas: a comparison between angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma, unspecified with and without associated B-cell proliferations. J Mol Diagn *8*, 466–475; quiz 527.

Tanaka, A., and Sakaguchi, S. (2017). Regulatory T cells in cancer immunotherapy. Cell Res. 27, 109–118.

Tang, J.C., Ho, F.C., Chan, A.C., and Srivastava, G. (1998). Clonality of lymphomas at multiple sites in SJL mice. Lab. Invest. 78, 205–212.

Tangye, S.G., Ma, C.S., Brink, R., and Deenick, E.K. (2013). The good, the bad and the ugly - TFH cells in human health and disease. Nat. Rev. Immunol. *13*, 412–426.

Tao, J.-H., Cheng, M., Tang, J.-P., Liu, Q., Pan, F., and Li, X.-P. (2017). Foxp3, Regulatory T Cell, and Autoimmune Diseases. Inflammation 40, 328–339.

Thorns, C., Bastian, B., Pinkel, D., Roydasgupta, R., Fridlyand, J., Merz, H., Krokowski, M., Bernd, H.-W., and Feller, A.C. (2007). Chromosomal aberrations in angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma unspecified: A matrix-based CGH approach. Genes, Chromosomes and Cancer *46*, 37–44.

Tisdale, E.J., Kelly, C., and Artalejo, C.R. (2004). Glyceraldehyde-3-phosphate Dehydrogenase Interacts with Rab2 and Plays an Essential Role in Endoplasmic Reticulum to Golgi Transport Exclusive of Its Glycolytic Activity. J. Biol. Chem. *279*, 54046–54052.

Tisdale, E.J., Azizi, F., and Artalejo, C.R. (2009). Rab2 Utilizes Glyceraldehyde-3-phosphate Dehydrogenase and Protein Kinase Ci to Associate with Microtubules and to Recruit Dynein. J. Biol. Chem. 284, 5876–5884.

Titov, A., Petukhov, A., Staliarova, A., Motorin, D., Bulatov, E., Shuvalov, O., Soond, S.M., Piacentini, M., Melino, G., Zaritskey, A., et al. (2018). The biological basis and clinical symptoms of CAR-T therapy-associated toxicites. Cell Death Dis *9*.

Troxell, M.L. (2005). Follicular dendritic cell immunohistochemical markers in angioimmunoblastic Tcell lymphoma. Applied Immunohistochemistry & Molecular Morphology : Aimm 13, 297–303. V Vallois, D., Dobay, M.P.D., Morin, R.D., Lemonnier, F., Missiaglia, E., Juilland, M., Iwaszkiewicz, J., Fataccioli, V., Bisig, B., Roberti, A., et al. (2016). Activating mutations in genes related to TCR signaling in angioimmunoblastic and other follicular helper T-cell-derived lymphomas. Blood *128*, 1490–1502.

Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. (2009a). Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science *324*, 1029–1033.

Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. (2009b). Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. Science *324*, 1029–1033.

Versele, M., Janssen, L., Geerts, T., Floren, W., Janssens, B., Millar, H., Jacoby, E., Gross, G., Ligny, Y., Simonnet, Y., et al. (2017). Abstract 4199: Inhibition of NF-kB inducing kinase (NIK) selectively abrogates NIK and TRAF3 mutant multiple myeloma tumor growth. Cancer Res 77, 4199–4199.

Voll, R.E., Jimi, E., Phillips, R.J., Barber, D.F., Rincon, M., Hayday, A.C., Flavell, R.A., and Ghosh, S. (2000). NF-kappa B activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. Immunity *13*, 677–689.

Vrsalovic, M.M., Korac, P., Dominis, M., Ostojic, S., Mannhalter, C., and Kusec, R. (2004). T- and Bcell clonality and frequency of human herpes viruses-6, -8 and Epstein Barr virus in angioimmunoblastic T-cell lymphoma. Hematol Oncol 22, 169–177.

 \mathcal{W}

Wang, C., McKeithan, T.W., Gong, Q., Zhang, W., Bouska, A., Rosenwald, A., Gascoyne, R.D., Wu, X., Wang, J., Muhammad, Z., et al. (2015). IDH2R172 mutations define a unique subgroup of patients with angioimmunoblastic T-cell lymphoma. Blood *126*, 1741–1752.

Wang, H., Wang, Y., Rayburn, E.R., Hill, D.L., Rinehart, J.J., and Zhang, R. (2007). Dexamethasone as a chemosensitizer for breast cancer chemotherapy: potentiation of the antitumor activity of adriamycin, modulation of cytokine expression, and pharmacokinetics. Int. J. Oncol. *30*, 947–953.

Weber, J.S., D'Angelo, S.P., Minor, D., Hodi, F.S., Gutzmer, R., Neyns, B., Hoeller, C., Khushalani, N.I., Miller, W.H., Lao, C.D., et al. (2015). Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol. *16*, 375–384.

Wee, Z.N., Li, Z., Lee, P.L., Lee, S.T., Lim, Y.P., and Yu, Q. (2014). EZH2-mediated inactivation of IFN-γ-JAK-STAT1 signaling is an effective therapeutic target in MYC-driven prostate cancer. Cell Rep *8*, 204–216.

Weinstein, J.S., Herman, E.I., Lainez, B., Licona-Limón, P., Esplugues, E., Flavell, R., and Craft, J. (2016). TFH cells progressively differentiate to regulate the germinal center response. Nat. Immunol. *17*, 1197–1205.

Weiss, L.M., Strickler, J.G., Dorfman, R.F., Horning, S.J., Warnke, R.A., and Sklar, J. (1986). Clonal T-cell populations in angioimmunoblastic lymphadenopathy and angioimmunoblastic lymphadenopathy-like lymphoma. Am. J. Pathol. *122*, 392–397.

Wen, D., Nong, Y., Morgan, J.G., Gangurde, P., Bielecki, A., Dasilva, J., Keaveney, M., Cheng, H., Fraser, C., Schopf, L., et al. (2006). A selective small molecule IkappaB Kinase beta inhibitor blocks nuclear factor kappaB-mediated inflammatory responses in human fibroblast-like synoviocytes, chondrocytes, and mast cells. J. Pharmacol. Exp. Ther. *317*, 989–1001.

Wiel, C., Le Gal, K., Ibrahim, M.X., Jahangir, C.A., Kashif, M., Yao, H., Ziegler, D.V., Xu, X., Ghosh, T., Mondal, T., et al. (2019). BACH1 Stabilization by Antioxidants Stimulates Lung Cancer Metastasis. Cell *178*, 330-345.e22.

Willenbrock, K., Roers, A., Seidl, C., Wacker, H.H., Küppers, R., and Hansmann, M.L. (2001). Analysis of T-cell subpopulations in T-cell non-Hodgkin's lymphoma of angioimmunoblastic lymphadenopathy with dysproteinemia type by single target gene amplification of T cell receptor- beta gene rearrangements. Am. J. Pathol. *158*, 1851–1857.

Willenbrock, K., Bräuninger, A., and Hansmann, M.-L. (2007). Frequent occurrence of B-cell lymphomas in angioimmunoblastic T-cell lymphoma and proliferation of Epstein–Barr virus-infected cells in early cases. British Journal of Haematology *138*, 733–739.

Willis, T.G., Jadayel, D.M., Du, M.Q., Peng, H., Perry, A.R., Abdul-Rauf, M., Price, H., Karran, L., Majekodunmi, O., Wlodarska, I., et al. (1999). Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. Cell *96*, 35–45.

van der Windt, G.J.W., and Pearce, E.L. (2012). Metabolic switching and fuel choice during T-cell differentiation and memory development. Immunol. Rev. 249, 27–42.

Wing, K., and Sakaguchi, S. (2010). Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nature Immunology *11*, 7–13.

Х

Xu, H., Li, X., Liu, D., Li, J., Zhang, X., Chen, X., Hou, S., Peng, L., Xu, C., Liu, W., et al. (2013). Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. Nature *496*, 523–527.

Xu, Y., Chaudhury, A., Zhang, M., Savoldo, B., Metelitsa, L.S., Rodgers, J., Yustein, J.T., Neilson, J.R., and Dotti, G. (2016). Glycolysis determines dichotomous regulation of T cell subsets in hypoxia. J. Clin. Invest. *126*, 2678–2688.

Υ

Yang, J.C., Hughes, M., Kammula, U., Royal, R., Sherry, R.M., Topalian, S.L., Suri, K.B., Levy, C., Allen, T., Mavroukakis, S., et al. (2007). Ipilimumab (anti-CTLA4 antibody) causes regression of metastatic renal cell cancer associated with enteritis and hypophysitis. J. Immunother. *30*, 825–830.

Yap, T.A., Winter, J.N., Leonard, J.P., Ribrag, V., Constantinidou, A., Giulino-Roth, L., Michot, J.-M., Khan, T.A., Horner, T., Carver, J., et al. (2016). A Phase I Study of GSK2816126, an Enhancer of Zeste Homolog 2(EZH2) Inhibitor, in Patients (pts) with Relapsed/Refractory Diffuse Large B-Cell Lymphoma (DLBCL), Other Non-Hodgkin Lymphomas (NHL), Transformed Follicular Lymphoma (tFL), Solid Tumors and Multiple Myeloma (MM). Blood *128*, 4203–4203.

Yemelyanov, A., Gasparian, A., Lindholm, P., Dang, L., Pierce, J.W., Kisseljov, F., Karseladze, A., and Budunova, I. (2006). Effects of IKK inhibitor PS1145 on NF- κ B function, proliferation, apoptosis and invasion activity in prostate carcinoma cells. Oncogene 25, 387.

Yin, M.J., Yamamoto, Y., and Gaynor, R.B. (1998). The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature *396*, 77–80.

Yoo, H.Y., Sung, M.K., Lee, S.H., Kim, S., Lee, H., Park, S., Kim, S.C., Lee, B., Rho, K., Lee, J.-E., et

al. (2014). A recurrent inactivating mutation in *RHOA* GTPase in angioimmunoblastic T cell lymphoma. Nature Genetics *46*, 371–375.

Yoo, H.Y., Kim, P., Kim, W.S., Lee, S.H., Kim, S., Kang, S.Y., Jang, H.Y., Lee, J.-E., Kim, J., Kim, S.J., et al. (2016). Frequent CTLA4-CD28 gene fusion in diverse types of T-cell lymphoma. Haematologica *101*, 757–763.

Younes, A., Santoro, A., Shipp, M., Zinzani, P.L., Timmerman, J.M., Ansell, S., Armand, P., Fanale, M., Ratanatharathorn, V., Kuruvilla, J., et al. (2016). Nivolumab for classical Hodgkin's lymphoma after failure of both autologous stem-cell transplantation and brentuximab vedotin: a multicentre, multicohort, single-arm phase 2 trial. Lancet Oncol. *17*, 1283–1294.

Yu, D., Tan, A.H.-M., Hu, X., Athanasopoulos, V., Simpson, N., Silva, D.G., Hutloff, A., Giles, K.M., Leedman, P.J., Lam, K.P., et al. (2007). Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. Nature 450, 299–303.

Yu, D., Rao, S., Tsai, L.M., Lee, S.K., He, Y., Sutcliffe, E.L., Srivastava, M., Linterman, M., Zheng, L., Simpson, N., et al. (2009). The Transcriptional Repressor Bcl-6 Directs T Follicular Helper Cell Lineage Commitment. Immunity *31*, 457–468.

Yu, Y., Ma, X., Gong, R., Zhu, J., Wei, L., and Yao, J. (2018). Recent advances in CD8+ regulatory T cell research. Oncol Lett 15, 8187–8194.

 \mathcal{Z}

Zang, S., Li, J., Yang, H., Zeng, H., Han, W., Zhang, J., Lee, M., Moczygemba, M., Isgandarova, S., Yang, Y., et al. (2017). Mutations in 5-methylcytosine oxidase TET2 and RhoA cooperatively disrupt T cell homeostasis. J. Clin. Invest. *127*, 2998–3012.

Zeng, H., and Chi, H. (2015). Metabolic control of regulatory T cell development and function. Trends Immunol. *36*, 3–12.

Zeng, H., Cohen, S., Guy, C., Shrestha, S., Neale, G., Brown, S.A., Cloer, C., Kishton, R.J., Gao, X., Youngblood, B., et al. (2016). mTORC1 and mTORC2 Kinase Signaling and Glucose Metabolism Drive Follicular Helper T Cell Differentiation. Immunity *45*, 540–554.

Zhang, B., Calado, D.P., Wang, Z., Fröhler, S., Köchert, K., Qian, Y., Koralov, S.B., Schmidt-Supprian, M., Sasaki, Y., Unitt, C., et al. (2015). An oncogenic role for alternative NF-κB signaling in DLBCL revealed upon deregulated BCL6 expression. Cell Rep *11*, 715–726.

Zhang, L., Yao, Y., Zhang, S., Liu, Y., Guo, H., Ahmed, M., Bell, T., Zhang, H., Han, G., Lorence, E., et al. (2019). Metabolic reprogramming toward oxidative phosphorylation identifies a therapeutic target for mantle cell lymphoma. Sci Transl Med *11*.

Zhang, Q., Siebert, R., Yan, M., Hinzmann, B., Cui, X., Xue, L., Rakestraw, K.M., Naeve, C.W., Beckmann, G., Weisenburger, D.D., et al. (1999). Inactivating mutations and overexpression of BCL10, a caspase recruitment domain-containing gene, in MALT lymphoma with t(1;14)(p22;q32). Nat. Genet. *22*, 63–68.

Zhang, X., Li, L., Choe, J., Krajewski, S., Reed, J.C., Thompson, C., and Choi, Y.S. (1996). Upregulation of Bcl-xL expression protects CD40-activated human B cells from Fas-mediated apoptosis. Cell. Immunol. *173*, 149–154.

Zheng, J. (2012). Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review).

Oncology Letters 4, 1151–1157.

Zheng, L., Roeder, R.G., and Luo, Y. (2003). Cell 114, 255-266.

Zhou, Y., Yi, X., Stoffer, J.B., Bonafe, N., Gilmore-Hebert, M., McAlpine, J., and Chambers, S.K. (2008). The Multifunctional Protein Glyceraldehyde-3-Phosphate Dehydrogenase Is Both Regulated and Controls Colony-Stimulating Factor-1 Messenger RNA Stability in Ovarian Cancer. Mol Cancer Res *6*, 1375–1384.

Zhu, Y., Zhao, Y., Zou, L., Zhang, D., Aki, D., and Liu, Y.-C. (2019). The E3 ligase VHL promotes follicular helper T cell differentiation via glycolytic-epigenetic control. J. Exp. Med. 216, 1664–1681.

Zotos, D., Coquet, J.M., Zhang, Y., Light, A., D'Costa, K., Kallies, A., Corcoran, L.M., Godfrey, D.I., Toellner, K.-M., Smyth, M.J., et al. (2010). IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J. Exp. Med. 207, 365–378.

Annexes

Article 3

SCIENTIFIC **Reports**

Received: 4 August 2017 Accepted: 12 December 2017 Published online: 10 January 2018

OPEN IL-34 and CSF-1 display an equivalent macrophage differentiation ability but a different polarization potential

Sonia Boulakirba¹, Anja Pfeifer¹, Rana Mhaidly¹, Sandrine Obba¹, Michael Goulard¹, Thomas Schmitt¹, Paul Chaintreuil¹, Anne Calleja¹, Nathan Furstoss¹, François Orange², Sandra Lacas-Gervais², Laurent Boyer¹, Sandrine Marchetti¹, Els Verhoeyen¹, Frederic Luciano¹, Guillaume Robert¹, Patrick Auberger¹ & Arnaud Jacquel¹

CSF-1 and IL-34 share the CSF-1 receptor and no differences have been reported in the signaling pathways triggered by both ligands in human monocytes. IL-34 promotes the differentiation and survival of monocytes, macrophages and osteoclasts, as CSF-1 does. However, IL-34 binds other receptors, suggesting that differences exist in the effect of both cytokines. In the present study, we compared the differentiation and polarization abilities of human primary monocytes in response to CSF-1 or IL-34. CSF-1R engagement by one or the other ligands leads to AKT and caspase activation and autophagy induction through expression and activation of AMPK and ULK1. As no differences were detected on monocyte differentiation, we investigated the effect of CSF-1 and IL-34 on macrophage polarization into the M1 or M2 phenotype. We highlighted a striking increase in IL-10 and CCL17 secretion in M1 and M2 macrophages derived from IL-34 stimulated monocytes, respectively, compared to CSF-1 stimulated monocytes. Variations in the secretome induced by CSF-1 or IL-34 may account for their different ability to polarize naïve T cells into Th1 cells. In conclusion, our findings indicate that CSF-1 and IL-34 exhibit the same ability to induce human monocyte differentiation but may have a different ability to polarize macrophages.

Monocytes are circulating blood leukocytes that play important role in tissue homeostasis and in the inflammatory response which is essential for the innate response to pathogens¹. They have the unique property among peripheral blood cells to migrate into tissues where they are subjected to differentiation into morphological and functionally heterogeneous cells that include macrophages, myeloid dendritic cells, and osteoclasts, depending on the stimulus². The differentiation of peripheral blood monocytes into resident tissue macrophages can be recapitulated ex vivo by incubation in the presence of colony-stimulating factor-1 (CSF-1)³. The biologic effects of CSF-1 are mediated by a unique receptor, the CSF-1R, which is encoded by the *c-fms* proto-oncogene⁴. Downstream signaling pathways activated by CSF-1R upon ligand binding include PI3K-AKT and AMPK pathways, which are implicated in the respective activation of caspases and autophagy, two key processes required for CSF-1-induced macrophage differentiation⁵. Our previous studies have established that physiological monocyte differentiation triggered by CSF-1R engagement is critically dependent on the oscillatory activation of the kinase AKT, which within 2-3 days leads to the formation of a multi-molecular platform that includes the adaptor Fas-associated death domain (FADD), the serine-threonine kinase RIP1, the long and short isoforms of FLIP, and procaspase-8⁶. Caspase-8 activation in this platform provokes a limited activation of several downstream caspases that cleave specific intracellular proteins^{7,8}. The contribution of these cleavages to the CSF-1-driven monocyte-to-macrophage differentiation remains poorly understood. More recently, we have also established that autophagy plays a crucial role during macrophage differentiation of monocytes⁹. We found that CSF-1 increases the expression of the purinergic receptor P2RY6 that in turn activates the CAMKK2-AMPK-ULK1

¹Université Côte d'Azur, C3M Inserm, U1065, Nice, France. ²Université Côte d'Azur, Centre Commun de Microscopie Appliquée, Nice, France. Patrick Auberger and Arnaud Jacquel contributed equally to this work. Correspondence and requests for materials should be addressed to P.A. (email: auberger@unice.fr) or A.J. (email: jacquel@unice.fr)

pathway leading to autophagy induction¹⁰. Notably, inhibition of this pathway abrogated both CSF-1-mediated induction of autophagy and differentiation.

IL-34 is a newly identified cytokine with only partially understood functions. IL-34 has been recently identified as the second ligand for CSF-1R through comprehensive proteomic analyses¹¹. Although it lacks appreciable similarity with CSF-1 or any other proteins, IL-34 tightly binds to CSF-1R and promotes the differentiation, proliferation and survival of monocytes, macrophages and osteoclasts as CSF-1 does^{12,13}. IL-34 actions have been rendered more complex by the discovery of two other distinct receptors: the receptor type protein-tyrosine phosphatase zeta (PTP- ζ)¹⁴ and CD138 (Syndecan 1)¹⁵, suggesting additional roles for IL-34, compared to CSF-1. Recently, IL-34 was found to be associated with the inflammation process seen in diseases such as rheumatoid arthritis (RA)¹⁶, inflammatory bowel disease¹⁷ and Sjogren's syndrome¹⁸. Interestingly, emerging findings indicate that IL-34 levels are abnormally increased in serum and synovial fluid in patients with reactive rheumatoid arthritis^{19,20}.

Here, we used human primary monocytes to characterize in details IL-34 and CSF-1-driven macrophage differentiation and polarization into the M1 or M2 phenotype. We demonstrate that IL-34, like CSF-1, induced caspase and autophagy activation and that both processes are required to induce differentiation of monocytes into macrophages in response to IL-34. Moreover, we report that IL-34 and CSF-1 macrophages display a different polarization potential when polarized by pro or anti-inflammatory stimulus, since we found some differentiated cells polarized into the M1 or M2 phenotype.

Results

CSF-1 and IL-34 exhibit similar signaling and differentiation properties in primary human monocytes. When stimulated with 100 ng/mL CSF-1, human monocytes differentiate into macrophages as shown by an increase in cell adherence and the acquisition of specific markers such as TFRC/CD71 and CD163. At day 2, 60–70% of myeloid cells were found to be positive for both CD71 and CD163 expression (Fig. 1a). This high rate of differentiation was maintained up to 6 days. The kinetic of macrophage differentiation induced by 100 ng/mL IL-34, the second known ligand of the CSF-1R, was nearly indistinguishable from that of CSF-1 (Fig. 1b and c, Sup Fig. 1a and b). As CSF-1 and IL-34 bind the CSF-1R with differential affinities, we performed a dose-response curve for each cytokine on monocyte differentiation (Sup Fig. 1c). We confirmed that for each cytokine, a 100 ng/mL concentration triggers maximal differentiation capacity.

We next analyzed the early signaling pathways associated with the engagement of the CSF-1 receptor by either CSF-1 or IL-34 in human primary monocytes. We show that both CSF-1 and IL-34 triggered a transient first wave of CSF-1R phosphorylation at 5 and 10 min and a second one at 16 and 24 h (Fig. 2a). For both cytokines, the second wave of phosphorylation was associated with an increase in CSF-1R protein expression. The same waves of phosphorylation and activation of AKT and ERK 1/2 were also observed in CSF-1 and IL-34-stimulated monocytes. We have previously shown that CSF-1 triggered long term phosphorylation and activation of AMPK and ÚLK1 that correlated with increased autophagy in primary human monocytes¹⁰. This increased phosphorylation and activation of AMPK and ULK1 also correlated with an enhanced expression of both AMPK and ULK1 proteins (Fig. 2a). CSF-1-mediated phosphorylation and activation of AMPK and ULK1 were confirmed in the present study, but we further established for the first time that IL-34 increased AMPK and ULK1 phosphorylation at 16 and 24 h. This activation of phosphorylation was accompanied with increased expression of AMPK and ULK1 (Fig. 2a). In addition, we also investigated the effect of increasing concentrations of both cytokines on the early signaling events triggered by CSF-1R engagement and we confirmed a slightly higher sensitivity to CSF-1 (Sup Fig. 2a, please see also Sup Fig. 1c). Nevertheless, a 100 ng/mL concentration was maximal for both cytokines. We next compared the ability of CSF-1 and IL-34 to promote differentiation of monocytes. Both cytokines equivalently increased the protein expression of CD71 and CD163, two macrophage differentiation markers at 48 h (Fig. 2b). CSF-1 and IL-34 induced the activation of both caspases 8 and 3, which are a prerequisite for monocyte differentiation into macrophage and for NPM cleavage (Fig. 2b)²¹. We also analyzed the lipidation and cleavage of LC3-l into LC3-ll, a hallmark of autophagy induction in monocytes stimulated with CSF-1 or IL-34. Autophagy induction, that was increased after 48 h of stimulation, correlated with Cathepsin B (CTSB) activation in both CSF-1 and IL-34 treated monocytes (Fig. 2b)9. In addition, caspase-3 activity at 3 and 6 days were found to be equivalent in CSF-1 or IL-34-treated monocytes (Fig. 2c). Furthermore, electron microscopy images of CSF-1 or IL-34 differentiated monocytes at day 3 showed accumulation of autolysosomes (Sup Fig. 2b), CTBS active fragment and increase in autophagic flux in the presence of Bafilomycin A1 (Sup Fig. 2c).

Finally, CSF-1 and IL-34 both triggered NPM cleavage, LC3-ll accumulation and CTSB activation at 2 days (Fig. 3a). Caspases or autophagy inhibition by qVD or 3MA, respectively, abolished NPM and LC3-l cleavage, CTSB activation and macrophage differentiation (Fig. 3a and b). Knock-down of caspase 8 was found to increase CSF-1 and IL-34-mediated LC3-ll accumulation while knock-down of BECLIN1 inhibited it (Fig. 3c). Importantly, caspase-8 or BECLIN1 knock-down both impaired macrophage differentiation of monocytes as shown by a reduction of the double positive CD71/CD163 population that corresponds to differentiated macrophages (Fig. 3d). In conclusion, we show that the signaling pathways involved in monocyte differentiation by CSF-1 or IL-34 are strictly identical.

CSF-1 and **IL-34** macrophages exhibit differences in their ability to polarize into M1 or M2 macrophages. Globally, the results described above indicate that CSF-1 and IL-34-mediated intracellular signaling pathways, including auto-phosphorylation of the CSF-1R, activation of AKT, ERK 1/2, AMPK and ULK1 and induction of caspase activities and autophagy are similar in human primary monocytes induced to differentiate into macrophages, suggesting that the effects of IL-34 are mediated via the CSF1-R. As no obvious differences have been reported so far in the effects of CSF-1 and IL-34 on monocyte differentiation (Figs 1–3), we investigated





IL-34



С



Figure 1. IL-34 and CSF-1 induce equivalent macrophagic differentiation of primary monocytes. Human peripheral blood monocytes from healthy donors were exposed to 100 ng/mL CSF-1 or 100 ng/mL IL-34 for the indicated times. (**a**,**b**) Macrophage differentiation was examined morphologically (fibroblastic shape) and by 2-color flow cytometric analysis. The percentage indicates cells that express both CD71 and CD163. (**c**) Macrophagic differentiation of monocytes from 3 different healthy donors was followed by 2-color flow cytometric analysis. The percentage indicates cells that express both CD71 and CD163. (**c**) Macrophagic differentiation of monocytes from 3 different healthy donors was followed by 2-color flow cytometric analysis. The percentage indicates cells that express both CD71 and CD163. (**c**) Macrophagic differentiation of monocytes from 3 different healthy donors was followed by 2-color flow cytometric analysis. The percentage indicates cells that express both CD71 and CD163. (**c**) Macrophagic differentiation of monocytes from 3 different healthy donors was followed by 2-color flow cytometric analysis. The percentage indicates cells that express both CD71 and CD163. (**c**) Macrophagic differentiation of monocytes from 3 different healthy donors was followed by 2-color flow cytometric analysis. The percentage indicates cells that express both CD71 and CD163. (**c**) Macrophagic differentiation of monocytes from 3 differentiation of monocytes from 4 differentiation flow cytometric analysis. The percentage indicates cells that express both CD71 and CD163. (**c**) Macrophagic differentiation flow cytometric analysis.

the effects of both cytokines on macrophage polarization. Macrophages are categorized as "classically activated" pro-inflammatory M1-macrophages or an "alternatively activated" anti-inflammatory M2-macrophages²². They can differentiate into various activation states owing to the cytokine balance in their microenvironment. M1 phenotypic activation (pro-inflammatory), in response to interferon gamma (lFN γ) and lipopolysaccharide (LPS), is



b



Figure 2. Caspases and autophagy are activated upon IL-34 or CSF-1 treatment. Human peripheral blood monocytes from healthy donors were exposed to 100 ng/mL CSF-1 or 100 ng/mL IL-34 for the indicated times. (a) Immunoblot analysis of indicated proteins in monocytes following CSF-1 or IL-34 stimulation. P indicate phosphorylated proteins. Each panel is representative of at least 3 independent experiments. (b) Immunoblot analysis of indicated proteins in monocytes following CSF-1 or IL-34 stimulation. The ratio of the LC3-II protein level to that of LC3-I protein level was determined using ImageJ software. Actin was detected as the loading control. Asterisks indicate cleavage fragments. Each panel is representative of at least 3 independent experiments. (c) Caspase activity was quantified by flow cytometry analysis using DEVD-FITC. The results are expressed as the fold induction compared with untreated cells and represent the mean ± SD of 3 independent experiments performed in duplicate. n.s. denotes non-significant according to a paired student *t* test.

characterized by up-regulation of interleukin IL-6 and enhancement of the Th1 immune response. M2 phenotypic activation (anti-inflammatory) is stimulated by IL-4 and characterized by increased expression of IL- 10^{23} . M1 or M2 polarization was initiated after 7 days of differentiation with either CSF-1 or IL-4 and next evaluated after a 2-day stimulation period with LPS + INF γ or IL-4, respectively. In a first series of experiments, the M1/M2





Figure 3. Caspases and autophagy are required for CSF-1 or IL-34 induced macrophagic differentiation. (**a**) Human monocytes were exposed for 2 days to 100 ng/mL CSF-1 or 100 ng/mL IL-34 alone or in combination with either 50 μM qVD or 3 mM 3MA (3-MethylAdenine), which were added 30 min before CSF-1 or IL-34 treatment. The expression of cleaved NPM (NPM*), LC3B and cleaved cathepsin B (CTSB*) was analyzed by immunoblotting. (**b**) Human monocytes were treated as in Fig. 3a. (**c**,**d**) Monocytes were transfected with siRNA targeting *LUCIFERASE* (LUC), *CASPASE-8* (CASP8) or *BECLIN* (BEC) and exposed for 2 days to 100 ng/mL CSF-1 or 100 ng/mL IL-34. (**c**) Expression of Caspase-8, Beclin and LC3B was analyzed by immunoblotting. Actin is used as a loading control. Asterisk indicates a cleavage fragment. (**d**) Differentiation was examined as previously described.

macrophage phenotype induced by lFN γ and LPS (M1 inducer) or IL-4 (M2 inducer) was analyzed by flow cytometry in macrophages differentiated with CSF-1 or lL-34. ln CSF-1 differentiated macrophages, LPS + lNF γ induced M1 polarization with both an increase in CD80 and CD86 expression and a decrease in CD200R and CD206 expression, whereas lL-4 promoted M2 polarization with an inhibition in CD80 and CD86 expression and

an increase in CD200R and CD206 expression (Fig. 4a). Interestingly, whereas there was no difference in the ability of CSF-1 and IL-34 differentiated macrophages to phagocyte bacteria (Fig. 4b), M2-macrophages derived from CSF-1-stimulated monocytes exhibited a higher capacity to phagocyte bacteria than M2-macrophages derived from IL-34 differentiated macrophages (Fig. 4c).

To identify the cytokines that could be important for the polarization of macrophages in IL-34 stimulated monocytes, we analyzed by both real time RT-PCR and Elisa the expression level of some cytokines/chemokines known to be modulated during the M1 or M2 polarization process²⁴⁻²⁶. Regarding M1 polarization, we found a significant increase in IL-10 and CXCL11 mRNA and protein levels when IL-34 macrophages were polarized in response to LPS + IFN_Y compared to CSF-1 polarized macrophages (Fig. 4d and Sup Fig. 3a). Some differences were also observed between macrophages polarized into the M2 phenotype by CSF-1 or IL-34. The most noticeable one were a very strong induction of CCL17, and CCL22, together with a decrease of IL-10 which was observed only when IL-34 differentiated monocytes were polarized into the M2 phenotype (Fig. 4e and Sup Fig. 3b). To investigate whether these differences in cytokine expression may be physiologically relevant, we analyzed the ability of M1 or M2 polarized macrophage secretomes (i.e from CSF-1 or IL-34 treated monocytes) to induce polarization of purified naïve T cells from two donors into the Th1 or Th2 phenotype. As no obvious differences were observed for Th2 polarization (not shown), we next focused on Th1 polarization. Therefore, we followed by flow cytometry the intracellular expression of IFN γ (Sup Fig. 3c and d) which is representative of Th1 cells. Of note, T cell polarization was higher in the presence of the secretome derived from M1-polarized CSF-1 macrophages as compared to M1-polarized IL-34 macrophages. Finally, the secretome derived from M2-polarized IL-34 macrophages strongly increased Th1 polarization compared to M2-polarized CSF-1 macrophages. In conclusion, although IL-34 and CSF-1 display an equivalent macrophage differentiation ability, the functionality of IL-34 and CSF-1 macrophages following polarization into the M1 or M2 phenotype is different, more specially regarding Th1 cell polarization.

Discussion

CSF-1 and IL-34 share the same receptor¹¹, the so-called CSF-1R and no obvious differences in the downstream signaling pathways triggered by both ligands in monocytes have been reported so far²⁷. However, some genetic evidence indicate that it could exist differences in the effect of both cytokines in myeloid cells and in the brain^{28,29}. This is exemplified by the fact that CSF-1 deficient ($CSF-1^{op/op}$) mice exhibit a less severe phenotype than their CSF-1R deficient counterpart suggesting that at least one part of the IL-34 effect could be triggered independently of the CSF-1R³⁰. This has been recently confirmed following the identification of a second receptor for IL-34 in the brain. Indeed, IL-34 binds to and signals by an alternative receptor, namely PTP-Q14. Moreover, it has also been reported minor differences in the production and secretion of some cytokines including eotaxin-2 in peripheral blood cells stimulated with CSF-1 or IL-3427. Although CSF-1 and IL-34 exert many redundant functions ex vivo, the profile of expression, the source and the kinetic of cytokine production are different in vivo. Indeed, in contrast to CSF-1, IL-34 shows specific expression by keratinocytes and neurons in mice and plays an important role in the development and maintenance of Langherans cells and microglia, respectively^{29,31}. In human, IL-34 is more widely/differently expressed, albeit also highly expressed in neuronal tissues and skin (see FANTOM5 and GTEX databases). There is also increasing evidence that IL-34 expression is upregulated in pathological conditions and may play important functions in autoimmune disorders, infections, inflammation and cancer³². Regarding cancer, IL-34 promotes the recruitment of M2-polarized tumor-associated macrophages by a direct effect on CSF-1R in macrophages, promoting new vessel formation and extravasation of immune cells, as CSF-1 does³³. However, clear evidence indicating different functions of both cytokines are still lacking.

In the present study, we scrutinized the effect of CSF-1 and IL-34 on human monocyte differentiation. We found that the early and late signaling pathways leading to the differentiation of monocytes into macrophages were strictly identical in both conditions. Moreover, we also described for the first time that IL-34 promotes caspase activation and the activation of the AMPK/ULK1 pathway in monocytes leading to LC3-I lipidation and cleavage into LC3-II, as CSF-1 does, confirming the similarity of action of both ligands on human monocyte differentiation and induction of autophagy.

Whereas monocyte differentiation proceeded similarly in CSF-1 and IL-34 stimulated human primary monocytes, we identified however, differences in the production of some cytokines/chemokines when cells were polarized into the M1 or M2 phenotype using LPS + IFN γ or IL-4 following macrophage differentiation by either CSF-1 or IL-34. The most noticeable examples, were increased production of IL-10 and CXCL11 at the mRNA and protein level in M1 polarized macrophages. Regarding M2 polarization, we also observed a striking induction of CCL17 and CCL22 and a decrease in IL-10 mRNA and protein expression, which were found exclusively in M2-polarized IL-34 macrophages. Therefore, despite a strictly identical profile of monocyte differentiation, the cytokines/chemokines produced by CSF-1 or IL-34 macrophages are somehow different, suggesting that macrophages generated in both conditions may have different role in pro or anti-inflammatory pathophysiological conditions.

CCL17 (TARC) was originally implicated in the selective attraction of Th2 lymphocytes and thus considered as a M2 cytokine. Recent findings in the literature have reported that human monocyte-derived macrophages grown in CSF-1 do induce CCL17 in response to IL-4 as do mouse bone-marrow-derived macrophages grown in CSF-1^{25,26}. It was also recently reported that GM-CSF drives CCL17 mRNA and protein expression in human and mice monocytes. Surprisingly, CCL17 seems to mediate at least some of the pro-inflammatory action of GM-CSF on arthritic pain and disease³⁴. Several other cytokines including IL-4³⁵ and IL-13³⁶ have been reported to be associated with upregulation of CCL17 mRNA and protein. The fact that CCL17 mRNA expression was selectively upregulated in macrophages differentiated in the presence of IL-34 and polarized into the M2 phenotype by IL-4 but not in their CSF-1 differentiated counterpart indicates that polarization rather than IL-4 itself is responsible



Figure 4. IL-34 macrophages have a different polarization potential as compared to CSF-1-macrophages. (a) Human monocytes were differentiated during 7 days with 100 ng/mL CSF-1 or 100 ng/mL IL-34 and then polarized into M1-macrophages (LPS + IFN γ) or M2-macrophages (IL-4) for 2 days. Macrophage polarization was evaluated by 2-color flow cytometric analysis. (b) Functional assay of monocytes exposed for 2 or 5 days to 100 ng/mL CSF-1 or 100 ng/mL IL-34. The results are expressed as MFI and represent the mean \pm SD of 3 independent experiments performed in duplicate. n.s. denotes not statistically significant according to a paired student *t* test. (c) Functional assay of monocytes exposed for 7 days with 100 ng/mL CSF-1 or 100 ng/mL IL-34 and then polarized into M2-macrophages (IL-4) for 2 days. The results are expressed as the fold induction compared to CSF-1 macrophages and represent the mean of 3 independent experiments performed in duplicate. **P* < 0.05 according to a paired student *t* test (versus CSF-1 or 100 ng/mL IL-34 and then polarized into M1-macrophages (IL-4) for 2 days. The results are expressed as the fold in duplicate. **P* < 0.05 according to a paired student *t* test (versus CSF-1 or 100 ng/mL IL-34 and then polarized into M1-macrophages (IL-4) for 2 days. The results are expression of the indicated into M1-macrophages (IL-4) for 2 days. The results are expressed as the fold in duplicate. **P* < 0.05 according to a paired student *t* test (versus CSF-1 or 100 ng/mL IL-34 and then polarized into M1-macrophages (IL-4) for 2 days. The expression of the indicated into M1-macrophages (IL-4) for 2 days. The expression of the indicated into M1-macrophages (IL-4) for 2 days. The expression of the indicated into M1-macrophages (IL-4) for 2 days. The expression of the indicated into M1-macrophages (IL-4) for 2 days. The expression of the indicated into M1-macrophages (IL-4) for 2 days. The expression of the indicated into M1-macrophages (IL-4) for 2 days. The expression of the indicated into M1-m

mRNA was analyzed by qPCR (mean \pm SEM of 5 independent experiments). *P < 0.05, ***P < 0.001 according to a paired student *t* test (versus CSF-1 macrophages). The production of IL-10 and CCL17 was analyzed using Multi-Analyte ELISArray kit as described in Material and Methods section. The results are expressed as ng/mL per million of cells and represent the mean \pm SD of 2 independent experiments performed in duplicate.

for CCL17 mRNA induction in our experiments. Globally, these findings indicate that IL-34 could resemble more GM-CSF than CSF-1 in its ability to trigger M2 polarization.

IL-10 is produced by Th2 lymphocytes and is known to suppress the differentiation and effector function of Th1^{37–39}. We showed here, that IL-10 protein expression is strikingly increased in M1-polarized IL-34 macrophages compared to M1-polarized CSF-1 macrophages. This may explain the impairment of Th1 polarization observed with the secretome of M1-polarized IL-34 macrophages.

The reason for the differential effect of CSF-1 and IL-34 regarding the polarization into M1 or M2 phenotypes is currently unknown. However, a likely explanation could rely on the fact that IFN γ R/TLR4 or IL-4 receptor expression is different in CSF-1 and IL-34 differentiated macrophages. We checked that it was not indeed the case (Sup Fig. 4). An alternative hypothesis would be that this specific effect of IL-34 is mediated independently of the CSF-1R. It has been described that IL-34 can bind CD138 and PTP- ζ that can act as putative IL-34 receptors. As monocytes and macrophages failed to express CD138 (syndecan 1) (Sup Fig. 4) and PTP- ζ expression seems restricted to neuronal tissues and skin, it would be interesting in future studies to investigate whether other proteoglycans such as serglycin, versican, perlecan or other syndecans^{40,41}, including syndecan 4, could explain the differential effect of IL-34 regarding the polarization into M1 or M2 phenotypes.

In conclusion, the signaling pathways induced by CSF-1 and IL-34 downstream CSF-1R are undistinguishable. However, some differences in chemokines/cytokines production do exist when CSF-1 or IL-34 differentiated monocytes are induced to polarize into the M1 or M2 phenotypes following LPS + INF γ or IL-4 treatments. Variations in the secretome induced by CSF-1 or IL-34 may account for their different ability to polarize naïve T lymphocytes into Th1 cells. Strikingly, the increase in IL-10 production in M1-polarized IL-34 macrophages promoted a decrease in Th1 cell polarization while the increase in CCL17 and CCL22 in M2-polarized IL-34 macrophages favored an increase in Th1 cell polarization. These findings suggest that in an inflammatory context, IL-34 and CSF-1 display equivalent macrophage differentiation ability but different polarization potential.

Material and Methods

Reagents and antibodies. Human IL-34 was from Biolegend (BLE577906). Human CSF-1 was purchased from Miltenyi (130-096-493). Bafilomycin A1 was from Tocris (1334). qVD was from Clinisciences (A1901-5mg). 3-MethylAdenine was purchased from Sigma Aldrich (M9281). Phospho-CSF-1R (Tyr723), Phospho-AKT, AKT, Phospho-ERK1/2, ERK1/2, Phospho-AMPK (Thr172), AMPK, Phospho-ULK1 (Ser555), ULK1, Caspase-8, Caspase-3, Nucleophosmin, LC3-B, and Beclin antibodies were purchased from Cell Signaling Technology (catalog numbers were 3155, 4060, 9272, 4370, 4695, 2535, 2532, 5869, 8054, 9746, 9662, 3542, 2775, and 3738 respectively). CSF-1R, ACTIN, CD71, and CD163 antibodies were from Santa Cruz Biotechnology (catalog numbers were sc-692, sc-1616, sc-51829 and sc-33559 respectively). Cathepsin B was from MERCK (IM27L). HRP-conjugated rabbit anti-goat or mouse was purchased from Dako (P0449 and P0260) and HRP-conjugated goat anti-rabbit was from Cell Signaling (5127).

Human monocyte culture and differentiation. Human peripheral volunteers were obtained from healthy donors with informed consent following the Declaration of Helsinki according to the recommendations of an independent scientific review board. The project has been validated by The Etablissement Français du Sang, the French national agency for blood collection (protocol N°ALM/PLER/FO/001). Blood samples were collected using ethylene diamine tetra-acetic acid–containing tubes. Mononucleated cells were first isolated using Ficoll Hypaque (Eurobio, CMSMSL0101). Then, we used the autoMACS[®] Pro Separator (Miltenyi, France) to perform cell enrichment. An initial positive selection, which included antibody targeting CD14, was used for monocyte enrichment (Miltenyi, 130-050-201). Purified monocytes from human were grown in RPMI 1640 medium with glutamax-I (Life Technologies, 61870044) supplemented with 10% (vol/vol) foetal bovine serum (Life Technologies). Monocytes were seeded in tissue culture treated flasks or wells. Macrophage differentiation was induced by adding into the culture medium 100 ng/mL CSF-1 or 100 ng/mL IL-34 and was visualized using standard optics (20x/0.35 Ph1) equipped with an AxioCam ERc camera (Zeiss, France). Phase images of the cultures were recorded with the Zen 2 software (Zeiss).

Flow cytometry. To analyze the macrophagic differentiation of monocytes, cells were washed with ice-cold phosphate buffered saline (PBS, Life Technologies, 14190169), incubated at 4 °C for 10 min in PBS/bovine serum albumin (BSA 0.5%, Dutscher, 871002) with anti-CD71 and anti-CD163 or an isotype control (Miltenyi and BD Biosciences, catalog numbers were 551374 and 130-097-628). Finally, cells were washed and fixed in 2% paraformaldehyde (EMS, 15710). To detect caspase activity, we used FITC-DEVD-FMK according to the manufacturer's instruction (Promokine, green caspase-3 staining kit, PK-CA577-K183). To perform macrophage polarization, purified monocytes were plated at 0.3×10^6 per mL in RPMI 1640 medium with glutamax-I supplemented with 10% (vol/vol) fetal bovine serum plus CSF-1 or IL-34 for 7 + 2 days to differentiate into M0 macrophages. 100 ng/mL lipopolysaccharide (LPS-EK Ultrapure, Invivogen, tlrl-peklps) and 20 ng/mL interferon gamma (Miltenyi, 130-096-484) were added after 7 days of differentiation for two days to polarize into

M1-macrophages. M2-macrophages were obtained by addition of 20 ng/mL IL-4 (Miltenyi, 130-094-117) for two days. To analyze the macrophage polarization, cells were detached using PBS/EDTA/BSA, washed with PBS, and incubated at 4°C for 10 min in PBS/ bovine serum albumin with anti-CD80 (Miltenyi, 130-097-204), anti-CD86 (Miltenyi, 130-094-877), anti-CD200R (Biolegend, 329308), and anti-CD206 (Miltenyi, 130-100-034) or isotype controls. Finally, cells were washed and fixed in 2% paraformaldehyde (EMS, 15710). Fluorescence was measured with a MACSQuant[®] Analyzer (Miltenyi, Paris, France). To analyze the ability of macrophages to phagocyte bacteria, we used Vybrant[®] Phagocytosis Assay Kit according manufacture's instruction (ThermoFisher, V-6694). Briefly, macrophages were detached and incubated with fluorescence, that indicate the internalization of particles, was measured with a MACSQuant[®] Analyzers (Miltenyi, Analyzers (Miltenyi, France). Trypan blue solution was used to quench the fluorescence from particles that were not internalized.

Immunoblot assays. Cells were lysed for 30 min at 4 °C in lysis buffer [50 mM HEPES pH 7.4, 150 mM NaCl, 20 mM EDTA, PhosphoSTOP (Sigma, 04906837001), complete protease inhibitor mixture (Sigma, 11836153001), 1% Triton X-100 (Sigma, T9284)]. Lysates were centrifuged at 20,000 g (15 min, 4 °C) and supernatants were supplemented with concentrated loading buffer (4X laemmli buffer). Fifty micrograms of proteins were separated and transferred following standard protocols before analysis with chemiluminescent detection kit (GE Healthcare, RPN2105).

siRNA knockdown. Small interfering (si) RNAs were introduced into monocytes by nucleoporation (Amaxa, VPA-1007) of 5×10^6 monocytes in 100 µL of nucleofector solution with 15 nmol of siRNA. Cells were incubated for 24h with 5 mL of prewarmed complete medium, and CSF-1 or IL-34 was subsequently added. We used siRNAs (Life Technologies) targeting *Caspase-8* (HSS141461), *BECLIN1* (HSS112741) and *LUCIFERASE* as a negative control (Sense: 5'-CUUACGCUGAGUACUUCGAtt-3').

Reverse-transcription and real-time polymerase chain reaction. RNA was prepared from 5×10^6 cells using the RNeasy Mini Kit according to manufacturer's protocol (Qiagen, 74104). Each cDNA sample was prepared using AMV RT and random primers (Promega, M510F and C1181). Real-time polymerase chain reaction (PCR) was performed using the SyBR Green detection protocol (Life Technologies, 4367659). Briefly, 5 ng of total cDNA, 500 nM (each) primers, and 5 μ L SyBR Green mixture were used in a total volume of 10 μ L. Detection of multiple endogenous controls (*ACTB, L32 and UBIQUITIN*) were used to normalize the results. Specific forward and reverse primers are accessible upon request.

Cytokine production assay. To perform macrophage polarization, purified monocytes were plated at 0.3×10^6 per mL in RPMI 1640 medium with glutamax-I supplemented with 10% (vol/vol) fetal bovine serum plus CSF-1 or IL-34 for 7 + 1 days to differentiate into M0 macrophages. 100 ng/mL lipopolysaccharide (LPS-EK Ultrapure, Invivogen, tlrl-peklps) and 20 ng/mL interferon gamma (Miltenyi, 130-096-484) were added after 7 days of differentiation for one day to polarize into M1-macrophages. M2-macrophages were obtained by addition of 20 ng/mL IL-4 (Miltenyi, 130-094-117) for one day. Next, cells were washed and seeded in RPMI 1640 medium with glutamax-I supplemented with 10% (vol/vol) fetal bovine serum during one day. The cytokines productions were finally evaluated using MultiAnalyte ELISArray kit (Qiagen, MEH-004A and MEH-009A).

Statistical analysis. Statistical analysis was performed using a paired Student *t* test and significance was considered when *P* values were lower than 0.05. The results are expressed as the mean \pm SEM.

References

- Ginhoux, F. & Jung, S. Monocytes and macrophages: developmental pathways and tissue homeostasis. Nature reviews. Immunology 14, 392–404 (2014).
- Jakubzick, C. V., Randolph, G. J. & Henson, P. M. Monocyte differentiation and antigen-presenting functions. *Nature reviews*. *Immunology* 17, 349–362 (2017).
- 3. Sordet, O. *et al.* Specific involvement of caspases in the differentiation of monocytes into macrophages. *Blood* **100**, 4446–4453 (2002).
- 4. Stanley, E. R. & Chitu, V. CSF-1 receptor signaling in myeloid cells. Cold Spring Harb Perspect Biol 6 (2014).
- Jacquel, A., Obba, S., Solary, E. & Auberger, P. Proper macrophagic differentiation requires both autophagy and caspase activation. Autophagy 8, 1141–1143 (2012).
- Jacquel, A. et al. Colony-stimulating factor-1-induced oscillations in phosphatidylinositol-3 kinase/AKT are required for caspase activation in monocytes undergoing differentiation into macrophages. Blood 114, 3633–3641 (2009).
- Cathelin, S. *et al.* Identification of proteins cleaved downstream of caspase activation in monocytes undergoing macrophage differentiation. *J Biol Chem* 281, 17779–17788 (2006).
 Rebe, C. *et al.* Caspase-8 prevents sustained activation of NF-kappaB in monocytes undergoing macrophagic differentiation. *Blood*
- **109**, 1442–1450 (2007).
- 9. Jacquel, A. *et al.* Autophagy is required for CSF-1-induced macrophagic differentiation and acquisition of phagocytic functions. *Blood* **119**, 4527–4531 (2012).
- Obba, S. *et al.* The PRKAA1/AMPKalpha1 pathway triggers autophagy during CSF1-induced human monocyte differentiation and is a potential target in CMML. *Autophagy* 11, 1114–1129 (2015).
- 11. Lin, H. et al. Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. Science 320, 807-811 (2008).
- Guillonneau, C., Bezie, S. & Anegon, I. Immunoregulatory properties of the cytokine IL-34. *Cell Mol Life Sci* 74, 2569–2586 (2017).
 Baud'huin, M. *et al.* Interleukin-34 is expressed by giant cell tumours of bone and plays a key role in RANKL-induced osteoclastogenesis. *J Pathol* 221, 77–86 (2010).
- 14. Nandi, S. *et al.* Receptor-type protein-tyrosine phosphatase zeta is a functional receptor for interleukin-34. *J Biol Chem* 288, 21972–21986 (2013).

- Segaliny, A. I. *et al.* Syndecan-1 regulates the biological activities of interleukin-34. *Biochim Biophys Acta* 1853, 1010–1021 (2015).
 Chemel, M. *et al.* Interleukin 34 expression is associated with synovitis severity in rheumatoid arthritis patients. *Ann Rheum Dis* 71, 100–1021 (2015).
- Jishirey, and war interleukin 34 expression is associated war synothic sectory in meanatoria a array parents. *Fun Artenia Dis* 7, 150–154 (2012).
 Zwicker, S. *et al.* Interleukin 34: a new modulator of human and experimental inflammatory bowel disease. *Clin Sci (Lond)* 129, 129.
- 281–290 (2015). 18. Ciccia, F. *et al.* IL-34 is overexpressed in the inflamed salivary glands of patients with Sjogren's syndrome and is associated with the
- local expansion of pro-inflammatory CD14(bright)CD16 + monocytes. *Rheumatology (Oxford*) 52, 1009–1017 (2013).
 Yang, S. *et al.* Interleukin 34 Upregulation Contributes to the Increment of MicroRNA 21 Expression through STAT3 Activation
- Associated with Disease Activity in Rheumatoid Arthritis. *J Rheumatol* **43**, 1312–1319 (2016). 20. Fan, Q. *et al.* IL-34 is associated with the presence and severity of renal dysfunction and coronary artery disease in patients with
- heart failure. Sci Rep 6, 39324 (2016).
- 21. Guery, L. et al. Fine-tuning nucleophosmin in macrophage differentiation and activation. Blood (2011).
- Murray, P. J. *et al.* Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 41, 14–20 (2014).
 Locati, M., Mantovani, A. & Sica, A. Macrophage activation and polarization as an adaptive component of innate immunity. *Adv Immunol* 120, 163–184 (2013).
- Spiller, K. L. et al. Differential gene expression in human, murine, and cell line-derived macrophages upon polarization. Exp Cell Res 347, 1–13 (2016).
- Martinez, F. O. *et al.* Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. *Blood* 121, e57–69 (2013).
- Martinez, F. O., Gordon, S., Locati, M. & Mantovani, A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *Journal of immunology* 177, 7303–7311 (2006).
 Chihara, T. *et al.* IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. *Cell death*
- Chihara, I. et al. IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. Cell death and differentiation 17, 1917–1927 (2010).
- Barve, R. A. *et al.* Transcriptional profiling and pathway analysis of CSF-1 and IL-34 effects on human monocyte differentiation. *Cytokine* 63, 10–17 (2013).
- Greter, M. et al. Stroma-derived interleukin-34 controls the development and maintenance of langerhans cells and the maintenance of microglia. *Immunity* 37, 1050–1060 (2012).
- Dai, X. M. et al. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. Blood 99, 111–120 (2002).
- Wang, Y. et al. IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. Nat Immunol 13, 753–760 (2012).
- Baghdadi, M., Endo, H., Tanaka, Y., Wada, H. & Seino, K. I. Interleukin 34, from pathogenesis to clinical applications. *Cytokine* 99, 139–147 (2017).
- Segaliny, A. I. et al. Interleukin-34 promotes tumor progression and metastatic process in osteosarcoma through induction of angiogenesis and macrophage recruitment. Int J Cancer 137, 73–85 (2015).
- Achuthan, A. et al. Granulocyte macrophage colony-stimulating factor induces CCL17 production via IRF4 to mediate inflammation. The Journal of clinical investigation 126, 3453–3466 (2016).
- Liddiard, K. et al. Interleukin-4 induction of the CC chemokine TARC (CCL17) in murine macrophages is mediated by multiple STAT6 sites in the TARC gene promoter. BMC Mol Biol 7, 45 (2006).
- Crapster-Pregont, M., Yeo, J., Sanchez, R. L. & Kuperman, D. A. Dendritic cells and alveolar macrophages mediate IL-13-induced airway inflammation and chemokine production. J Allergy Clin Immunol 129, 1621–1627 e1623 (2012).
- Wang, X., Wong, K., Ouyang, W. & Rutz, S. Targeting IL-10 Family Cytokines for the Treatment of Human Diseases. Cold Spring Harb Perspect Biol (2017).
- Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. & O'Garra, A. IL-10 inhibits cytokine production by activated macrophages. *Journal of immunology* 147, 3815–3822 (1991).
- Mosser, D. M. & Zhang, X. Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* 226, 205–218 (2008).
 Chang, M. Y. et al. Monocyte-to-macrophage differentiation: synthesis and secretion of a complex extracellular matrix. *J Biol Chem*
 - **287**, 14122–14135 (2012).
- Wegrowski, Y. et al. Cell surface proteoglycan expression during maturation of human monocytes-derived dendritic cells and macrophages. Clin Exp Immunol 144, 485–493 (2006).

Acknowledgements

This work was supported by Inserm, the Ligue Nationale Contre le Cancer, the foundation ARC (team labelled 2014–2017 and 2017–2019), the Fondation pour la Recherche Médicale, the Association Laurette Fugain and the French Government (National Research Agency, ANR) through the "Investments for the Future" Labex Signalife (ANR-11-LABX-0028-01). The authors are indebted to the Etablissement Francais du sang for providing us with human blood from healthy donors. We also thank the C3M facilities (ISO-9001 certified cytometry facility). S.B., A.P., and S.O. were supported by a fellowship from the Association pour la Recherche contre le Cancer, the Ligue Nationale Contre le Cancer, and the Fondation pour la Recherche Médicale, respectively.

Author Contributions

S.B. and A.P. designed and performed the experimental work; R.M., S.O., M.G., T.S., P.C., A.C. and N.F. contributed to some experiments; F.O. and S.L.G. realized the EM; L.B. performed functional assays; S.M., E.V., F.L., and G.R. participated in helpful discussion; and P.A. and A.J. designed the study, directed the work and wrote the paper.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-18433-4.

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018

Article 4

Article

Cell Metabolism

Low-Protein Diet Induces IRE1*α*-Dependent Anticancer Immunosurveillance

Graphical Abstract



Highlights

- Feeding mice with a diet presenting a decrease in protein limits tumor growth
- Low PROT diet increases tumor-infiltrating lymphocytes
- Feeding mice with a Low PROT diet induces the IRE1α/RIG1 pathway in tumor cells
- IRE1α/RIG1 activation in tumor cells results in an anticancer immune response

Authors

Camila Rubio-Patiño, Jozef P. Bossowski, Gian Marco De Donatis, ..., Béatrice Bailly-Maitre, Eric Chevet, Jean-Ehrland Ricci

Correspondence ricci@unice.fr

In Brief

Dietary restriction (DR) slows down tumor growth by increasing tumor immunosurveillance. Rubio-Patiño et al. show that a moderate reduction in dietary protein intake, rather than carbohydrate reduction, without overall calorie changes, activates the IRE1α/RIG1 pathway in tumor cells resulting in an anticancer immune response in mice.



Rubio-Patiño et al., 2018, Cell Metabolism 27, 828–842 April 3, 2018 © 2018 Elsevier Inc. https://doi.org/10.1016/j.cmet.2018.02.009



Low-Protein Diet Induces IRE1a-Dependent Anticancer Immunosurveillance

Camila Rubio-Patiño,^{1,9} Jozef P. Bossowski,^{1,9} Gian Marco De Donatis,¹ Laura Mondragón,¹ Elodie Villa,¹ Lazaro E. Aira,¹ Johanna Chiche,¹ Rana Mhaidly,¹ Cynthia Lebeaupin,¹ Sandrine Marchetti,¹ Konstantinos Voutetakis,^{2,3} Aristotelis Chatziioannou,^{2,4} Florence A. Castelli,⁵ Patricia Lamourette,⁵ Emeline Chu-Van,⁵ François Fenaille,⁵ Tony Avril,^{6,7} Thierry Passeron,¹ John B. Patterson,⁸ Els Verhoeyen,¹ Béatrice Bailly-Maitre,¹ Eric Chevet,^{6,7} and Jean-Ehrland Ricci^{1,10,*}

¹Université Côte d'Azur, INSERM U1065, C3M, 151 route de St Antoine de Ginestière, BP 23194, 06204 Nice, France

²Institute of Biology, Medicinal Chemistry and Biotechnology, NHRF, 11635 Athens, Greece

³Department of Biochemistry and Biotechnology, University of Thessaly, Larissa, Greece

⁴e-NIOS Applications PC, 17671 Kallithea-Athens, Greece

⁵Service de Pharmacologie et Immunoanalyse (SPI), Laboratoire d'Etude du Métabolisme des Médicaments, CEA, INRA, Université Paris Saclay, MetaboHUB, 91191 Gif-sur-Yvette, France

⁶Inserm U1242 «Chemistry, Oncogenesis, Stress, Signaling», Université Rennes 1, 35042 Rennes, France

⁷Centre de Lutte Contre le Cancer Eugène Marquis, 35042 Rennes, France

Centre de Lutte Contre le Cancer Eugene Marquis, 35042 Rennes, Fran

⁸Fosun Orinove, Newbury Park, CA 91320, USA

⁹These authors contributed equally

¹⁰Lead Contact

*Correspondence: ricci@unice.fr

https://doi.org/10.1016/j.cmet.2018.02.009

SUMMARY

Dietary restriction (DR) was shown to impact on tumor growth with very variable effects depending on the cancer type. However, how DR limits cancer progression remains largely unknown. Here, we demonstrate that feeding mice a low-protein (Low PROT) isocaloric diet but not a low-carbohydrate (Low CHO) diet reduced tumor growth in three independent mouse cancer models. Surprisingly, this effect relies on anticancer immunosurveillance, as depleting CD8⁺ T cells, antigen-presenting cells (APCs), or using immunodeficient mice prevented the beneficial effect of the diet. Mechanistically, we established that a Low PROT diet induces the unfolded protein response (UPR) in tumor cells through the activation of IRE1 α and RIG1 signaling, thereby resulting in cytokine production and mounting an efficient anticancer immune response. Collectively, our data suggest that a Low PROT diet induces an IRE1 a-dependent UPR in cancer cells, enhancing a CD8-mediated T cell response against tumors.

INTRODUCTION

Dietary restriction (DR) without malnutrition, which includes caloric restriction (CR) and fasting, is well recognized as one of the most reliable methods to enhance life span and reduce the incidence of a wide variety of diseases, including human cancers (Fontana and Partridge, 2015). DR has a beneficial impact on health and life span, affects cancer development (Longo and

828 Cell Metabolism 27, 828–842, April 3, 2018 © 2018 Elsevier Inc.

Mattson, 2014; Lu et al., 2017; Meynet and Ricci, 2014), and sensitizes tumor cells to chemotherapy (Meynet et al., 2013; Rubio-Patiño et al., 2016), notably by increasing tumor immunosurveillance (Di Biase et al., 2016). The identification of new methods to induce immunosurveillance has become crucial for the development of effective therapeutic protocols against cancers, the inhibition of tumor development and progression, and the enhancement of long-term protection. However, using DR interventions, such as acute fasting or prolonged CR, to reduce tumor growth can be very difficult to tolerate for most cancer patients receiving treatment due to the development of cachexia and DR-related weight loss (Porporato, 2016). Therefore, developing alternative methods to benefit from DR-mediated reduction in tumor growth without impacting total caloric intake is an area of intense research (Bénéteau et al., 2012; Pietrocola et al., 2016). Indeed, it has been suggested that macronutrient modulation rather than calorie intake determines the effect of DR on health and aging (Levine et al., 2014; Solon-Biet et al., 2014). Interestingly, several studies have demonstrated that the balance of protein in the diet is especially important for these effects, as a low-protein (Low PROT) diet produces beneficial metabolic effects similar to DR, such as reductions in insulinlike growth factor 1 (IGF-1) and cancer incidence and an increase in longevity (Fontana et al., 2013; Levine et al., 2014; Solon-Biet et al., 2014, 2015).

Macronutrient modulation might also impact protein homeostasis, which is also referred to as proteostasis, and in particular endoplasmic reticulum (ER) functions (Cubillos-Ruiz et al., 2017). Consequently, the adaptive unfolded protein response (UPR) is stimulated when ER proteostasis is disturbed. Accumulating evidence indicates that chronic activation of the UPR supports the main hallmarks of cancer by favoring cancer cell autonomous and non-autonomous processes, which ultimately condition the immunosuppressive and pro-tumorigenic microenvironment (Cubillos-Ruiz et al., 2017). However, certain forms of ER stress


⁽legend on next page)

Cell Metabolism 27, 828-842, April 3, 2018 829

(environmentally or therapy-induced) can elicit immunogenic cancer cell death (ICD), which enables the release of key immunostimulatory (such as interferon γ [INF γ]) and danger signals, eventually driving efficient antitumor immunity (Galluzzi et al., 2015; Garg et al., 2015).

The UPR is controlled by three main ER resident sensors: Inositol Requiring Enzyme 1a (IRE1a), Activating Transcription Factor 6a (ATF6a), and PKR-like endoplasmic reticulum kinase (PERK) (Hetz et al., 2015). Upon ER stress, IRE1α oligomerizes and auto-transphosphorylates, thus activating the endoribonuclease domain that subsequently catalyzes the non-conventional splicing of X-box binding protein 1 (XBP1) (Calfon et al., 2002; Lee et al., 2002) together with the tRNA ligase RtcB (Lu et al., 2014). Spliced XBP1 (sXBP1) is an active transcription factor that regulates the expression of genes encoding proteins involved in protein folding and quality control, ER-associated degradation, and phospholipid synthesis. IRE1a RNase activity is also involved in regulated IRE1-dependent decay (RIDD) of mRNA, rRNA, and microRNAs (Maurel et al., 2014). Interestingly, in response to ER stress, RIDD-mediated RNA cleavage into single-strand fragments lacking markers of self was shown to activate RIG1 (Retinoic Acid Inducible Gene 1). This RIDD-RIG1 pathway affects in turn adaptive immunity in humans (Eckard et al., 2014). Similar to IRE1a, on ER stress, PERK dimerizes and trans-autophosphorylates, thus leading to the phosphorylation of the translation initiation factor eIF2a and global protein synthesis attenuation. This also leads to translational activation of ATF4, a transcription factor that controls the expression of genes whose products are involved in amino acid transport, autophagy, and redox control (Pakos-Zebrucka et al., 2016). Finally, ER stress induces ATF6 export from the ER and its transmembrane cleavage by Site-1 protease (S1P) and Site-2 protease (S2P), two Golgi resident proteases. The released cytosolic ATF6 fragment acts as an active transcription factor (Hetz et al., 2015).

Based on these premises, we analyzed the mechanism by which macronutrient modulation can mimic the antitumoral properties of DR by feeding mice *ad libitum* with isocaloric custom diets. Our results demonstrate that an isocaloric Low PROT diet but not a low-carbohydrate (Low CHO) diet can induce an IRE1 α /RIG1-dependent increase in immunosurveillance, suggesting that such a diet can represent a clinically interesting alternative to DR interventions in the context of cancer development, tumor immunity, and treatment.

RESULTS

A Low-Protein Diet Limits Tumor Development through the Induction of Immunosurveillance

To analyze the effect of macronutrient modulation on tumor development, we fed mice bearing myc-driven lymphoma isolated from Eµ-Myc mice (Adams et al., 1985) custom engineered isocaloric diets containing either 25% less proteins (Low PROT) or 25% less carbohydrates (Low CHO) compared with the control diet. Mice were fed ad libitum with the different diets, and the regimens' impact on mouse survival was monitored over time (Figure 1A). We established that only Low PROT diet increased mouse survival, whereas global food intake and mouse weight were equivalent for all diets (Figures S1A and S1B). The increase in survival observed under the Low PROT diet was associated with a reduction of spleen weight compared with the other groups, thus indicating reduced lymphoma development (Figure 1B). Moreover, lymphoma-bearing lymph nodes from immunocompetent mice fed with a Low PROT diet presented an increase in IFN γ expression (Figure S1C). Given that IFN γ is a key cytokine for innate and adaptive immunity, we hypothesized that the Low PROT-dependent increase in survival could, at least in part, be due to an enhanced anticancer immune response. To test this hypothesis, we depleted cytotoxic CD8⁺ T cells using an anti-CD8-specific antibody (Figures S1D and S1I). Strikingly, the impairment of the cytotoxic immune response prevented the beneficial effect induced by the Low PROT diet, suggesting a central role for CD8⁺ T cells (Figure 1A).

To further confirm the role of Low PROT diet-induced immunosurveillance, we transplanted primary lymphoma cells isolated from Eµ-Myc mice into immunodeficient NOD-Scid gamma $c^{-/-}$ (NSG) mice. As shown in Figure 1A, a Low CHO diet did not impact mouse survival compared with the control diet (Figure 1C). Conversely, a Low PROT diet led to a dramatic decrease in mouse survival compared with the control diet (Figure 1D). Importantly, the co-injection of C57BL/6 splenocytes (containing mainly T and B cells syngeneic with the Eµ-Myc lymphoma cells injected) resulted in an increase in mouse survival under a Low PROT diet (Figure 1D). These findings further confirm the key role of the immune system in the beneficial effect of the Low PROT diet on tumor development.

We extended our observations *in vivo* using two additional mouse models: a murine colorectal carcinoma (CRC) cell line (CT26) subcutaneously injected into syngeneic

Figure 1. A Low PROT Diet Affects Tumor Development in a CD8-Dependent Manner

(A) Survival curve of syngeneic C57BL/6 mice that were intravenously injected with E μ -Myc lymphoma cells. Mice were fed *ad libitum* with Ctl, Low CHO, and Low PROT diets and were intraperitoneally injected with PBS or α CD8 antibody (Ctl, n = 8; Low CHO, n = 9; Low PROT, n = 10; Low PROT α CD8, n = 6). (B) Spleen weight of C57BL/6 mice intravenously injected with E μ -Myc lymphoma cells and fed *ad libitum* with Ctl, Low CHO and Low PROT diets (4 mice per group).

(E) Tumor growth curve of syngeneic BALB/c mice that were subcutaneously injected with CT26 colorectal carcinoma cells. Mice were fed *ad libitum* with Ctl and Low PROT (-12.5%, -25%, and -40%) diets (Ctl, n = 7; Low PROT -12,5%, n = 7; Low PROT -25%, n = 8; Low PROT -40%, n = 8).

(F) Tumor volume at day 14 of data shown in (E).

(G) Tumor growth curve of syngeneic BALB/c mice that were subcutaneously injected with CT26 colorectal carcinoma cells. Mice were fed *ad libitum* with Ctl and Low PROT diets and were intraperitoneally injected with PBS or α CD8 antibody (Ctl, n = 11; Low PROT, n = 10; Ctl α CD8, n = 8; Low PROT α CD8, n = 8). (H) Tumor volume at day 12 of data shown in (G).

*p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent SEM. Comparisons of every group versus Ctl group, except where specified otherwise. When not mentioned, differences are not significant. All experiments are representative of two performed. See also Figure S1.

830 Cell Metabolism 27, 828-842, April 3, 2018

⁽C and D) Survival curve of NSG mice that were intravenously injected with $E\mu$ -Myc lymphoma cells with or without splenocytes and fed *ad libitum* with Ctl, Low CHO and Low PROT diets (Ctl, n = 5; Low CHO, n = 5; Low PROT, n = 5; Ctl + Splenocytes, n = 3; Low PROT + Splenocytes, n = 3).



Figure 2. A Low PROT Diet Increases CD8 Tumor-Infiltrating Lymphocytes

(A and B) BALB/c mice were subcutaneously injected with CT26 colorectal carcinoma cells and were fed *ad libitum* with CtI and Low PROT diets. Upon sacrifice, CD3⁺ cells were isolated from spleens and incubated with (A) live CT26 cells or (B) B16 cells for 4 hr. The ability of T cells to kill tumor cells was determined by flow cytometry. Cell death of CT26 and B16 cells was determined by DAPI staining (at least seven mice per group).

(legend continued on next page)

Cell Metabolism 27, 828-842, April 3, 2018 831

immunocompetent BALB/c mice and a murine melanoma cell line (B16) subcutaneously injected into syngeneic immunocompetent C57BL/6 mice. Given that a Low CHO diet did not have any effect on mouse survival using the Eµ-Myc model (Figures 1A and 1B), we focused our attention on the Low PROT diet. We first tested the impact of diet-protein content on tumor growth in the CT26-BALB/c model by generating isocaloric diets presenting a reduction of 12.5%, 25% (as in Figures 1A-1D), or 40% of protein content. The global food intake and mouse weight were equivalent for all diets (Figures S1E and S1F). While -12.5% and -25% protein diets resulted in similar tumor growth reduction, the -40% diet did not show any beneficial effect (Figures 1E and 1F). We therefore decided to use the -25% Low PROT diet (named Low PROT diet) for the rest of the study. Then, using the CT26-BALB/c model (Figures 1G and 1H) and the B16-C57BL/6 model (Figures S1G and S1H), we established that a Low PROT diet attenuated tumor development in an immune-dependent manner, as the depletion of CD8⁺ T cells (shown in Figure S1I) prevented the beneficial effects mediated by the Low PROT diet (Figures 1G, 1H, S1G, and S1H). Importantly, we verified that food intake or mouse body weight were not affected by the different diets in both models (Figures S1J-S1M). We also verified that glycemia was not affected by the Low PROT diet (Figure S1N).

Altogether, we established using three independent and different mouse cancer models that a Low PROT diet attenuates tumor growth through the induction of an anticancer immune response.

To directly address whether a Low PROT diet can induce an efficient and specific anticancer immune response, we isolated T cells from the spleens of BALB/c mice previously injected with CT26 cells and fed with a control or a Low PROT diet and from C57BL/6 mice previously injected with Eµ-Myc cells and fed a control, Low CHO, or Low PROT diet. We then tested the ability of isolated cytotoxic T cells to kill tumor cells ex vivo. In both models, T cells isolated from the spleens of Low PROTfed mice were more efficient in killing syngeneic tumor cells compared with T cells isolated from control and Low CHO diet-fed mice (Figures 2A, S2A, and S2B). To test whether cytotoxic activity of T cells was specific for the syngeneic tumor cells, we incubated ex vivo the T cells isolated from the CT26-BALB/c model with B16 melanoma cells. T cells isolated from the CT26-BALB/c model fed with a Low PROT diet, while efficiently killing CT26 cells (Figure 2A), were unable to kill B16 cells (Figure 2B), thus suggesting that a Low PROT diet leads to the expansion of tumor antigen-specific T cells. Although the percentage of tumor-infiltrating T regulatory cells (Treg, CD3⁺CD4⁺CD25⁺ CD127⁻) did not vary in CT26 tumors, intratumoral natural killer cells (NK, CD3⁻ NK1.1⁺) and CD3⁺/CD8⁺ T lymphocytes were increased (Figure 2C). Importantly, this beneficial effect of the Low PROT diet was confirmed and correlated with an increase in CD8⁺ tumor-infiltrating lymphocytes (TILs) that were visualized by immunofluorescence on tumor sections of BALB/c bearing

CT26 tumors (Figures 2D and 2E). The increase in CD8⁺ TILs was associated with an increase in the mRNA levels of *IFN* γ and its target *CXCL10* in tumors *in vivo* (Figures S2C and S2D).

We demonstrated that a Low PROT diet can limit tumor development not by affecting tumor cell proliferation capacity or by inducing tumor cell death directly but rather through the increase in TILs that results in the induction of an efficient and specific anticancer immune response.

Low-Protein-Diet-Induced Immunosurveillance Requires APCs and Involves INF_{γ} Production by Tumor Cells

Antigen-presenting cells (APCs), like dendritic cells (DCs) or macrophages, are central for antigen presentation (Joffre et al., 2009; Kroemer et al., 2013). We therefore investigated their involvement *in vivo* by depleting phagocytic cells from the myeloid lineage (DCs and macrophages) using liposome clodronate (Van Rooijen and Sanders, 1994) (Figures S3A and S3B). Interestingly, the Low PROT diet-mediated protective effect was lost upon APC depletion (Figures 3A and 3B).

To further support our conclusions, we neutralized CD86, a co-stimulatory protein expressed on APCs that is required for T cell activation, using a blocking antibody *in vivo* (Figure S3C). As for APCs depletion, CD86 blockade *in vivo* prevented the effect of the Low PROT diet on tumor growth (Figures 3C and 3D). Cytokine production by tumor cells, including INF_Y, plays a key role in ICD (Galluzzi et al., 2015). We established that reduction of *IFN*_Y expression by tumor cells (Figure 3E) prevents the effects of a Low PROT diet on tumor growth (Figure 3F.)

Collectively our data suggest that a Low PROT diet can induce an antitumoral immune response that relies on APCs and, at least in part, on IFN γ production by tumor cells.

A Low-Protein Diet Induces IRE1*a*-Dependent ER Stress

Since we found that a Low PROT diet induced an antitumoral effect in three independent mouse tumor models, we then sought to investigate the underlying molecular mechanism by monitoring key signaling pathways previously reported as affected by such a regimen. We did not observe significant modulation of the mammalian target of rapamycin (mTOR) pathway or of GCN2 (general control nonderepressible 2) activation, two key sensors of the amino acid content in the cells (Kim et al., 2017; Wek et al., 1995) (Figures S4A-S4C). We also did not observe any modulation of the Akt pathway (Figures S4A and S4B). Moreover, recently, autophagy induction by CR mimetics was associated with an increase in anticancer immunosurveillance (Pietrocola et al., 2016). However, we did not observe any sign of autophagy induction as judged by LC3 conversion in tumor cells isolated from Low PROT-fed mice compared with the control diet (Figures S4D and S4E). Hence, in our model of an isocaloric, mild reduction of protein intake, the increase in immunosurveillance is not mediated by the modulation of macroautophagy or mTOR/GCN2/Akt pathways.

⁽C) Effect of Ctl and Low PROT diets on the intratumoral frequency of infiltrating Treg, NK, CD3⁺, and CD8⁺ cells in CT26 tumors as analyzed by flow cytometry (at least three mice per group).

⁽D) Immunofluorescent staining of CD8⁺ T cells in BALB/c CT26 bearing tumors after 25 days of diet. Scale bar is equivalent to 50 μ m.

⁽E) Corresponding quantification (three mice per group).

^{*}p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent SEM. When not mentioned, differences are not significant. See also Figure S2.



Figure 3. Role of Macrophages and Dendritic Cells on the Antitumor Effect of a Low PROT Diet

BALB/c mice were subcutaneously injected with CT26 colorectal carcinoma cells and were fed ad libitum with Ctl and Low PROT diets.

(A) Mice were intraperitoneally injected with clodronate liposomes (Lipo Clod) or PBS liposomes (Lipo PBS) (Ctl-Lipo PBS, n = 8; Low PROT-Lipo PBS, n = 8; Ctl-Lipo Clod, n = 6; Low PROT-Lipo Clod, n = 6).

(B) Tumor volume at day 14 of data shown in (A).

(C) Mice were intraperitoneally injected with PBS or CD86 antibody (Ctl - PBS, n = 8; Low PROT - PBS, n = 7; Low PROT - CD86, n = 7).

(D) Tumor volume at day 15 of data shown in (C).

(E) Knockdown in CT26 cells of IFN γ using the shRNA technique. IFN γ expression was analyzed by qPCR.

(F) BALB/c mice were subcutaneously injected with CT26 shRNA of interferon γ colorectal carcinoma cells and were fed *ad libitum* with Ctl and Low PROT diets. Tumor volume at day 13.

*p < 0.05, **p < 0.01. Error bars represent SEM. Comparisons of every group versus Ctl group, except where specified otherwise. When not mentioned, differences are not significant. See also Figure S3.

Cell Metabolism 27, 828–842, April 3, 2018 833



(legend on next page)

We then reasoned that reducing protein intake could limit amino acid availability in tumor cells. Knowing that alterations in certain amino acids (such as proline) can control ER stress induction (Jeon et al., 2015; Sahu et al., 2016), we monitored the content of amino acids in tumors. We observed a decrease in most of the amino acids present in tumors isolated from Low PROT diet-fed mice compared with tumors isolated from the control diet group (Figure S4F). We therefore investigated the activation status of different UPR markers in tumors isolated from mice fed with the control or Low PROT diets. GRP78 and CHOP expression were increased and IRE1a phosphorylation was detected in tumor cells isolated from mice fed a Low PROT diet (Figures 4A and 4B), indicating that a Low PROT diet induced the UPR in cancer cells. We did not observe any modulation of the AFT4 and ATF6 branches of the UPR (Figures S5A-S5C).

Given that a Low PROT diet induced the expression of cytokines (*IFN* γ and *CXCL10*) in tumor cells (Figures S1C, S2C, and S2D) and that IRE1 α is a central player in this phenomenon (Martinon et al., 2010), we further investigated this pathway. We observed an increase in sXBP1 protein levels in tumor cells obtained from mice fed a Low PROT diet (Figures 4A and 4B). ER stress-dependent IRE1 α induction mediated by a Low PROT diet in tumors in vivo was further supported by the degradation of RIDD target mRNAs (Figure 4C). RIG1 (retinoic acid inducible gene 1) senses the small RNA fragments generated by RIDD, leading to its activation (Cho et al., 2013). We observed the induction of RIG1 protein levels within the tumors isolated from Low PROT-fed mice (Figures 4D and 4E). Importantly, the activation of IRE1a signaling, as judged by the generation of sXBP1, was not observed in TILs nor in DCs, thereby suggesting it is a tumor cell-specific activation (Figure 4F).

Collectively, our data indicate that feeding tumor-bearing mice a Low PROT diet induces an IRE1 α -dependent UPR in tumor cells.

IRE1α-Mediated RIG1 Activation in Tumor Cells Is Required for a Low PROT-Induced Anticancer Immune Response

If IRE1 α signaling in tumor cells is a central event in the Low PROT-induced anticancer immune response, we reasoned that attenuation of ER stress should prevent the beneficial effect provided by this diet. To test this hypothesis, we first injected CT26 cells in syngeneic immunocompetent BALB/c mice fed *ad libitum* with the control or Low PROT diets. As observed in Figure 1, feeding the mice a Low PROT diet decreased tumor progression compared with mice fed with a control diet (Figures 5A and 5B).

Eleven days after subcutaneous injection of tumor cells, half of the mice in each group were treated either with the pan ER stress inhibitor TUDCA or with PBS (vehicle). Strikingly, TUDCA treatment prevented the antitumoral effect of the Low PROT diet, thereby indicating that ER stress is necessary to achieve such response. To further support this observation, we then used the IRE1a-specific RNase inhibitor MKC4485 to block IRE1a-downstream signaling events. Although tumor burden was reduced by the Low PROT diet, this effect was impaired upon treatment with MKC4485 (Figures 5C and 5D). We verified the efficacy of both inhibitors by assessing the reduction in CHOP (for tauroursodeoxycholic acid [TUDCA]) or sXBP1 expression (for MKC4485) (Figures 5E and 5F) in tumor cells of mice fed with a Low PROT diet. Consistent with our hypothesis, both inhibitors prevented CD8⁺ T cell infiltration in tumors (Figures 5G, S6A, and S6B), and this effect was associated with a drastic reduction in Low PROT-induced IFN_Y mRNA levels in tumors (Figure S6C). Importantly, neither the Low PROT diet nor the inhibitors affected T cell populations in lymphoid organs of tumor-bearing mice, suggesting a local modulation of the immune cell infiltrate rather than a global impact on whole immune cell populations (Figures S6D and S6E).

To demonstrate that the Low PROT diet-induced anticancer immune response is controlled by the modulation of IRE1 α in cancer cells, we used CT26 cells that were stably silenced for IRE1 α (using two independent shRNA sequences, Figure 6A). Importantly, we verified that IRE1 α knockdown did not impact on cell proliferation (Figure S7A). We again validated that a Low PROT diet limited tumor growth (Figure 6B). Very importantly, our *in vivo* data also demonstrated that IRE1 α knockdown is sufficient to prevent the beneficial effect of a Low PROT diet on tumor growth (Figures 6C, 6D, S7B, and S7C).

To further support our conclusions and to prevent any offtarget effects of both small hairpin RNA (shRNA) targeting IRE1a, we invalidated IRE1a in CT26 cells using the CRISPR/ Cas9 technology (Figure 6E). Again, IRE1a invalidation did not have an impact on cell proliferation in vitro (Figure S7D) but blunted the Low PROT diet-dependent reduction in tumor growth (Figures 6F-6H). Finally, to elucidate the role of RIG1 on the IRE1a-dependent antitumor effect of the Low PROT diet, we invalidated RIG1 in CT26 cells using the CRISPR/Cas9 technique (Figure 6E). As for IRE1a, RIG1 invalidation did not modulate cell proliferation in vitro (Figure S7D) but prevented the reduction in tumor growth induced by the Low PROT diet (Figures 6F–6H). We then confirmed that IRE1 a invalidation in tumor cells limited the splicing of XBP1 and RIG1 activation induced by the Low PROT diet (Figure 6I), confirming that RIG1 activation is downstream of IRE1 $\!\alpha$ activation.

Figure 4. A Low PROT Diet Causes Unresolved ER Stress in CT26 Tumors

Syngeneic BALB/c mice were subcutaneously injected with CT26 colorectal carcinoma cells and fed ad libitum with Ctl and Low PROT diets.

(A) Tumors were harvested after 25 days of diets and lysates were prepared. Expressions of proteins related to the IRE1α pathway were analyzed by western blot.
(B) Average quantification of GRP78, pIRE1α, sXBP1, and CHOP compared with ERK2 levels (used as a loading control) or the corresponding total protein.

(C) mRNA levels of RIDD targets were measured in tumors by qPCR (at least five mice per group).

(D) Expression of RIG1 in tumor tissues was analyzed by western blot.

(E) Average quantification of RIG1 compared with ERK2 levels.

(F) CD4^{+/}CD8⁺ TILs were isolated from CT26 tumors and DCs were isolated from spleens of BALB/c mice fed with Ctl and Low PROT diets. mRNA levels of *sXBP1* were determined by gPCR and normalized by *uXBP1*.

*p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent SEM. When not mentioned, differences are not significant. See also Figures S4 and S5.

Cell Metabolism 27, 828–842, April 3, 2018 835



Figure 5. Treatment with ER Stress Inhibitors Reverses the Antitumor Immune Response Induced by a Low PROT Diet

(A–D) Tumor growth curves of syngeneic BALB/c mice that were subcutaneously injected with CT26 colorectal carcinoma cells and were fed *ad libitum* with Ct1 and Low PROT diets. Mice were (A) intraperitoneally injected with TUDCA (five mice per group) (C) or administered MKC4485 by gavage from day 11–16 after subcutaneous injection with CT26 cells (at least five mice per group). Average tumor size at day 16 of mice treated with (B) TUDCA or (D) MKC4485 of data shown in (A) and (C).

(E) Tumors were harvested after 30 days of diets and lysates were prepared. Expression of CHOP and sXBP1 were analyzed by western blot.

(F) Corresponding average quantification of CHOP and sXBP1 compared with ERK2 levels (used as a loading control).

(G) Quantification of immunofluorescent staining of CD8⁺ T cells in tumors shown in Sup Figure 6A (three mice per group).

*p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent SEM. Comparisons of every group versus Ctl group, except where specified otherwise. When not mentioned, differences are not significant. All experiments are representative of two performed. See also Figure S6.

836 Cell Metabolism 27, 828-842, April 3, 2018



⁽legend on next page)

Cell Metabolism 27, 828-842, April 3, 2018 837

Altogether, these results indicate that a Low PROT diet induces IRE1 α -mediated RIG1 activation in tumor cells and that this represents a central event in the Low PROT diet-induced immunosurveillance.

Increased IRE1 α Signaling Is Associated with Increased Antitumor T Cell Response in Human Cancers

We then decided to corroborate our mouse findings to human data. For that matter, we investigated whether tumor IRE1a activity could be associated with signatures of antitumoral responses. Recently, a gene signature reflecting IRE1a activation was established in glioblastoma (Lhomond et al., 2018). Using TGCA datasets, we stratified glioblastoma (n = 523), melanoma (n = 293), and colorectal cancers (n = 456) based on IRE1 α activity and identified for each cancer type IRE1 α^{high} and IRE1 α^{low} populations (Figures S7E-S7G). Then we investigated the expression of a series of T cell markers (Figures 7A-7C) in both populations in the three cancer types. Interestingly, in both glioblastoma and melanoma, high IRE1 α activity correlated with increased levels of T cell markers (Figures 7B-7D). Surprisingly, when the same analysis was carried out in CRC, the enrichment in T cell markers was exclusively observed in grade I tumors (Figures 7A and 7D).

These results suggested that the association between an increase in IRE1 α signaling and the increase in T cell recruitment is a common feature observed in human tumors from various origins (Figure 7).

DISCUSSION

In this study, we examined the effect of Low CHO or Low PROT isocaloric diets on tumor growth using three independent immunocompetent syngeneic mouse models: the Eµ-Myc-C57BL/6 B lymphoma model, the B16-C57BL/6 melanoma model, and the CT26-BALB/c CRC model. We established that a 25% reduction in protein but not in carbohydrate intake with no change in calories resulted in a marked decrease in tumor growth. All diets used in our study were isocaloric but had very different impacts on tumor growth (Figure 1). This suggested that the class of nutrients rather than the amount of energy present in the food is essential to limit tumor growth. Very importantly, this effect was not related to the inhibition of cancer cell proliferation or to the induction of cancer cell death per se but was rather due to the induction of an efficient antitumoral immune response. Depletion of CD8⁺ T cells (Figures 1A, 1G, 1H, S1G, and S1H), the use of immunodeficient mice (Figures 1C and 1D) or depletion/blocking of APCs (Figures 3A–3D) prevented the beneficial effect of the Low PROT diet. We also established that feeding tumor-bearing mice with a Low PROT diet induced a tumor-specific cytotoxic T cell response (Figures 2, S2A, and S2B). Therefore, our data challenge the common dogma that lowering protein in the diet limits tumor development by decreasing tumor proliferation. Instead, we demonstrate that a mild reduction in protein intake without a change in total calories ingested induces an adaptive IRE1 α -dependent signal in the tumor cells, leading to RIG1 activation. We have also shown that this IRE1 α /RIG1-dependent anticancer immune response goes in hand with cytokine production and that this plays a role in the Low PROT diet-induced anticancer immune response as shown for *IFN* γ (Figures 3E and 3F).

Nutrients are main regulators of circulating IGF-1, the levels of which are correlated with increased cancer risk. Consistently, dietary restriction-mediated reduction in IGF-1 is largely described as preventing cancer incidence (Klement and Fink, 2016; Meynet and Ricci, 2014). Restriction of protein intake or some amino acids is more efficient than CR in reducing IGF-1, which consequently inhibits the PI3K/mTOR pathway and therefore limits tumor cell proliferation (Fontana et al., 2008, 2013; Norat et al., 2007; Underwood et al., 1994). However, in our experiments, a mild reduction of dietary protein content (by 25%) slightly, but significantly, reduced the amino acid concentration within tumor tissues but was not associated with a significant modulation of the mTOR pathway (Figures S4A, S4B, and S4F). In addition, we demonstrated that the Low PROT diet-dependent reduction in tumor development was effectively abrogated upon CD8⁺ T cell or APC depletion (Figures 1A, 1G, 1H, 3A, and 3B) or when using immunodeficient mice (Figures 1C and 1D). Moreover, IRE1 α and RIG1 inhibition or invalidation in tumor cells reverted the Low PROT-dependent reduction in cancer development (Figures 5 and 6). Altogether, our results argue against a role of circulating IGF-1 in our settings but indicate that a Low PROT diet induces a tumor cell IRE1a-dependent activation of anticancer-specific T cells in a PI3K/mTOR-independent manner.

The UPR has been described as being either pro- or antitumoral depending on the tumor type, the intensity of the stress, and the nature of the microenvironment (Cubillos-Ruiz et al., 2017; Obacz et al., 2017). We made the observation that mild dietary reduction of protein intake leads to the induction of the UPR in tumor cells (Figure 4). We demonstrated that the beneficial effect of the Low PROT diet is dependent on the endoribonuclease activity of IRE1 α (using the MKC4485 RNase inhibitor, Figure 5). Our data therefore suggest that IRE1 α activity can

Figure 6. A Low PROT Diet Induces an Anticancer Immune Response in an IRE1a-Dependent Manner

(A) Knockdown in CT26 cells of IRE1 α using two different shRNAs. IRE1 α expression was analyzed by western blot.

(E) Invalidation in CT26 cells of IRE1 a and RIG1 using the CRISPR/Cas9 technique. IRE1 and RIG1 expression was analyzed by western blot.

(F) Tumor growth curve of BALB/c mice that were subcutaneously injected with CRISPR Ctl, CRISPR IRE1 a and CRISPR RIG1 CT26 cells and were fed ad libitum with Ctl and Low PROT diets (at least eight mice per group).

(G) Tumor volume at day 13 of data shown in (F).

(H) Picture of representative dissected tumors for each group.

(I) Tumors were harvested after 17 days of diets and lysates were prepared. Expressions of proteins related to the IRE1 α pathway were analyzed by western blot. *p < 0.05, **p < 0.01. Error bars represent SEM. Comparisons of every group versus Ctl group, except where specified otherwise. When not mentioned, differences are not significant. See also Figure S7.

838 Cell Metabolism 27, 828-842, April 3, 2018

⁽B and C) (B) Tumor growth curve of BALB/c mice that were subcutaneously injected with CT26 (Empty Vector) colorectal carcinoma cells (C) and with shIRE1 a CT26 colorectal carcinoma cells and were fed ad libitum with Ctl and Low PROT diets (at least eight mice per group).

⁽D) Picture of representative dissected tumors for each group.



D





Low High IRE1a IRE1a Low High IRE1α IRE1α







Cell Metabolism 27, 828-842, April 3, 2018 839

selectively contribute to the modulation of tumor outcome by impacting on the tumor cells themselves and/or on the nature of the tumor microenvironment.

It was recently established that activation of XBP1 in tumor associated DCs led to a decrease in the anticancer immune response (Cubillos-Ruiz et al., 2015). The authors therefore suggested that targeting ER stress responses could have some relevance in cancer treatment. In our model of Low PROT diet-induced immunosurveillance, we observed the activation of the IRE1 α pathway in tumor cells but not in DCs or TILs (Figure 4F). This suggests that this is a tumor cell-specific ER stress response, probably due to the high metabolic demand and sensitivity of tumor cells to ER stress and the UPR. Moreover, this also coincides with tumor IRE1-dependent mechanisms recently described to modulate the nature of the tumor microenvironment (Lhomond et al., 2018). However, our study points toward a careful use of IRE1 α inhibitors for cancer treatment.

In the context of our current observations, how IRE1 α is activated in tumor cells upon a Low PROT diet remains to be elucidated. One possibility would be that a Low PROT diet, leading to a decrease in specific amino acids in tumors (Figure S4F) results in IRE1a activation, as it was previously suggested (Jeon et al., 2015; Sahu et al., 2016). However, which amino acids are involved remains to be clarified. Another hypothesis could be that somehow a Low PROT diet modulates glycemia that is known to be a central regulator of the UPR (Cubillos-Ruiz et al., 2017). However, we did not observe a difference in glycemia between the two groups (Figure S1N). The exact nature of how IRE1a is activated in tumor cells upon a Low PROT diet remains to be identified, in particular the specific signaling balance between sXBP1 and RIDD. Indeed, it was recently demonstrated that both arms of the IRE1a signaling pathway play antagonistic roles through the remodeling of tumor microenvironment. As such, one might postulate that a Low PROT diet could condition a specific IRE1a signaling code in tumor cells that would favor the recruitment of cytotoxic T cells to the tumor site and prevent the generation of protumoral stroma.

Recently, the use of caloric restriction mimetics (CRm) was shown to induce an anticancer immune response through the induction of autophagy (Pietrocola et al., 2016). However, we did not observe any modulation of autophagy in our settings (Figures S4D and S4E), suggesting that CRm and Low PROT diets utilize independent and possibly complementary pathways to increase immunosurveillance. Here, we describe that a 25% reduction in protein intake is sufficient to induce an efficient anticancer immune response. Of note, this diet did not have a systemic impact on the proportion of immune system populations (Figures S6D and S6E), suggesting a local tumor microenvironment modulation that results in T cell recruitment.

Limitations of Study

Limitation of our work is brought by the fact that there is no established or consensus definition of the percentage of protein reduction that is sufficient to generate a Low PROT diet, a factor that might be individual, tissue, and tumor dependent. Therefore, it remains a challenge to compare studies using strong protein reduction or prolonged fasting with studies using a mild reduction in protein intake. Very low-protein conditions will impact body weight, which may not be suitable for therapeutic approaches (Di Biase et al., 2016). Importantly, in most of these studies, the role of the immune system was not addressed, as experiments were performed in immunodeficient xenograft models. We observed that a drastic reduction in protein content (by 40%) did not lead to a protection toward tumor growth (Figure 1E) in our model, underlying the notion that a mild reduction in protein content may not only be more easily tolerated by patients but also that a massive reduction in protein may prevent the beneficial effect of the diet.

Stimulating the ability of the immune system to fight and limit/ eradicate tumor development is among the most promising treatment strategies. The enthusiasm for immunomodulating therapies targeting immune checkpoints results from the success observed in patients suffering from several types of cancer (Callahan et al., 2016). However, this approach is restricted to some types of cancers or some mutations within a cancer type. Here, we describe a simple and efficient method to stimulate the targeted killing of cancer cells by T cells. However, given that mice have a metabolic rate 7-fold higher compared with humans and that they appear to be more resistant to cachexia than humans (Bozzetti and Zupec-Kania, 2016; Demetrius, 2005), the effect of such DR interventions on improvements in immunosurveillance in human cancer prevention should be investigated in future studies. Translating the relevance of our work to humans can be hard to address, as data from cancer patients receiving a Low PROT regimen are not available. For this reason, we investigated the link between IRE1 α activation and the increase in antitumor T cells markers (Figure 7). Importantly, regardless of the type of tumor analyzed (CRC, melanoma, or glioblastoma), we observed a strict correlation between a high IRE1 a signature and an increase in markers associated with immunosurveillance, suggesting that our results could be relevant to cancer patients.

STAR * METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- - $\,\circ\,$ Cell Lines and Cell Culture Conditions

(A–C) mRNA expression of Th1-, cytotoxic mechanisms-, chemokines-, adhesion-, T cell, and MHC class I and II genes based on the groups defined relative to IRE1 α activity (High or Low). Probe analysis was carried out from CRC stage I (A), melanoma (B). and glioblastoma (C) tumors from patients according to tumor IRE1 α status (i.e., IRE1 α high [gray boxes] and IRE1 α low [white boxes] groups).

(D) Significant representative genes modulated in IRE1 α low versus high tumors. *PRF1*: Perforin-1, *HLA-DRA*: HLA class II histocompatibility antigen, DR alpha chain, *CD3D*: T cell surface glycoprotein CD3 delta chain, *CD8A*: T cell surface glycoprotein CD8 alpha chain. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent SEM. See also Figure S7.

840 Cell Metabolism 27, 828–842, April 3, 2018

• METHOD DETAILS

- Cytotoxicity Assay
- $\odot~$ Flow Cytometry Analysis
- Confocal Laser Scanning Microscopy
- Western Blot Analysis
- Reverse Transcriptase Quantitative-PCR (RT-qPCR) Analysis
- $\odot~$ Generation of shIFN γ and shIRE1 α -Transduced Cells
- Generation of CRISPR/Cas9 Cells
- O Sampling of Intracellular Metabolites
- Analysis of Amino Acid Residues by Liquid Chromatography Coupled to High Resolution Mass Spectrometry (LC-MS)
- Bioinformatic Analysis
- Statistics

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at https://doi.org/10.1016/j.cmet.2018.02.009.

ACKNOWLEDGMENTS

We gratefully acknowledge the Centre Méditerranéen de Médecine Moléculaire (C3M) animal and confocal facilities. We thank Maeva Gesson and Drs. Claudio Hetz, Bruno Segui, Frederic Bost and Cédric Chaveroux, Sophie Tartare-Deckert. Benjamin Salucki, Frederic Larbret, and Caetano Reis e Sousa for reagents and help. This work was supported by the Fondation ARC pour la Recherche sur le Cancer, the Agence Nationale de la Recherche (LABEX SIGNALIFE ANR-11-LABX-0028-01) and Inserm. This study was partly supported by research funding from the Canceropôle PACA, Institut National du Cancer and Conseil Régional PACA, by CEA and the French Ministry of Research and National Research Agency as part of the French metabolomics and fluxomics infrastructure (MetaboHUB, ANR-11-INBS-0010 grant), L.M. was supported by la Fondation pour la Recherche Médicale (FRM) and E. Villa by La Ligue contre le Cancer. E.C. is funded by grants from Institut National Du Cancer (INCa PLBIO: 2015-111 and INCA-7981), Ligue Contre le Cancer (Comité des Landes, LARGE project), and EU H2020 MSCA ITN-675448 (TRAINERS). B.B.-M. was supported by grants from Société Francophone du Diabète and Association Française pour l'Etude du Foie (AFEF)/Aptalis and by the Fondation ARC.

AUTHOR CONTRIBUTIONS

C.R.-P. and J.P.B performed the majority of research described herein and were assisted by G.M.D.D, L.M., E. Villa, L.E.A., J.C., R.M., and E. Verhoeyen, S.M., C.L., B.B.-M., E.C., and T.P. provided intellectual input and experiments. J.B.P. and E.C. provided invaluable reagents. K.V., A.C., T.A., and E.C. performed bioinformatic analysis of the IRE1 α gene signature. F.A.C., P.L., E.C.-V., and F.F. performed amino acid measurements. J.-E.R. designed research, secured funding, and wrote the manuscript.

DECLARATION OF INTERESTS

E.C. is a founding member of Cell Stress Discoveries. J.B.P. is an employee of and owns stock in Fosun Orinove. The IRE1 signature is patented: EP17306855.2. Remaining authors declare no competing interests.

Received: June 26, 2017 Revised: December 19, 2017 Accepted: February 12, 2018 Published: March 15, 2018

REFERENCES

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D., and Brinster, R.L. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature *318*, 533–538.

Bénéteau, M., Zunino, B., Jacquin, M.A., Meynet, O., Chiche, J., Pradelli, L.A., Marchetti, S., Cornille, A., Carles, M., and Ricci, J.E. (2012). Combination of glycolysis inhibition with chemotherapy results in an antitumor immune response. Proc. Natl. Acad. Sci. USA *109*, 20071–20076.

Bozzetti, F., and Zupec-Kania, B. (2016). Toward a cancer-specific diet. Clin. Nutr. 35, 1188–1195.

Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., Clark, S.G., and Ron, D. (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature *415*, 92–96.

Callahan, M.K., Postow, M.A., and Wolchok, J.D. (2016). Targeting T cell coreceptors for cancer therapy. Immunity 44, 1069–1078.

Cho, J.A., Lee, A.H., Platzer, B., Cross, B.C., Gardner, B.M., De Luca, H., Luong, P., Harding, H.P., Glimcher, L.H., Walter, P., et al. (2013). The unfolded protein response element IRE1 alpha senses bacterial proteins invading the ER to activate RIG-I and innate immune signaling. Cell Host Microbe *13*, 558–569. Cubillos-Ruiz, J.R., Bettigole, S.E., and Glimcher, L.H. (2017). Tumorigenic and immunosuppressive effects of endoplasmic reticulum stress in cancer. Cell *168*, 692–706.

Cubillos-Ruiz, J.R., Silberman, P.C., Rutkowski, M.R., Chopra, S., Perales-Puchalt, A., Song, M., Zhang, S., Bettigole, S.E., Gupta, D., Holcomb, K., et al. (2015). ER stress sensor XBP1 controls anti-tumor immunity by disrupting dendritic cell homeostasis. Cell *161*, 1527–1538.

Demetrius, L. (2005). Of mice and men. When it comes to studying ageing and the means to slow it down, mice are not just small humans. EMBO Rep. 6 (Spec No), S39–S44.

Di Biase, S., Lee, C., Brandhorst, S., Manes, B., Buono, R., Cheng, C.W., Cacciottolo, M., Martin-Montalvo, A., de Cabo, R., Wei, M., et al. (2016). Fasting-mimicking diet reduces HO-1 to promote T cell-mediated tumor cytotoxicity. Cancer Cell *30*, 136–146.

Eckard, S.C., Rice, G.I., Fabre, A., Badens, C., Gray, E.E., Hartley, J.L., Crow, Y.J., and Stetson, D.B. (2014). The SKIV2L RNA exosome limits activation of the RIG-I-like receptors. Nat. Immunol. *15*, 839–845.

Fontana, L., Adelaiye, R.M., Rastelli, A.L., Miles, K.M., Ciamporcero, E., Longo, V.D., Nguyen, H., Vessella, R., and Pili, R. (2013). Dietary protein restriction inhibits tumor growth in human xenograft models. Oncotarget *4*, 2451–2461.

Fontana, L., and Partridge, L. (2015). Promoting health and longevity through diet: from model organisms to humans. Cell *161*, 106–118.

Fontana, L., Weiss, E.P., Villareal, D.T., Klein, S., and Holloszy, J.O. (2008). Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. Aging Cell 7, 681–687.

Frecha, C., Fusil, F., Cosset, F.L., and Verhoeyen, E. (2011). In vivo gene delivery into hCD34+ cells in a humanized mouse model. Methods Mol. Biol. 737, 367–390.

Galluzzi, L., Buque, A., Kepp, O., Zitvogel, L., and Kroemer, G. (2015). Immunological effects of conventional chemotherapy and targeted anticancer agents. Cancer Cell 28, 690–714.

Garg, A.D., Galluzzi, L., Apetoh, L., Baert, T., Birge, R.B., Bravo-San Pedro, J.M., Breckpot, K., Brough, D., Chaurio, R., Cirone, M., et al. (2015). Molecular and translational classifications of DAMPs in immunogenic cell death. Front. Immunol. *6*, 588.

Hetz, C., Chevet, E., and Oakes, S.A. (2015). Proteostasis control by the unfolded protein response. Nat. Cell Biol. *17*, 829–838.

Jeon, Y.J., Khelifa, S., Ratnikov, B., Scott, D.A., Feng, Y., Parisi, F., Ruller, C., Lau, E., Kim, H., Brill, L.M., et al. (2015). Regulation of glutamine Carrier proteins by RNF5 determines breast cancer response to ER stress-inducing chemotherapies. Cancer Cell *27*, 354–369.

Joffre, O., Nolte, M.A., Spörri, R., and Reis e Sousa, C. (2009). Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. Immunol. Rev. 227, 234–247.

Kim, L.C., Cook, R.S., and Chen, J. (2017). mTORC1 and mTORC2 in cancer and the tumor microenvironment. Oncogene 36, 2191–2201.

Cell Metabolism 27, 828-842, April 3, 2018 841

Klement, R.J., and Fink, M.K. (2016). Dietary and pharmacological modification of the insulin/IGF-1 system: exploiting the full repertoire against cancer. Oncogenesis 5, e193.

Kroemer, G., Galluzzi, L., Kepp, O., and Zitvogel, L. (2013). Immunogenic cell death in cancer therapy. Annu. Rev. Immunol. *31*, 51–72.

Lee, K., Tirasophon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., Yoshida, H., Mori, K., and Kaufman, R.J. (2002). IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. Genes Dev. *16*, 452–466.

Levine, M.E., Suarez, J.A., Brandhorst, S., Balasubramanian, P., Cheng, C.W., Madia, F., Fontana, L., Mirisola, M.G., Guevara-Aguirre, J., Wan, J., et al. (2014). Low protein intake is associated with a major reduction in IGF-1, cancer, and overall mortality in the 65 and younger but not older population. Cell Metab. *19*, 407–417.

Lhomond, S., Avril, T., Dejeans, N., Voutetakis, K., Doultsinos, D., McMahon, M., Pineau, R., Obacz, J., Papadodima, O., Jouan, F., et al. (2018). Dual IRE1 RNase functions dictate glioblastoma development. EMBO Mol. Med., in press, pii: e7929. https://doi.org/10.15252/emmm.201707929.

Longo, V.D., and Mattson, M.P. (2014). Fasting: molecular mechanisms and clinical applications. Cell Metab. *19*, 181–192.

Lu, Y., Liang, F.X., and Wang, X. (2014). A synthetic biology approach identifies the mammalian UPR RNA ligase RtcB. Mol. Cell *55*, 758–770.

Lu, Z., Xie, J., Wu, G., Shen, J., Collins, R., Chen, W., Kang, X., Luo, M., Zou, Y., Huang, L.J., et al. (2017). Fasting selectively blocks development of acute lymphoblastic leukemia via leptin-receptor upregulation. Nat. Med. 23, 79–90. Martinon, F., Chen, X., Lee, A.H., and Glimcher, L.H. (2010). TLR activation of

the transcription factor XBP1 regulates innate immune responses in macrophages. Nat. Immunol. *11*, 411–418. Maurel, M., Chevet, E., Tavernier, J., and Gerlo, S. (2014). Getting RIDD of

RNA: IRE1 in cell fate regulation. Trends Biochem. Sci. 39, 245-254.

Meynet, O., and Ricci, J.E. (2014). Caloric restriction and cancer: molecular mechanisms and clinical implications. Trends Mol. Med. 20, 419–427.

Meynet, O., Zunino, B., Happo, L., Pradelli, L.A., Chiche, J., Jacquin, M.A., Mondragon, L., Tanti, J.F., Taillan, B., Garnier, G., et al. (2013). Caloric restriction modulates McI-1 expression and sensitizes lymphomas to BH3 mimetic in mice. Blood *122*, 2402–2411.

Norat, T., Dossus, L., Rinaldi, S., Overvad, K., Grønbaek, H., Tjønneland, A., Olsen, A., Clavel-Chapelon, F., Boutron-Ruault, M.C., Boeing, H., et al. (2007). Diet, serum insulin-like growth factor-I and IGF-binding protein-3 in European women. Eur. J. Clin. Nutr. *61*, 91–98.

Obacz, J., Avril, T., Rubio-Patiño, C., Bossowski, J.P., Igbaria, A., Ricci, J.E., and Chevet, E. (2017). Regulation of tumor-stroma interactions by the unfolded protein response. FEBS J. https://doi.org/10.1111/febs.14359.

Pakos-Zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A., and Gorman, A.M. (2016). The integrated stress response. EMBO Rep. *17*, 1374–1395.

Pietrocola, F., Pol, J., Vacchelli, E., Rao, S., Enot, D.P., Baracco, E.E., Levesque, S., Castoldi, F., Jacquelot, N., Yamazaki, T., et al. (2016). Caloric restriction mimetics enhance anticancer immunosurveillance. Cancer Cell *30*, 147–160.

Pluquet, O., Dejeans, N., Bouchecareilh, M., Lhomond, S., Pineau, R., Higa, A., Delugin, M., Combe, C., Loriot, S., Cubel, G., et al. (2013). Posttranscriptional regulation of PER1 underlies the oncogenic function of IRE α . Cancer Res. 73, 4732–4743.

Porporato, P.E. (2016). Understanding cachexia as a cancer metabolism syndrome. Oncogenesis 5, e200.

Rubio-Patiño, C., Bossowski, J.P., Villa, E., Mondragon, L., Zunino, B., Proics, E., Chiche, J., Bost, F., Verhoeyen, E., and Ricci, J.E. (2016). Low carbohydrate diet prevents McI-1-mediated resistance to BH3-mimetics. Oncotarget 7, 73270–73279.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. *43*, e47.

Sahu, N., Dela Cruz, D., Gao, M., Sandoval, W., Haverty, P.M., Liu, J., Stephan, J.P., Haley, B., Classon, M., Hatzivassiliou, G., et al. (2016). Proline starvation induces unresolved ER stress and hinders mTORC1-dependent tumorigenesis. Cell Metab. *24*, 753–761.

Solon-Biet, S.M., McMahon, A.C., Ballard, J.W., Ruohonen, K., Wu, L.E., Cogger, V.C., Warren, A., Huang, X., Pichaud, N., Melvin, R.G., et al. (2014). The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. Cell Metab. *19*, 418–430.

Solon-Biet, S.M., Mitchell, S.J., Coogan, S.C., Cogger, V.C., Gokarn, R., McMahon, A.C., Raubenheimer, D., de Cabo, R., Simpson, S.J., and Le Couteur, D.G. (2015). Dietary protein to carbohydrate ratio and caloric restriction: comparing metabolic outcomes in mice. Cell Rep. *11*, 1529–1534.

Underwood, L.E., Thissen, J.P., Lemozy, S., Ketelslegers, J.M., and Clemmons, D.R. (1994). Hormonal and nutritional regulation of IGF-I and its binding proteins. Horm. Res. 42, 145–151.

Van Rooijen, N., and Sanders, A. (1994). Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J. Immunol. Methods *174*, 83–93.

Villeneuve, J., Lepreux, S., Mulot, A., Bérard, A.M., Higa-Nishiyama, A., Costet, P., De Ledinghen, V., Bioulac-Sage, P., Balabaud, C., Nurden, A.T., et al. (2010). A protective role for CD154 in hepatic steatosis in mice. Hepatology *52*, 1968–1979.

Wek, S.A., Zhu, S., and Wek, R.C. (1995). The histidyl-tRNA synthetase-related sequence in the eIF-2 alpha protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. Mol. Cell. Biol. *15*, 4497–4506.

STAR***METHODS**

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC Anti-CD19	Miltenyi Biotec	130-102-494; RRID:AB_2661108
FITC Anti-CD45R	Miltenyi Biotec	130-110-845; RRID:AB_2658273
FITC Anti-CD49B	BD Biosciences	553857; RRID:AB_395093
FITC Anti-CD11b	BD Biosciences	553310; RRID:AB_394774
FITC Anti-TER-119	BD Biosciences	557915; RRID:AB_396936
VioBlue Anti-CD4	BD Biosciences	558107; RRID:AB_397030
PE Anti-CD8	BD Biosciences	553032; RRID:AB_394570
PE Anti NK1.1	BD Biosciences	557391; RRID:AB_396674
PE Anti-CD11c	BD Biosciences	557401; RRID:AB_396684
PECy7 Anti-CD86	BD Biosciences	560582; RRID:AB_1727518
APC Anti-CD127	BD Biosciences	564175
FITC Anti-CD3	eBioscience	11-0031-85; RRID:AB_464883
VioBlue Anti-F4/80	eBioscience	48-4801-82; RRID:AB_1548747
APCCy7 Anti-CD25	BD Biosciences	557658; RRID:AB_396773
Mouse monoclonal Anti-XBP1	Santa Cruz	sc-8015; RRID:AB_628449
Mouse monoclonal Anti-ERK2	Santa Cruz	sc-1647; RRID:AB_627547
Rabbit monoclonal Anti-IRE1α	Cell Signaling	3294; RRID:AB_823545
Rabbit monoclonal Anti-pS6K	Cell Signaling	9234; RRID:AB_2269803
Rabbit polyclonal Anti-S6K	Cell Signaling	9202; RRID:AB_331676
Rabbit polyclonal Anti-pAKT	Cell Signaling	9271; RRID:AB_329825
Mouse monoclonal Anti-AKT	Cell Signaling	2967; RRID:AB_331160
Rabbit monoclonal Anti-LC3B	Cell Signaling	3868; RRID:AB_2137707
Rabbit polyclonal Anti-elF2α	Cell Signaling	9722; RRID:AB_2230924
Rabbit polyclonal Anti-pelF2α	Cell Signaling	9721; RRID:AB_330951
Rabbit monoclonal Anti-RIG1	Cell Signaling	3743; RRID:AB_2269233
Rabbit monoclonal Anti-ATF4	Cell Signaling	11815; RRID:AB_2616025
Mouse monoclonal Anti-CHOP	Cell Signaling	2895; RRID:AB_2089254
Rabbit polyclonal Anti-GCN2	Cell Signaling	3302; RRID:AB_2277617
Mouse monoclonal Anti-ATF6	Novus biologicals	NBP1-40256; RRID:AB_2058774
Rabbit polyclonal Anti-pIREα	Novus biologicals	NB100-2323; RRID:AB_10145203
Rabbit polyclonal Anti-GRP78	Abcam	ab21685; RRID:AB_2119834
Rabbit monoclonal Anti-pGCN2	Abcam	ab75836; RRID:AB_1310260
anti-CD8-depleting antibody (clone53-6.7)	Bioxcell	BE0004-1; RRID:AB_1107671
anti-CD86-blocking antibody (clone GL-1)	Bioxcell	BE0025; RRID:AB_1107678
Purified Anti-mouse CD8a (clone 53-6.7)	Biolegend	100701; RRID:AB_312740
Alexa Fluor 594 anti-rat secondary antibody	Molecular Probes	A11007; RRID:AB_141374
Chemicals, Peptides, and Recombinant Proteins		
jetPEI DNA transfection Reagent	PolyPlus Transfection	POL101-10N
Clodronate Liposomes and PBS Liposomes	Liposoma B.V.	PBS-02
TUDCA	Sigma-Aldrich	T0266
MKC4485	John B. Patterson	N/A
Recombinant mouse IL-2	AbD Serotech	PMP38
DAPI	Sigma-Aldrich	D9542
Fast Sybr Green	Applied Biosystems	4385616

(Continued on next page)

Cell Metabolism 27, 828-842.e1-e7, April 3, 2018 e1

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Taqman Fast Universal PCR Master Mix	Applied Biosystems	4352042
O.C.T. compound	Tissue-Tek	4583
amino acids mixture (98 atom % 13C, 98 atom % 15N)	Sigma-Aldrich	608254
Critical Commercial Assays		
Mouse Tumor Dissociation Kit	Miltenyi Biotec	130-096-730
Mouse CD4/CD8 (TIL) Microbeads	Miltenyi Biotec	130-116-480
Mouse CD11c Microbeads ultrapure	Miltenyi Biotec	130-108-338
ECL Western Blotting Detection Reagents	GE Healthcare	RPN2106
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23225
RNAeasy minikit	Qiagen	74104
Omniscript RT kit	Qiagen	205113
Experimental Models: Cell Lines		
Mouse B16-F1 cells	ATCC	CRL-6323
Mouse CT26.WT cells	ATCC	CRL-2638
Human Embrionic Kidney-293T Cells	ATCC	CRL-1573
Experimental Models: Organisms/Strains		
Mouse: Eµ-Myc	The Jackson Laboratory	002728
Mouse: C57BL/6JOlaHsd	ENVIGO	N/A
Mouse: BALB/cOlaHsd	ENVIGO	N/A
Mouse: NOD scid gamma c ^{-/-} (NSG)	The Jackson Laboratory	005557
Oligonucleotides		
Atf3 (Mm00476033_m1)	Applied Biosystems	4331182
Ero1lb (Mm00470754_m1)	Applied Biosystems	4331182
Sars (Mm00803379_m1)	Applied Biosystems	4331182
Trib3 (Mm00454879_m1)	Applied Biosystems	4331182
Hsp90b1 (Mm00441926_m1)	Applied Biosystems	4331182
Hyou1 (Mm00491279_m1)	Applied Biosystems	4331182
Rn18s (Mm03928990_g1)	Applied Biosystems	4331182
Scara3 Forward TGCATGGATACTGACCCTGA	This paper	N/A
Scara3 Reverse GCCGTGTTACCAGCTTCTTC	This paper	N/A
Blos1 Forward CAAGGAGCTGCAGGAGAAGA	This paper	N/A
Blos1 Reverse GCCTGGTTGAAGTTCTCCAC	This paper	N/A
Col6 Forward TGCTCAACATGAAGCAGACC	This paper	N/A
Col6 Reverse TTGAGGGAGAAAGCTCTGGA	This paper	N/A
IFNγ Forward TCAAGTGGCATAGATGTGGAAGAA	This paper	N/A
IFNγ Reverse TGGCTCTGCAGGATTTTCATG	This paper	N/A
CXCL10 Forward GCTGATGCAGGTACAGCGT	This paper	N/A
CXCL10 Reverse 5'- CACCATGAATCAAACTGCGA	This paper	N/A
βactin Forward TGGAATCCTGTGGCATCCATGAAA	This paper	N/A

(Continued on next page)

e2 Cell Metabolism 27, 828–842.e1–e7, April 3, 2018

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
βactin Reverse TAAAACGCAGCTCAGTAACAGTCCG	This paper	N/A
sXBP1 Forward GCTGAGTCCGCAGCAGGTG	Villeneuve et al., 2010	N/A
uXBP1 Forward GAGTCCGCAGCACTCAGACT	Villeneuve et al., 2010	N/A
XBP1 Reverse GTGTCAGAGTCCATGGGAAGA	Villeneuve et al., 2010	N/A
shRNA target sequence: IRE1α sh#1: CCAAGATGCTGGAGAGATT	This paper	N/A
shRNA target sequence: IRE1α sh#2: GCTCGTGAATTGATAGAGA	This paper	N/A
shControl	Santa Cruz	sc-108060
shIFNγ	Santa Cruz	sc-39607-SH
Recombinant DNA		
psPAX2 plasmid	Addgene	12260
pSUPER	Oligoengine	VEC-PRT-0005/0006
MLV-Gag-Pol	Els Verhoeyen	N/A
CRISPR-Cas9-control plasmid	Santa Cruz	sc-418922
CRISPR–Cas9-IRE1α plasmid	Santa Cruz	sc-429758
CRISPR-Cas9-RIG1 plasmid	Santa Cruz	sc-432915
Software and Algorithms		
Bioinfominer	e-NIOS	www.bioinfominer.com
GraphPad Prism 7	GraphPad software	https://www.graphpad.com/scientific- software/prism/
Image J	NIH	https://imagej.nih.gov/ij/
Xcalibur 2.1	Thermo Fisher Scientific	N/A
Other		
Control Diet	ENVIGO	TD.130931
Low CHO Diet	ENVIGO	TD.130932
Low PROT Diet -12,5%	ENVIGO	TD.170630
Low PROT Diet -25%	ENVIGO	TD.130933
Low PROT Diet -40%	ENVIGO	TD.170631
Dulbecco's MEM (DMEM) w/o Amino Acids	USBiological	D9800-13

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jean-Ehrland Ricci (ricci@unice.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee and the regional ethics committee (approval reference PEA-232 and PEA-233). All experiments used age-matched female littermates. Eµ-Myc/wild-type (WT) mice were obtained from The Jackson Laboratory (#002728). Five-week-old WT syngeneic C57BL/6 mice and BALB/c mice were obtained from ENVIGO. NOD scid gamma $c^{-/-}$ (NSG) mice were obtained from The Jackson Laboratory (#005557) and housed in our animal facilities (C3M-Nice, France).

Mice were fed with isocaloric diets generated by ENVIGO: Control (Ctl: TD.130931), low carbohydrates (Low CHO: TD.130932) and low protein diet (Low PROT or Low PROT -25%: TD.130933). When specified, two other low protein diets were used (Low

PROT -12.5%: TD.170630 and Low PROT -40%: TD.170631). % energy CHO:PROT:FAT: Ctl - (70.9%:19.5%:9.6%); Low CHO - (54%:26.9%:19.2%); Low PROT -25% - (73.7%:14.9%:11.5%); Low PROT -12.5% - (72.2%:17%:10.8%); Low PROT -40% - (76.4%:12.2%:11.4%).

WT syngeneic C57BL/6 mice were intravenously injected with 0.1×10^6 Eµ-Myc cells. At day four after injection, the food was replaced with isocaloric diets generated by ENVIGO for 2 weeks: Ctl, Low CHO or Low PROT. BALB/c and C57BL/6 mice were fed with isocaloric Ctl and Low PROT diets (-12.5%, -25%, -40%) one week before subcutaneous injection with 0.5×10^6 CT26 cells or 0.25×10^6 B16 cells. NOD scid gamma c^{-/-} (NSG) mice were fed with Ctl, Low CHO or Low PROT isocaloric diets generated by ENVIGO one week before intravenous injection with 0.1×10^6 Eµ-Myc cells. For the groups with splenocyte co-injection, splenocytes from wild-type C57BL/6 mice were freshly isolated and washed in PBS. Then, 2×10^6 splenocytes were co-injected with 0.1×10^6 Eµ-Myc cells.

Syngeneic C57BL/6 mice and NSG mice injected with E_{μ} -Myc cells were monitored for lymphoma development and systemic signs of illness, including apathy, breathing problems, precipitous weight loss, and limited ability to reach food or water. Animals were euthanized as soon as they exhibited any signs of illness. After subcutaneous B16 and CT26 tumor cell injection syngeneic C57BL/6 and BALB/c mice were inspected daily for tumor development. Increase in tumor size was measured with a caliper. Tumor volume was calculated as follows: (Length x width to the power of 2)/2, where L is the longer of the 2 measurements.

For antibody-mediated depletion experiments *in vivo*, mice were intraperitoneally injected with 100 µg of an anti-CD8-depleting antibody (Bioxcell, clone53-6.7, #BE0004-1) or vehicle (PBS) every second day for seven doses during 2 weeks after tumor cell injection. For antibody-mediated blockade experiments *in vivo*, mice were intraperitoneally injected with 100 µg of anti-CD86-blocking antibody (Bioxcell, clone GL-1, #BE0025) or vehicle (PBS) every second day for seven doses during 2 weeks after tumor cell injection. For dendritic cell and macrophage depletion *in vivo*, mice were injected intraperitoneally with 200 µL of a 5mg/mL clodronate-loaded liposome suspension (Liposoma B.V., #PBS-02) every second day for seven doses during 2 weeks after tumor cell injection. Control mice were injected with 200 µL PBS-loaded liposomes using the same schedule.

TUDCA (Sigma-Aldrich, #T0266) was intraperitoneally administered in PBS (250 µg/gram of mouse body weight). MKC4485 was administered by oral gavage at a dose of 10 mL/kg from a 30 mg/mL suspension in 1% microcrystalline cellulose in a simple sugar at 300 mg/kg daily (Provided by John B. Patterson). Both inhibitors were administered from day 11 until day 16 after subcutaneous tumor cell injection. Then, the mice were euthanized for further analysis. When described glycemia was measured after a few hours of fasting by using a freestyle Optium blood glucose monitoring device.

Cell Lines and Cell Culture Conditions

Lymphoma-bearing C57BL/6 Eµ-Myc mice were killed by cervical dislocation as soon as they presented signs of illness. A single-cell suspension was prepared from lymph nodes by teasing them on a 70-µm nylon filter. Cells were either resuspended in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS), 10 mM Hepes, 0.1 mM L-asparagine, and 50 µM β -mercaptoethanol for further ex vivo analysis or reimplantation in wild-type mice. B16 cells were obtained from the ATCC (#CRL-6323) and cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS). CT26 cells were obtained from the ATCC (#CRL-2638) and cultured in RPMI-1640 medium (GIBCO) supplemented with 10% FBS and 1% sodium pyruvate. When indicated CT26 cells were cultured for 24 hr in amino acid (AA)-deprived medium (USBiological, #D9800-13). All cell lines were incubated at 37°C in a 5% CO^2 atmosphere.

METHOD DETAILS

Cytotoxicity Assay

CD3⁺ cells were negatively sorted from mice spleens using autoMACS (Miltenyi Biotec) with FITC antibodies against CD19 (Miltenyi Biotec, #130-102-494), CD45R (Miltenyi Biotec, #130-110-845), CD49b (BD Biosciences, #553857), CD11b (BD Biosciences, #553310) and Ter-119 (BD Biosciences, #557915). The resulting purified cells were co-incubated with CT26 or B16 cells at a ratio 1:5 in the presence of IL-2 (0.1 ng/mL, AbD Serotech, #PMP38) for 4 hr or 48 hr at 37 °C. Flow cytometry (MACS-Quant Analyzer, Miltenyi Biotec) was used to analyze the cell viability of CT26 and B16 cells. CD3-negative population and back gating was used to confirm the difference in forward scatter and side scatter parameters between cells. Cell death was evaluated by looking at plasma membrane permeabilization of CT26 and B16 cells using 4',6-diamidino-2-phenylindole staining (DAPI, Sigma-Aldrich #D9542).

Flow Cytometry Analysis

To obtain a single-cell suspension from tumors, lymph nodes, and spleens were filtered through a 70-μm strainer, stained, and analyzed on MACS-Quant Analyzer (Miltenyi Biotec). The following fluorochrome-conjugated anti-mouse antibodies were used: CD4 (VioBlue, #558107), CD8 (PE, #553032), NK1.1 (PE, #557391), CD11c (PE, #557401), CD86 (PECy7, #560582), CD127 (APC, #564175), CD25 (APCCy7, #557658) (BD Biosciences). CD3 (FITC, #11-0031-85), F4/80 (VioBlue, #48-4801-82) (eBioscience). Percentage of CD4⁺ and CD8⁺ cells are calculated within CD3⁺ cells. T regs were defined as the CD3⁺CD4⁺CD25⁺CD127⁻ T-cell

population. NK cells were defined as CD3⁻NK1.1⁺ cells. DCs were defined as F4/80⁻ CD11c⁺ cells and macrophages were defined as F4/80⁺ cells. Percentage of CD86⁺ cells was calculated within CD11c⁺ cells.

Confocal Laser Scanning Microscopy

Tumoral tissue was snap-frozen in O.C.T. compound (Tissue-Tek, #4583). Then, 5-µm cryosections were prepared and fixed in acetone. Purified anti-mouse CD8a (Biolegend, clone 53-6.7, #100701) was used for CD8 staining and was visualized using Alexa Fluor 594 anti-rat secondary antibody (Molecular Probes, #A11007). All sections were stained with DAPI. For each condition at least three measurements were performed. The number of CD8-positive cells was determined in optical fields of 40× on individual sections. Samples were imaged using a Nikon A1R confocal microscope and processed with ImageJ software analysis.

Western Blot Analysis

Tissue samples were collected and lysed using a Precellys 24 (Bertin Instruments) homogenizer (3×30 s, 6500 \times g) in Laemmli buffer. Proteins were immunoblotted with the indicated antibodies. Immunoblots were visualized (FUJIFILM LAS4000) using the ECL Western Blotting Detection Reagents (GE Healthcare, #RPN2106), and quantification was performed using ImageJ software. anti-XBP1 (#sc-8015), and anti-ERK2 (#sc-1647) antibodies were purchased from Santa Cruz Biotechnology. Anti-IRE1 α (#3294), Anti-pS6K (#9234), anti-S6K (#9202), anti-pAKT (#9271), anti-AKT (#2967), anti-LC3B (#3868), anti-eIF2 α (#9722), anti-peIF2 α (#9721), anti-RIG1 (#3743), anti-ATF-4 (#11815), anti-CHOP (#2895) and GCN2 (#3302) antibodies were purchased from Cell Signaling. Anti-ATF6 (#NBP1-40256) and anti-pIRE1 α (#NB100-2323) were purchased from Novus Biologicals. Anti-GRP78 (#ab21685) and anti-pGCN2 (#ab75836) was purchased from Abcam.

Reverse Transcriptase Quantitative-PCR (RT-qPCR) Analysis

CT26 tumors were dissociated with the mouse Tumor Dissociation Kit (Miltenyi Biotec, #130-096-730) and CD4⁺/CD8⁺ TILs were sorted from the obtained single cell suspension using mouse CD4/CD8 (TIL) MicroBeads (Miltenyi Biotec, #130-116-480) in an autoMACS (Miltenyi Biotec). CD11c⁺ DC were sorted from spleens of tumor-bearing BALB/c mice using mouse CD11c Microbeads ultrapure in an autoMACS (Miltenyi Biotec, #130-108-338). For total tumor tissue samples were collected and lysed using a Precellys 24 (Bertin Instruments) homogenizer (3 × 30 s, 6500 × g) and total RNA was isolated from cells and tissue using the RNAeasy minikit (Qiagen, #74104) according to the manufacturer's protocol. Reverse transcription was performed using the Omniscript RT Kit (Qiagen, #205113). Quantitative-PCR was performed with Fast SYBR Green (Applied Biosystems, Life Technologies, #4385616) or TaqMan Fast Universal PCR Master Mix (Applied Biosystems, #4352042) using the 7500 Fast and the Step One real-time PCR systems (Applied Biosystems) following the manufacturers' instructions.

The following primers were used for SYBR Green qPCR: *Scara3* Forward 5'-TGCATGGATACTGACCCTGA-3' and Reverse 5'-GCCGTGTTACCAGCTTCTC-3'; *Blos1* Forward 5'-CAAGGAGCTGCAGGAGAAGA-3' and Reverse 5'-GCCTGGTTGAA GTTCTCCAC-3' *Col6* Forward 5'-TGCTCAACATGAAGCAGAACC-3' and Reverse 5'-TTGAGGGAGAAAGCTCTGGA-3' *IFN* γ Forward; 5'-TCAAGTGGCATAGATGTGGAAGAA-3' and Reverse 5'-TGGCTCTGCAGGATTTTCATG-3'; *CXCL10* Forward 5'-GCTGATGCAGGTACAGCGT-3' and Reverse 5'-CACCATGAAACTGCGA-3'; *βactin* Forward 5'-TGGAATCCTGTGGCATC CATGAAA-3' and Reverse 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'. *sXBP1* Forward 5'-GCTGAGTCCGCAGCAGCAGGTG-3'; *uXBP1* Forward 5'-GAGTCCGCAGCACTCAGAACT-3' and *XBP1* Reverse 5'-GTGTCAGAGTCCATGGGAAGA-3' (Villeneuve et al., 2010). The housekeeping gene β -actin was used as a control for RNA quality and for normalization.

The following Taqman assay primer sets from Applied Biosystems were used: *Atf*3 Mm00476033_m1; *Ero1lb* Mm00470754_m1; *Sars* Mm00803379_m1; *Trib3* Mm00454879_m1; *Hsp90b1* Mm00441926; *Hyou1* Mm00491279_m1. The housekeeping gene Rn18s was used as a control for RNA quality, and used for normalization: Mm03928990_g1.

All analyses were performed in triplicate, and melting curve analysis was performed for SYBR Green to control product quality and specificity.

Generation of shIFNγ- and shIRE1α-Transduced Cells

Self-inactivating viruses were generated by transient transfection of 293T cells (ATCC, #CRL-1573) and tittered as described previously (Frecha et al., 2011). Briefly For VSV-G preparation, 3 microgram of envelope plasmid was co-transfected using the classical calcium phosphate method with a 8,6 microgram Gag-Pol packaging plasmid (psPAX2, Adgene, #12260) and 8,6 microgram of a plasmid encoding a control shRNA plasmid (Santa Cruz, #sc-108060) and a self-inactivating mouse lentiviral shIFN_Y plasmid (Santa Cruz, #sc-39607-SH). Eighteen hours after transfection, the medium was replaced by Opti-MEM supplemented with HEPES (Invitrogen). Viral supernatants were harvested 48 hr after transfection and filtered. The vectors were concentrated at low speed by overnight centrifugation of the viral supernatants at 3000g at 4°C.

For the generation of stable CT26 with silenced IRE1α, we used the pSUPER retroviral vector with neo+GFP (Oligoengine, #VEC-PRT-0005/0006). The target sequences were as follows: sh#1= 5'-CCAAGATGCTGGAGAGATT-3' and sh#2= 5'-GCTCGTGAATT GATAGAGA-3'. Oligonucleotides were cloned into the pSUPER vector following the manufacturer's protocol. Double-stranded DNA templates encoding siRNA oligonucleotides for IRE1α were synthesized. The specific oligonucleotide sequence contained a sense strand of 19 nucleotides followed by a short spacer (TTCAAGAGA) and the reverse complement of the sense strand. Five thymidines were added at the end of the synthesized oligonucleotide as an RNA polymerase III transcriptional stop signal. Oligonucleotides were annealed and ligated into the pSUPER vector digested by BgIII and HindIII, and insertion was confirmed by EcoRI–HindIII digestion via migration in an agarose gel. For VSV-G preparation, 3 microgram of envelope plasmid was co-transfected using the classical calcium phosphate method with a 8,6 microgram MLV-Gag-Pol packaging plasmid and 8,6 microgram of the empty pSUPER plasmid or the shIRE1α containing pSUPER plasmids. CT26 cells were transduced and sorted using a SONY sorter SH800 based on GFP expression, resulting in >95% purity.

Generation of CRISPR/Cas9 Cells

For the generation of stable CT26 with invalidated IRE1 α or RIG1 cells were transfected with 3 µg of CRISPR–Cas9-expressing knockout plasmids (control, sc-418922; IRE1 α , sc-429758; RIG1, sc-432915; all from Santa Cruz) using the jetPEI DNA transfection Reagent (PolyPlus Transfection, #POL101-10N) as described by the manufacturer. The knockout plasmids are a mixture of three plasmids, each carrying a different guide RNA specific for the target gene, as well as the Cas- and GFP-coding regions. GFP+ cells were selected by sorting on a SH800S Cell Sorter (Sony Biotechnology) 24 hr after transfection, and depletion of target proteins was verified by immunoblotting.

Sampling of Intracellular Metabolites

Tumor samples were resuspended in 170 μ L of ultrapure water, manually crushed with a micro potter, vortexed, and then sonicated 5 times for 10 s using a sonication probe (vibra cell, Bioblock Scientific). At this step, 20 μ L of each sample were withdrawn for further determining the total protein concentration (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, #23225). Then, we added 350 μ L of methanol to the remaining 150 μ L of lysate and we sonicated again twice for 10 s each using a sonication probe. Cell debris were then removed by centrifugation for 30 min at 4°C and 20,000g. Supernatant was recovered and incubated 1h30 on ice before a second centrifugation step for 15 min at 4°C and 20,000g. The resulting metabolic extracts were dried under a stream of nitrogen using a TurboVap instrument (Thermo Fisher Scientific) and stored at -80° C until analysis.

Dried extracts were dissolved using a given volume of 95 % mobile phase A / 5% mobile phase B to give in a 1000 ng/mL total protein concentration for alanine, arginine, proline methionine, tyrosine, isoleucine, leucine, phenylalanine, and tryptophan quantification. The extract was then diluted 2-fold for aspartic acid, glutamine, glycine, and valine quantification, while another 4-fold dilution was performed for asparagine, serine, threonine, glutamic acid, lysine, and histidine quantification. A defined concentration* of a labeled amino acids mixture 98 atom % 13C, 98 atom % 15N (Sigma-Aldrich, #608254) was added to each sample in order to normalize the signals and estimate endogenous amino acid concentrations. *13C 415N -Asn , 0.21 µg/mL; *13C3, 15N-Ser, 0.27 µg/mL; *13C4,15N-Asp, 0.64 µg/mL; *13C5,15N2-Gln, 0.24 µg/mL; *13C2,15N-Gly, 0.34 µg/mL; *13C4,15N-Thr, 0.32 µg/mL; *13C5,15N-Glu, 0.58 µg/mL; *13C3,15N-Ala, 0.54 µg/mL; *13C6,15N2-Lys, 0.26 µg/mL; *13C6,15N3-His, 0.06 µg/mL; *13C6,15N4-Arg, 0.35 µg/mL; *13C5,15N-Pro, 0.2 µg/mL; *13C5,15N-Val, 0.31 µg/mL; *13C5,15N-Met, 0.12 µg/mL; *13C6,15N3-His, 0.06 µg/mL; *13C6,15N4-Arg, 0.35 µg/mL; *13C6,15N3-Leu, 0.56 µg/mL; *13C9,15N-Phe, 0.26 µg/mL; *13C11,15N2-Trp, 0.34 µg/mL.

Analysis of Amino Acid Residues by Liquid Chromatography Coupled to High Resolution Mass Spectrometry (LC-MS)

LC-MS experiments were performed using a Dionex Ultimate chromatographic system (Thermo Fisher Scientific) coupled to an Exactive (Orbitrap) mass spectrometer (Thermo Fisher Scientific) fitted with an electrospray ion source. The mass spectrometer was externally calibrated before each analysis using the manufacturer's predefined methods and provided recommended calibration mixture. Chromatographic separation was performed on a Discovery HS F5 PFPP 5 µm, 2.1 × 250 mm column (Sigma-Aldrich) at 30°C. The chromatographic system was equipped with an on-line prefilter (Thermo Fisher Scientifics). Mobile phases were 100% water (A) and 100% aceonitrile (B), both of which containing 0.1% formic acid. Chromatographic elution was achieved with a flow rate of 250 µL/min. After sample injection (20 µL), elution started with an isocratic step of 2 min at 5% phase B, followed by a linear gradient from 5 to 100% of phase B in 18 min. These proportions were kept constant for 4 min before returning to 5% of phase B and letting the system equilibrate for 6 min. The column effluent was directly introduced into the electrospray source of the mass spectrometer, and analyses were performed in the positive ion mode. Source parameters were as follows: capillary voltage set at 5 kV, capillary temperature at 300°C; sheath and auxiliary gas (nitrogen) flow rates at 50 and 25 arbitrary units, respectively; mass resolution power of the analyzer set at 50,000 at m/z 200 (full width at half maximum, FWHM) for singly charged ions. The acquisition was achieved from m/z 50 to 250 in the positive ionization mode during the first 12 min of the run. Under these conditions, we achieved a good chromatographic separation and detection (with an average mass accuracy better than 3ppm) of the 19 targeted amino acids (under their [M+H]+ form). These species were readily identified and quantified by the isotope dilution method using 13C, 15N-labeled homologues (see above). Corresponding extracted ion chromatograms were generated and resulting peaks integrated using the Xcalibur software (version 2.1, Thermo Fisher Scientific) for alanine ([M+H]+ at theoretical m/z 90.05496, retention time 2.98 min), arginine (m/z 175.11895, 3.19 min), asparagine (m/z 133.06077, 2.81 min), aspartate (m/z 134.04478, 2.84 min), glutamate (m/z 148.06043, 2.95 min), glutamine (m/z 147.07642, 2.84 min), glycine (m/z 76.03931, 2.84 min), histidine (m/z 156.07675, 3.03 min), isoleucine (m/z 132.10191, 5.73 min), leucine (m/z 132.10191, 6.37 min), lysine (m/z 147.11280, 3.00 min), methionine (m/z 150.05833, 4.17 min), proline (m/z 116.07061, 3.22 min), phenylalanine (m/z 166.08626, 8.57 min), serine (m/z 106.04987, 2.81 min), threonine (m/z 120.06552, 2.88 min), tryptophan (m/z 205.09715, 10.58 min), tyrosine (m/z 182.08117, 5.51 min), and valine (m/z 118.08626, 3.79 min). P-values were calculated by applying a Mann Whitney test using the GraphPad Prism Software.

Bioinformatic Analysis

Patients were clustered according to IRE1 α activity based on the normalized z-score of gene expression for the *BioInfoMiner* signature of 38 genes (Lhomond et al., 2018). The z-score was calculated by the equation (X - m)/s, X stands for normalized log2

e6 Cell Metabolism 27, 828-842.e1-e7, April 3, 2018

expression data of each gene in each sample; m stands for mean of expression of each gene among all samples; and s stands for standard deviation. Raw data (*.CEL files) of the GSE27306 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=GSE27306) from (Pluquet et al., 2013) were processed into R/Bioconductor by using the RMA normalization and Limma package (Ritchie et al., 2015). The deferentially expressed genes (DEGs) between DN and WT U87 cells, were selected by using a corrected p value threshold of 0.05 and fold change threshold of $|log2(fc)| \ge 1.5$. 1051 deferentially expressed (D.E.) genes were then introduced into the *BioInfoMiner* tool and gene prioritization was executed based on the biomedical ontologies of the four-different functional and phenotype databases (GO), Reactome, MGI and HPO, separately. For the annotation process was used the "complete" version (this version amplifies the annotation of each gene with the ancestors of every direct correlated ontological term, exploiting the structure of ontological tree) and the hypergeometric pvalue threshold was set to 0.05. 227 highly prioritized genes including their proximal interactors was the union of the BioInfoMiner output from the four databases and 38 hub-genes were highlighted as the intersection with the IRE1 α signature of 97 genes of (Pluquet et al., 2013). *The BioInfoMiner* signature was composed of 19 genes; highly up-regulated in WT versus DN U87 cells (ASS1, C3, CCL20, COL4A6, CXCL2, CXCL5, CXCL8, IFI44L, IL1B, IL6, KCNN2, MMP1, MMP12, MMP3, PLA2G4A, PPP4R4, SERPINB2, TFP12, ZNF804A), and 19 genes; highly down-regulated in WT versus DN U87 cells (ASS1, C0L3A1, COL8A1, DACH1, DCN, FHL1, GAS1, LUM, OXTR, PLAC8, RGS4, TAGLN, TGFB2, THBS1, TIMP3, TMEM255A).

This 38-genes signature was used to stratify 3 different types of tumors including glioblastoma multiform (GBM; TCGA and GBMmark, Lhomond et al., 2018), melanoma (TCGA) and colorectal cancer (TCGA) into IRE1α high and IRE1α low activity tumors. Then based on these 2 tumor groups, the expression of the following T-cell markers was evaluated in the two groups using the transcriptome data: *IFNG*, *IL12*, *TBX21*, *IRF1*, *STAT1*, *GZMA*, *GZMB*, *GZMH*, *PRF1*, *GNLY*, *NKG7*, *CXCL9*, *CXCL10*, *CCL5*, *CX3CL1*, *CXCR3*, *CCL2*, *CCL4*, *CXCL11*, *MADCAM1*, *ICAM1*, *VCAM1*, *CD3D*, *CD8A*, *GBP1*, and all the available *HLAs*.

Statistics

Statistical analysis was conducted using GraphPad Prism 7 software. Differences in calculated means between groups were assessed by two-sided Student's t tests. For experiments involving more than two groups, differences in the calculated mean values between the groups were assessed by one-way analysis of variance (ANOVA) followed by Fisher's exact test. Kaplan-Meier survival analyses were performed, and survival curves were compared using log-rank tests. A P-value less than 0.05 was considered significant. Error bars represent the means ± standard error of the mean (SEM).

Article 5



Regular Article

LYMPHOID NEOPLASIA

HSP110 sustains chronic NF-KB signaling in activated B-cell diffuse large B-cell lymphoma through MyD88 stabilization

Christophe Boudesco,^{1,2} Els Verhoeyen,^{3,4} Laurent Martin,⁵ Catherine Chassagne-Clement,⁶ Leila Salmi,^{1,2} Rana Mhaidly,⁴ Céline Pangault,⁷ Thierry Fest,⁷ Selim Ramla,⁵ Fabrice Jardin,⁸ Olaf-Oliver Wolz,⁹ Alexander N. R. Weber,⁹ Carmen Garrido,^{1,10} and Gaetan Jego^{1,2}

¹INSERM, LNC Unité Mixte de Recherche (UMR) 1231, Equipe Labellisée Ligue Nationale Contre le Cancer, Dijon, France; ²Faculté des sciences de santé, Université Bourgogne Franche-Comté, Dijon, France; ³INSERM U1111, Centre National de la Recherche UMR 5308, CIRI, EVIR Team, Université de Lyon-1, ENS de Lyon, Lyon, France; ⁴Université Côte d'Azur, INSERM, C3M, Contrôle Métabolique des Morts Cellulaires, Nice, France; ⁵Service Pathologie du Plateau de Biologie du Centre Hospitalier Universitaire (CHU) Dijon, France; ⁴Service de Biopathologie, Centre Léon Bérard, Lyon, France; ⁷INSERM, UMR 11236, Université Rennes 1, Etablissement Français du Sang Bretagne, CHU de Rennes, Laboratoire d'Hématologie, Rennes, France; ⁸INSERM, U918, Rouen, France; ⁹Department of Immunology, University of Tübingen, Germany; and ¹⁰Centre Georges François Leclerc, Dijon, France

KEY POINTS

- HSP110 sustains chronic NF-кB signaling in ABC-DLBCL through MyD88 stability.
- HSP110 is highly expressed in cells of patients with ABC-DLBCL and correlates with MyD88 expression.

Activated B-cell diffuse large B-cell lymphoma (ABC-DLBCL) is an aggressive lymphoproliferative disorder involving chronic NF-κB activation. Several mutations in the BCR and MyD88 signaling pathway components, such as MyD88 L265P, are implicated in this aberrant activation. Among heat shock proteins, HSP110 has recently been identified as a prosurvival and/or proliferation factor in many cancers, but its role in ABC-DLBCL survival mechanisms remained to be established. We observed that short hairpin RNA-mediated HSP110 silencing decreased the survival of several ABC-DLBCL cell lines and decreased immunoglobulin M-MyD88 co-localization and subsequent NF-κB signaling. Conversely, overexpression of HSP110 in ABC-DLBCL or non-DLBCL cell lines increased NF-κB signaling, indicating a tight interplay between HSP110 and the NF-κB pathway. By using immunoprecipitation and proximity ligation assays, we identified an interaction between HSP110 and both wild-type MyD88 and MyD88 L265P. HSP110 stabilized both MyD88

forms with a stronger effect on MyD88 L265P, thus facilitating chronic NF-κB activation. Finally, HSP110 expression was higher in lymph node biopsies from patients with ABC-DLBCL than in normal reactive lymph nodes, and a strong correlation was found between the level of HSP110 and MyD88. In conclusion, we identified HSP110 as a regulator of NF-κB signaling through MyD88 stabilization in ABC-DLBCL. This finding reveals HSP110 as a new potential therapeutic target in ABC-DLBCL. (*Blood*. 2018;132(5):510-520)

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of aggressive non-Hodgkin lymphoma (NHL) in adults. Although therapy with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) has led to an improvement in survival, a significant percentage of patients are still refractory to the treatment or they eventually relapse.1 Therefore, it is important to find new molecular targets for treating patients with DLBCL. Advanced genome sequencing studies have unveiled the complexity of DLBCL and revealed a heterogeneous group of tumors characterized by specific genetic alterations.² One way to overcome the heterogeneity of this lymphoma is to identify and target mechanisms that drive or sustain oncogenic signaling and survival of lymphoma cells, regardless of their somatic mutations. Interestingly, a body of literature has highlighted the fact that tumor cells acquire a biological addiction to heat shock proteins (HSPs) and stress proteins during malignant transformation.³⁻⁵ HSPs are highly conserved molecular chaperone proteins whose expression is induced in response to multiple physiological and environmental stresses.⁶ In these contexts, they control a large array of cellular functions to rescue the cell from debilitating conditions. Cancer cells can be considered highly stressed cells because of their numerous mutations, translocation or polyploidy, and microenvironment.^{7,8} Therefore, HSPs are often overexpressed in cancer cells, where they contribute to cancer cell resistance, notably to apoptosis and to the clearance of cancer cells by immune cells.^{9,10} As a consequence, targeting HSPs in various types of cancer has been proposed. Several articles have shown that HSPs are also highly expressed in hematologic malignancies, in which they are involved in several key proliferative and survival functions.¹¹ In particular, in lymphoma, HSP90 has been shown to have multiple roles in key DLBCL oncogenic pathways. Indeed, its capacity to enhance B-cell-like 6 (BCL6) oncogenesis⁴ and to interact with and stabilize key B-cell receptor (BCR) protein complexes (CD79A/B-SYK-BTK-PLC γ 2)³ suggests that targeting HSP90 could represent an interesting additional option for treating DLBCL. Unfortunately, a recent clinical trial using the HSP90 inhibitor AUY922 in DLBCL (NCT01485536) was halted early because of limited response and adverse effects in 100% of patients, prompting the search for other potential targets.

Among HSPs, HSP110 is a high-molecular-weight chaperone with antiaggregation properties and, through its interaction with HSP70, participates in the correct folding of newly synthesized or misfolded proteins.^{12,13} We have recently demonstrated that HSP110 is a major factor in colon cancer growth through its intraand extracellular functions.^{14,15} At the intracellular level, HSP110 binds directly to STAT3, facilitating its phosphorylation by JAK2 and contributing to STAT3-dependent tumor growth. We have demonstrated that colon cancer cells at the extracellular level secrete HSP110 to promote macrophage polarization toward a procancer phenotype (M2).¹⁵ Furthermore, we have identified a truncated form of HSP110 called HSP110DE9 in colorectal tumors with microsatellite instability that acts as an endogenous HSP110 inhibitor.¹⁶ The high expression of HSP110DE9 and/or the low expression of wild-type (WT) HSP110 in patients with colon cancer was associated with a highly effective response to chemotherapy.¹⁷ Recent studies have shown major new roles for HSP110 in human NHL. In particular, HSP110 was shown to be an immunogenic antigen in a xenograft model of NHL, and expression of HSP110 was shown to correlate with the aggressiveness and proliferation index of tumor cells from patients.¹⁸ A significant positive correlation between HSP110 and BCL6 expression was also found in germinal center (GC) BCL/BCL6+ DLBCL, which could be explained by the stabilization of BCL6.¹⁹

Given the emerging role of HSP110 in cancer and NHL in particular, we investigated the role of HSP110 in ABC-DLBCL, which relies on the NF- κ B signaling pathway for growth and survival.²⁰ Mutations affecting MyD88 L265P and the CD79/CARD11/MALT1/BCL10 signaling cascade are mostly responsible for the aberrant NF- κ B activation.^{21,22} Here we show that HSP110 is an essential survival factor for these tumor cells. Furthermore, we demonstrate that HSP110 binds to and stabilizes wild-type and mutated MyD88 in cell lines and in patients' samples and thereby amplifies the aberrant NF- κ B signaling pathway. Thus, we have identified a new role for HSP110 in tumor cells and have confirmed that HSP110 is a potential target for the development of future therapies for NHL.

Methods

Primary tumors and cell lines

ABC-DLBCL cell lines were purchased from American Type Culture Collection (U2932/OCI-Ly3/OCI-Ly10/SUD-HL2) or kindly provided by D. Krappmann (Helmholtz Zentrum München, Munich, Germany) (HBL1/TMD8) and cultured in RPMI 1640 (Dominique Dutscher, Brumath, France) plus fetal bovine serum (Dominique Dutscher) at 10% (U2932/TMD8), 15% (OCI-Ly3), or 20% (HBL1/OCI-Ly10 and SUD-HL2). Tumors for immunoblots and immunohistochemistry were provided by the Ferdinand Cabanne Biological Resource Center (BB-0033-00044), Dijon, France, and by the Département de Biopathologie, Centre Léon Bérard, Lyon, France. Samples for messenger RNA (mRNA) analysis were from the Centre de Ressources Biologiques-Santé (BB-0033-00056) of Rennes Hospital. Patients with DLBCL were classified according the World Health Organization classification using the Hans immunohistochemical algorithm. The study using human biopsies was approved by each relevant institutional review board or ethics committee, and all human participants gave informed consent.

Transfection

ABC-DLBCL cell lines were transfected with the AMAXA Nucleofector 2b device (Lonza, Basel, Switzerland) and the Nucleofector kit T (HBL1, OCI-Ly3, OCI-Ly10) or V (U2932, SU-DHL2, TMD8). Short interfering RNA (siRNA) control (MISSION siRNA Universal Negative Control #1, Sigma-Aldrich, Lyon, France) or hsph1 targeting siRNA (Silencer Select Pre-Designed siRNAs, Life Technologies, Saint-Aubin, France) were used. Specific AMAXA programs were applied (U-15 for U2932; P005 for SU-DHL2, TMD8; G016 for HBL1, OCI-Ly3, OCI-Ly10). For other HSPs, Silencer Pre-Designed siRNA for HSP27 (ID: 122371-HSPB1) and HSP70 (IDs6966-HSPA1A) or the specific functional inhibitor of HSP90, 1 µM PU-H71 16 hours (Axon Medchem, Groningen, The Netherlands) were used. HEK293T transfections were performed using Genjet (Ver. II) reagent (TebuBio, Le Perray-en-Yvelines, France). Green fluorescent protein (GFP), HSP110-GFP, and HSP110DE9-mutant GFP were homemade plasmids. Myd88 WT and L265P plasmid were used as described,²³ pGL4.32(luc2P/NF-ĸB-RE/Hygro) vector (Promega E849A) was kindly provided by P. Gaulard (Mondor Institute of Biomedical Research, Paris, France).

Retroviral particles

Human short hairpin RNA (shRNA) lentiviral vectors pseudotyped with measles virus glycoproteins or baboon retroviral glycoproteins have been described elsewhere.24-26 These lentiviral vectors were produced in 293T cells with GFP-expressing shRNA control (Sigma MISSION SHC016-1EA) or hsph1targeting shRNA lentiviral constructs (Sigma MISSION shRNA SHCLND-NM_006644, clone TRCN0000275617). HBL1, Ly3, TMD8, and U2932 were incubated using viral supernatant at multiplicity of infection 2 for 24 hours before washing. At 48 hours after transduction, cells were subjected to puromycin selection for 96 hours. The viability of GFP+ cells over time was observed by using an LSRII Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). For rescue experiments, the retroviral IKK2-EE vector was a gift from Stephan Ludwig (Zentrum für Molekularbiologie der Entzündung, Munster, Germany).27 This murine retroviral vector was produced by transfection in 293T cells together with murine leukemia virus gag-pol plasmid and the BAEV-encoding plasmid. HBL1 was infected 3 times every 12 hours, selected at 96 hours, and then transduced with the lentiviral vector-bearing shRNA control or HSP110 shRNA.

Immunoblot analysis

Primary antibodies used in this study were anti-Bcl-xL (2764S), c-Myc (9605S), cyclin D1 (2926S), p-STAT3 (9145S), STAT3 (9139S), and p-I κ B α Ser32/36 (9246) from Cell Signaling Technologies (Danvers, MA); Mcl-1 (sc-819), HSC70 (sc-7298), HSP110 (sc-6241), GFP (sc-8334), BcL10 (331.3), and p-IRAK1 (sc-325147) from Santa Cruz Biotechnologies (Dallas, TX); anti-HSP110 (ab109624) and anti-MyD88 (ab119048) for IHC staining, anti-BcL10 (ab199011), immunoglobulin M (IgM; ab20054), I κ B α (ab32518), p-I κ B α (ab133462), Myd88 (ab119048), HSP90 (ab59459), HA (ab130275), HSP70 (ab5439), ubiquitin



Figure 1. HSP110 expression has an impact on ABC-DLBCL cell survival. (A) Kinetics of cell survival determined by annexin-V/7-aminoactinomycin D (7AAD) staining on shRNA-expressing cells, data are presented as the mean \pm standard deviation (n = 3). (B) Representative annexin-V/7AAD staining on shRNA-expressing cells determined at day 9. (C) Immunoblot analysis of HSP110, Bcl-xL, Bcl2, and cleaved caspase-3 (Casp3) at 48 hours after transfection of HBL1 with an shRNA targeting HSP110 or a control shRNA (n = 3). β -actin served as a loading control. ns, not significant. ***P < .001.

(linkage-specific K63) (ab179434) from Abcam (Paris, France); anti-IkBa (#4812), IRAK1 (#4504), Myd88 (#4283), c-Rel (#4727), TRAF6 (#8028), p65 (#8242), BcL2 (#2870), caspase-3 Asp175 (#9664), total caspase-3 (#9662), lamin A/C (#4777), and HSP60 (#12165) from Cell Signaling Technologies; anti-p50 (616702) from BioLegend (San Diego, CA); HSP27 (ADI-SPA-800) from Enzo Life (Villeurbanne, France), and monoclonal anti-β-actin-peroxidase antibody (A3854) from Sigma-Aldrich. Normal mouse serum IgG (sc-2025) and normal rabbit IgG (sc-2027) from Santa Cruz Biotechnologies were used as nonrelevant antibodies for control immunoprecipitation and Duolink assay. Cytoplasmic and nuclear extracts were obtained using the NE-PER Nuclear and Cytoplasmic Extraction reagent kit (Thermo Fisher Scientific, Waltham, MA). Immunoprecipitations were performed as previously described,²¹ and the HSP110 primary antibody (ab109624) was used.

NF-кВ reporter assay

HEK293T cells were transfected using Genjet Ver. II reagent, 0.4 μ g of pGL4.32 (luc2P/NF- κ B-RE/Hygro) vector (Promega, Madison, WI), 0.4 μ g Myd88 WT or L265P-hemagglutinin (HA) tagged, increasing amounts of HSP110-HA-tagged plasmid (0.1 to 0.7 μ g), and 0.3 μ g of GFP vector for normalization. Samples were prepared according to the Promega protocol (Luciferase Assay System E1500). Luminescence from equivalent amounts of lysate was read in triplicate on an EnVision Multilabel Plate Reader (PerkinElmer). All readings were normalized to the mean fluorescence intensity of GFP expression.

Immunofluorescence and Duolink proximity ligation assay (PLA)

Primary antibodies were anti-IgM (IM260), anti-I κ B α (ab32518), anti-HSP105 (EPR4576), anti-HSP70 (ab5439), and anti-Myd88 (ab119048). Duolink experiments were performed according

to the manufacturer's protocol (Sigma-Aldrich, St Louis, MO). Microscopy images were taken on an Axio Imager 2 (Carl Zeiss Microscopy GmbH, Jena, Germany), and images were acquired by using an AxioCam MRm CCD camera (Carl Zeiss). The Spot detector plugin of Icy software was used to count the number of spots per cell. Statistical analysis was performed with GraphPad Prism 6 (unpaired Mann-Whitney *U* test; at least 100 cells counted for each condition).

Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR for reactive lymph nodes and patients was performed using the TaqMan Universal Master Mix and specific TaqMan Gene Expression Assays (Life Technologies-Applied Biosystems): HSP110/hs-00971475-m1 and ABL/hs001104728-m1 (for internal standard gene). Statistical analyses were performed with Prism software (GraphPad Software Inc., San Diego, CA) using a Mann-Whitney nonparametric *U* test. For other RT-PCRs, PrimePCR SYBR Green Assay (RPS18, Human [Biorad]), MyD88 F (GTTGTCTCTGATGAT TACCTG), MyD88 R (GGGGAACTCTTTCTTCATTG; Sigma-Aldrich), and the HSP110 primers used were described earlier.¹⁷

Results

HSP110 expression has an impact on B-cell lymphoma cell survival and NF-κB signaling

To explore the role of HSP110 in ABC-DLBCL cell lines, we transduced them with a lentivirus carrying HSP110 shRNA that was optimized to have high transduction capacities toward hard-to-transfect human B-cell lines.^{24,28} We observed a decreased survival in the HSP110 shRNA-infected cell lines we tested: mean percentage of viable cells in shRNA HSP110 vs shRNA control at day 9 for HBL1 was 30 \pm 4 and 83 \pm 1.5; for OCI-Ly3, 41 \pm 13 and 83 \pm 1.5; and for TMD8, 32 \pm 0.7 and 80 \pm 1.5; but no

From www.bloodjournal.org at INSERM DISC on August 9, 2019. For personal use only.



Figure 2. HSP110 expression has an impact on NF-kB signaling. (A) Immunoblot analysis of HSP110, p-IkB α , p-p65, IkB α , and p65 at 48 hours after transfection with an siRNA targeting HSP110 or a control siRNA. β -actin served as a loading control. (B) p-IkB band intensity from immunoblots shown in (A) and determined from all cell lines (except U2932) relative to the loading control, data are presented as the mean \pm standard deviation (n = 5; not U2932; P < .01). (C) Immunoblot analysis of HSP110, p65, and p50 content in the nucleus and cytosol of OCI-Ly3 and SU-DHL2 48 hours after transfection with an siRNA targeting HSP110 or a control siRNA. β -actin served as a loading control (n = 3). (D) Immunoblot analysis of HSP110, p-IkB, and IkB in SU-DHL2 cells 48 hours after transfection with plasmids coding for HSP110-GFP (n = 3). HSC70 served as a loading control. (E) Kinetics of cell survival determined by annexin-V/7AAD staining on HBL1 stably expressing either shRNA HSP110, shRNA control plus IKK2-EE, or shRNA HSP110 plus IKK2-EE, data are presented as the mean \pm standard deviation (n = 3). **P < .01; ***P < .001.

decrease for U2932 cells, 9.72 \pm 16 and 76 \pm 4 (Figure 1A-B). A decrease in the antiapoptotic proteins Bcl-xL and Bcl2, together with an increase in caspase-3 cleavage, suggested that depletion of HSP110 is deleterious in certain ABC-DLBCL cell lines and induced apoptotic cell death (Figure 1C). Because hyperactivation of the NF-KB signaling pathway is primarily responsible for ABC-DLBCL proliferation and survival, we studied the impact of HSP110 on this pathway in the following experiments. Interestingly, siRNA-mediated HSP110 knockdown induced a decrease in IkB and p65 phosphorylation in all ABC-DLBCL cell lines tested, with the exception of U2932 (Figure 2A-B). The silencing of HSP110 did not modulate AKT activation, another important signaling pathway in ABC-DLBCL (supplemental Figure 1A, available on the Blood Web site). Next, we compared the effect observed with HSP110 depletion with that observed by depleting or inhibiting other main HSPs (HSP27, HSP70, and HSP90). We used either an siRNA approach (for HSP27, HSP70, and HSP110 [specificity of HSP110 siRNA is shown in supplemental Figure 1A]) or a chemical inhibitor (PU-H71 for HSP90). As shown in supplemental Figure 1B, inhibition of HSP90, as already reported,³ induced a decrease in IkB phosphorylation similar to that obtained upon HSP110 depletion. In contrast, HSP27 or HSP70 knockdown did not affect p-IkB content. In keeping with the decrease in IkB phosphorvlation induced by HSP110 knockdown, the nuclear localization of p65 and p50 were also decreased in cells depleted in HSP110 (Figure 2C; supplemental Figure 1C). Conversely, transfection of SU-DHL2 by increasing amounts of HSP110 induced an increase in IkB phosphorylation (Figure 2D). Finally, HBL1 cells were rescued from HSP110 shRNA-induced cell death by co-expression of the

constitutively active IKK2-EE (Figure 2E). Taken together, these data suggest that HSP110 is involved in ABC-DLBCL survival through NF- κ B signaling modulation.

Recently, Louis M. Staudt pointed out that BCR components colocalized with MyD88, TLR9, and CARD11-BCL10-MALT1 (CBM) complex in endolysosomes, where they activate the NF-κB pathway (Louis M. Staudt, oral communication, American Association for Cancer Research Annual Meeting, 3 April 2017). To obtain more insights on the stage of the NF-KB pathway at which HSP110 interferes, we looked at the IgM-MyD88 complex formation when HSP110 is downregulated. By using PLA, we detected close proximity of IgM and p-IkB in the cytosol of ABC-DLBCL control cells (Figure 3A-B). Upon siRNA-mediated HSP110 down-expression, IgM and p-I κB proximity was strongly reduced in the cell lines bearing dual MyD88 and CBM mutations (HBL1 and OCI-Ly3), but not in the U2932 cell line, which is devoid of these mutations. This suggested that HSP110 could play a role in the IgM-MyD88 complex. We sought to confirm this hypothesis by studying the effect of HSP110 depletion in the IRAK1-TRAF6 complex formation immediately downstream of IgM and MyD88. Upon HSP110 downregulation, we found that IRAK1 was less phosphorylated in HBL1 and OCI-Ly3 (but not in U2932) (Figure 3C), and p-IRAK1 was less bound to TRAF6 (supplemental Figure 1D). Under normal IRAK1-TRAF6 complex formation, the auto-K63 ubiquitination of TRAF6 seems to enable the recruitment and activation of downstream signaling.²⁹ Here, this TRAF6 K63 ubiguitination was also decreased (Figure 3D). These data suggest that HSP110 could intervene upstream of IRAK1-TRAF6 complex formation at an



Figure 3. HSP110 intervenes upstream of the IRAK1-TRAF6 complex formation. (A) In cellulo interaction of IgM with $p-I\kappa$ B α was determined in HBL1, OCI-Ly3, and U2932 by Duolink technology after transfection with an siRNA targeting HSP110 or a control siRNA (n = 3; 1 representative image is shown). Scale bar represents 20 μ m. (B) Quantitation of IgM- μ kB α spots per cell determined by Duolink technology as in (A). (C) Immunoblot analysis of HSP110, $p-I\kappa$ B α that HRL1, OCI-Ly3, and U2932 cells at 48 hours after transfection with an siRNA targeting HSP110 or a control siRNA. (h = 3; 1 representative image is shown). Scale bar represents 20 μ m. (B) Quantitation of IgM- μ kB α spots per cell determined by Duolink technology as in (A). (C) Immunoblot analysis of HSP110, $p-I\kappa$ B α (X) and U2932 cells at 48 hours after transfection with an siRNA targeting HSP110 or a control siRNA. (h = 3; 1 representative image is shown). Scale bar represents 20 μ m. (B) Quantitation of IgM- μ kB α spots per cell determined by Duolink technology as in (A). (C) Immunoblot analysis of HSP110, $p-I\kappa$ B α (X) and U2932 cells at 48 hours after transfection with an siRNA targeting HSP110 or a control siRNA. (h = 3; 1 representative image is shown). Scale bar representation of Lysine 63-linked polyubiquitin chain (K63-ub) in SU-DHL2 and OCI-Ly10 cells 48 hours after transfection with an siRNA targeting HSP110 or a control siRNA, followed by immunoblot using a TRAF6 antibody. Immunoblot of total cell extract (input) shows HSP110 and TRAF6 analysis (n = 3). ***P < .001.

early stage of the NF- κB signaling pathway when IgM and MyD88 co-localize.

HSP110 enhances MyD88-mediated NF-ĸB signaling through direct interactions

MyD88 activation upon BCR-TLR complex stimulation of B cells leads to NF- κ B signaling and subsequent proliferation (Louis M. Staudt, oral communication, American Association for Cancer Research annual meeting, 3 April 2017). Furthermore, oncogenically active MyD88 in 30% of patients with ABC-DLBCL accounts for spontaneous assembling of the IRAK1-IRAK4 complex, leading to a strong and sustained NF-κB signal. Therefore, we hypothesized that HSP110 could enhance MyD88-mediated NF-кВ signaling. We overexpressed HSP110 together with WT MyD88 or with the most common mutated form of MyD88 (MyD88 L265P) in a Burkitt lymphoma cell line (BJAB), that in contrast to ABC-DLBCL cells, is easy to transfect and does not bear either the MyD88 mutation or NF-ĸB hyperactivation. As shown in Figure 4A and supplemental Figure 1E, HSP110, MyD88, and MyD88 L265P alone enhanced IkB phosphorylation. As expected, MyD88 L265P promoted a stronger phosphorylation,²³ which was enhanced by HSP110 overexpression.

To functionally test whether HSP110 was able to promote NF- κ B target gene transcription in an MyD88-dependent manner, we used a luciferase reporter gene driven by an NF- κ B promoter. HEK293 cells were co-transfected with increasing amounts of HSP110, with or without MyD88 WT or MyD88 L265P. We observed an HSP110 dose-dependent induction of luciferase activity (Figure 4B). Strikingly, whereas MyD88 L265P alone was able to promote this activity, the addition of HSP110 induced a 100-fold increase at the maximal concentration (Figure 4B). Overall, these data show that HSP110 enhances both MyD88 WT and mutated signaling. This suggests that HSP110 may interact directly with MyD88 to reinforce its action.

As shown in Figure 4C-D, HSP110 and MyD88 co-localized in ABC-DLBCL cells and associated in cellulo. Of note, in all 3 tested cell lines, HSP110-MyD88 binding spots were more abundant than HSP110-HSP70 binding spots, used here as a positive control because HSP70 is a well-known partner of HSP110. No close proximity of HSP70 and MyD88 was observed (data not shown). This interaction was confirmed by co-immunoprecipitation experiments in OCI-Ly3 and SU-DHL2 (Figure 4E). Because both cell lines expressed a mutated form of MyD88, we wondered whether HSP110 could also bind to the WT form. Co-immunoprecipitation experiments with HSP110 and MyD88 WT in HEK293 cells showed similar binding capacities (Figure 4F).



Figure 4. HSP110 interacts with MyD88 to enhance NF-кB signaling. (A) Immunoblot analysis of p-IkB, IkB, HSP110, and MyD88 in Burkitt lymphoma (BJAB) cells 48 hours after transfection with plasmids coding for HSP110-GFP, and/or MyD88 WT, or MyD88 L265P. HSC70 served as a loading control (n = 3). (B) Luciferase reporter experiment in HEK293T cells. Cells co-transfected with GFP reporter (internal control) and NF-kB luciferase reporter plasmid with or without increasing concentrations of untagged HSP110 and with or without 0.4 μ g of Myd88 L265P or Myd88 WT plasmids (n = 3). Shown are relative luciferase values (luciferase:GFP ratio). Data are presented as the mean \pm standard deviation. (C) In cellulo interaction of HSP110 with MyD88 was determined in HBL1, OCI-Ly3, and U2932 by Duolink technology in the presence or not of siRNA HSP110. HSP70 and HSP110-MyD88 spots per cell determined by Duolink technology as in (A). Boxplots depict the mean (line), the 25-75 percentiles (box) and the 10-90 percentiles (whiskers). (E) Immunoprecipitation (IP) of HSP110 and MyD88 in SU-DHL2 and OCI-Ly3 cells followed by immunoblot using an anti-MyD88 and anti-HSP110 antibody. A nonrelevant antibody was used as a control (IP control) (n = 3). (F) Immunoprecipitation of HSP110-GFP (IP-GFP) and MyD88-HA (IP-HA) in HEK293 cells 48 hours after transfection with plasmids coding for HSP110-GFP with or without MyD88-HA L265P (0.7 μ g) or MyD88-HA WT (0.7 μ g) (n = 3). Endo, endogenous. ***P* < .01; ****P* < .001.

HSP110 stabilizes MyD88

Expression of increasing amounts of HSP110 in HEK293 cells induced a concomitant increase in the amount of MyD88 protein (Figure 5A-B; supplemental Figure 2A) for both L265P and WT MyD88. This phenomenon was not the result of transcriptional control of the *MyD88* gene because MyD88 mRNA content did not increase (supplemental Figure 2B). Furthermore, expression of the HSP110 dominant negative mutant HSP110DE9 (known to inhibit HSP110 WT at a 1:1 ratio¹⁷) inhibited the expression of MyD88 (Figure 5A-B). Expression of an HSP110



Figure 5. HSP110 stabilizes MyD88. Immunoblot analysis of HSP110-GFP, total HSP110, (A) MyD88 L265P, or (B) MyD88 WT 48 hours after transfection with plasmids coding for MyD88 L265P (0.7 μg) together with control GFP, HSP110 DE9GFP, or increasing concentrations of HSP110-GFP in HEK293 cells (n = 3). HSC70 served as a loading control. (C) Immunoblot analysis of HSP110-GFP and MyD88 L265P at 48 hours after transfection with plasmids coding for MyD88 L265P (0.5 μg) together with control GFP, increasing concentrations of HSP110-GFP in HEK293 cells (n = 3). HSC70 served as a loading control. (C) Immunoblot analysis of HSP110-GFP and MyD88 L265P at 48 hours after transfection with plasmids coding for MyD88 L265P (0.5 μg) together with control GFP, increasing concentrations of the cytoplasmic-only mutant of HSP110 (HSP110 deficient in the nuclear localization signal [HSP110-NLS]) in HEK293 cells (n = 3). (D) Immunoblot analysis of HSP110-GFP, total HSP110, MyD88 L265P, or MyD88 WT in HEK293 cells treated with cycloheximide (CHX; 100 μg/mL) during the indicated times, 24 hours after transfection with control-GFP or HSP110-GFP plasmids. HSC70 served as a loading control. Lower panel shows the MyD88 immunoblot intensity from the above experiment. (E) Immunoblot analysis of HSP110 and MyD88 in HBL1, OCI-Ly3, and U2932 60 hours after transfection by a universal control siRNA or with an siRNA that targets HSP110 (n = 3). conc. concentration. (F) Immunoblot analysis of total HSP110 and MyD88 in HBL1 transfected as in (E) and treated or not with MG132 (10 μM) during the last 3 or 5 hours of the culture (n = 3).

mutant deficient in its nuclear localization signal also increased MyD88 protein expression, suggesting that HSP110 acts directly on MyD88 in the cytosol (Figure 5C). The study of MyD88 half-life in the presence of the protein synthesis inhibitor cycloheximide showed that HSP110 stabilizes MyD88 WT and MyD88 L265P in a similar way (Figure 5D). These observations were confirmed in HBL1 and OCI-Ly3 because HSP110 down expression by specific siRNA reduced the expression of MyD88 but not in U2932, which is in keeping with the previous results (Figure 5E). Finally, in the presence of the proteasome inhibitor MG132, the MyD88 level was restored, suggesting that HSP110 stabilized MyD88 by interfering with its proteasomal degradation (Figure 5F).

HSP110 is highly expressed in ABC-DLBCL patients and correlates with the amount of MyD88

Strong HSP110 expression has been found in samples from patients with different cancers, including those with GC-DLBCL. To get clinical insight on the role of HSP110 in ABC-DLBCL, we measured HSP110 mRNA levels in purified B cells from a cohort of patients with GC-DLBCL (n = 6) (as a positive control for HSP110), a cohort of patients with ABC-DLBCL (n = 9), and samples from nonmalignant reactive lymph nodes (n = 4). As for the GC-DLBCL samples, we observed stronger although heterogeneous HSP110 mRNA expression in ABC-DLBCL in comparison with reactive lymph node samples (a 21-fold increase; P = .009) (supplemental Figure 3A). This elevated HSP110 expression was then confirmed by IHC in biopsies from 35 patients with the ABC subtype (Figure 6A-B), as well as in biopsies from 7 patients with the GC subtype (provided for comparison) (supplemental Figure 3B). In all cases, more than 80% of cells were positive, with a score 3 to 4 for the staining intensity of cells from 57% of patients with the ABC subtype. Interestingly, although HSP110 was always present in the cytosol, frequent nuclear localization was also observed. HSP110 staining in reactive lymph nodes (n = 5) was limited to the GC, confirming its association with B-cell activation. We confirmed the HSP110 and MyD88 association in 2 ABC-DLBCL biopsies cells by PLA (Figure 6C). MyD88 expression was present in more than 80% of cells from this cohort with 3 levels of intensity (supplemental Figure 3C). Strong MyD88 expression was more frequent for biopsies with high HSP110 intensity (a score >2; 37%) compared with biopsies with low HSP110 intensity (score <2; 15%), but it was not statistically significant (supplemental Figure 3D). To get a more quantitative insight into MyD88-HSP110 expression, we used another cohort of patients with ABC-DLBCL; we were able to extract proteins from their tumor biopsies and we observed that the level of HSP110 correlated significantly with that of MyD88 (${\it R}^2$ = 0.7577 [n = 13]) (Figure 6D-E) and with that of p-I κ B α ($R^2 = 0.5781$ [n = 9]; supplemental Figure 3E). These patient data support our results showing that HSP110 plays a role in MyD88 protein expression in ABC-DLBCL.

Discussion

Genomic and functional investigations have shown that ABC-DLBCL tumors are driven by either chronic BCR and/or TLR9-MyD88 signaling in contrast to GC-DLBCL. Many downstream effectors are therefore activated, leading to sustained NF- κ B activation, which ABC-DLBCL tumors rely on for survival. There is important genetic heterogeneity in ABC-DLBCL tumors that accounts for this chronic BCR and MyD88 signaling, because

mutations in CD79a/b, MyD88, CARD11, and A20 have been detected in patients' samples. In particular, 29% of tumors harbor a single nucleotide variant that changes a leucine residue at position 265 of the MyD88 coding region to a proline, leading to a mutant protein (MyD88 L265P) that spontaneously assembles with IRAK1 and IRAK4 into so-called myddosomes.²³ We showed here that HSP110 directly binds to MyD88, independent of L265P mutation, and stabilizes both protein forms leading to the amplification of NF-KB signaling and to subsequent improved survival in ABC-DLBCL lines. We also observed a correlation between HSP110 and MyD88 levels in patients, thus confirming our in vitro results and suggesting that HSP110 could be a potential target for lymphoma therapy in the future. This effect seems specific for HSP110 because HSP27 and HSP70 do not alter NF-κB signaling. In contrast, HSP90 inhibition alters the BCR-dependent NF-KB pathway because HSP90 is a key component of the BCR signalosome.³ The involvement of HSP90 in MyD88 signaling, alone or in association with HSP110, deserves further investigation.

As an adaptor of Toll-like receptor signaling, MyD88 is regulated by endogenous mechanisms as well as pathogens. In macrophages, the E3 ubiquitin ligase Nrdp1 directly binds and polyubiquitinates MyD88, leading to its proteasomal degradation, thus limiting the overproduction of proinflammatory cytokines.³⁰ In the same way, TGF-β1 drives MyD88 degradation through the Smad6-Smurf pathway.³¹ Pathogens have many ways to escape immune surveillance. For example, they can drive MyD88 through the Ubproteasome pathway as shown for Kaposi's sarcoma–associated herpesvirus.³² Because an adequate inflammatory response to pathogens must be balanced, molecular machinery that opposes MyD88 degradation must exist, and our work suggests that HSP110 could be involved because it promotes MyD88 stability.

HSPs have multiple roles in cancer cells that relate to their chaperone function; among these, stabilization of mutated or overexpressed proteins is common. In addition, proliferation- or survival-dependent activating mutations of signaling molecules show increased dependence on HSPs.^{3,33,34} In GC BCL/BCL6+ DLBCL, HSP90 interacts with the oncogene BC-6 and stabilizes BCL6 mRNA and protein.³⁴ A significant positive correlation between HSP110 and BCL6 expression has also been found in GC-DLBCL cell lines and in patients with GC-DLBCL.¹⁸ In addition, in Burkitt lymphoma, HSP110 prevents degradation of the overexpressed oncogene c-Myc.¹⁹ Therefore, through their multiple oncogene targets in lymphomas, HSPs, and particularly HSP110, seem to be crucial to their proliferation and survival, thus confirming the overall addiction of tumors to HSPs. HSP110 plays an important role in GC-DLBCL, ABC-DLBCL, and Burkitt lymphoma, in which it acts on different oncogenes frequently associated with these particular diseases.

Beyond ABC-DLBCL, the missense mutation of MyD88 has been described in other diseases such as benign monoclonal IgM gammopathy of undetermined significance (47%), Waldenström macroglobulinemia (in almost 100% of patients), and chronic lymphocytic leukemia (less than 10%).^{35,36} It is very likely that HSP110 could also have the same MyD88 stabilizing function in these diseases, although this hypothesis needs to be investigated.

Tumor microenvironment may influence DLBCL progression because enriched expression of T-cell and natural killer cell



Figure 6. High expression of HSP110 correlates with MyD88 expression in patients. (A) Representative images of reactive lymph nodes (n = 5) and ABC-DLBCL (n = 35) patients' lymph node sections stained with HSP110 antibody by IHC. The 2 original magnifications are ×2.8 for A and C (scale bar represents 425 µm) and ×40 for B and D (scale bar represents 30 µm). Representative images of 4 HSP110 intensities are shown beside sections from patients with ABC-DLBCL (original magnification ×20; scale bar represents 60 µm). (B) Schematic representation of HSP110 IHC intensity in the cohort of patients analyzed (n = number of patients with each intensity). (C) Representative image of in cellulo interaction of HSP110 with MyD88 (determined by Duolink technology) in lymph node sections from 2 patients (scale bar represents 40 µm (upper panel] or 20 µm (lower panel]). (D) Immunoblet analysis of HSP110 and MyD88 in biopsies from 13 patients with ABC-DLBCL. HSC70 served as a loading control. (E) HSP110 immunoblot intensity relative to MyD88 from (C).

activation molecules and CD4⁺ T-cell tumor infiltration are associated with good prognosis.³⁷⁻³⁹ The ability of extracellular recombinant HSP110 to improve antigen presentation and T-cell responses has been described.⁴⁰⁻⁴³ But the question remains of whether the cell-intrinsic antitumor effects of HSP110 targeting will be offset by reductions in T-cell responses.

Recent reports suggest that p-I κ B and/or MyD88 expression can be used as prognosis factors.^{44,45} Given that HSP110 expression

shows a good positive correlation with both markers, we can hypothesize that HSP110 is associated with more aggressive clinical behavior.

HSP110 protein level was found to be low in normal nonactivated B cells outside the GC (Figure 6A), and the currently available HSP110 knockout model shows no alteration of B-cell number in homeostatic conditions (not shown). This information could be of particular interest for the rationale of HSP110

From www.bloodjournal.org at INSERM DISC on August 9, 2019. For personal use only.

inhibition in DLBCL because the current R-CHOP-based regimen targets normal and pathologic B cells. In contrast, HSP110 inhibition, because of its overexpression in NHL may selectively target lymphoma cells but not naive normal B cells. If new therapies use lymphoma oncogenic cell signaling to selectively kill DLBCL cells in preference to normal B cells, HSP110 inhibition could represent a promising new approach. Of course future therapies will need to combine strategies (BTK inhibitors) to completely eradicate tumor cells because some remaining cells persist despite HSP110 down expression. Once available, HSP110-specific inhibitors will probably play a preponderant role in the therapeutic arsenal for ABC-DLBCL and beyond.

Acknowledgments

The authors thank the fonds européen de développement régional (FEDER) for their support; the Plateforme de Cytométrie Dijon and Cellimap Dijon for technical support; Gisèle Froment, Didier Nègre, and Caroline Costa from the lentivectors production facility SFR BioSciences Lyon (UMS3444/US8); and Thierry Defrance (INSERM U1111, Lyon, France) and Karen Leroy (Paris Descartes University, Paris, France) for helpful discussions and support.

This work was supported by grants from the Ligue Nationale Contre le Cancer (G.J. and C.G.), the Conseil Regional de Bourgogne, the French National Research Agency under the Investissements d'Avenir Program (ANR-11-LABX-0021-01-LipSTIC Labex), the German Research Foundation CRC685 Immunotherapy (A.W. and O.-O.W.), and the Juniorprofessoren-Programm of the Federal State of Baden-Württemberg, Germany (A.W. and O.-O.W.).

REFERENCES

- Li S, Young KH, Medeiros LJ. Diffuse large B-cell lymphoma. *Pathology*. 2018;50(1): 74-87.
- Schmitz R, Wright GW, Huang DW, et al. Genetics and pathogenesis of diffuse large B-cell lymphoma. N Engl J Med. 2018;378(15): 1396-1407.
- Goldstein RL, Yang SN, Taldone T, et al. Pharmacoproteomics identifies combinatorial therapy targets for diffuse large B cell lymphoma. J Clin Invest. 2015;125(12): 4559-4571.
- Marullo R, Rutherford SC, Leonard JP, Cerchietti L. Therapeutic implication of concomitant chromosomal aberrations in patients with aggressive B-cell lymphomas. *Cell Cycle*. 2016;15(17):2241-2247.
- Whitesell L, Santagata S, Mendillo ML, Lin NU, Proia DA, Lindquist S. HSP90 empowers evolution of resistance to hormonal therapy in human breast cancer models. Proc Natl Acad Sci USA. 2014;111(51):18297-18302.
- Saibil H. Chaperone machines for protein folding, unfolding and disaggregation. Nat Rev Mol Cell Biol. 2013;14(10):630-642.
- Watson IR, Takahashi K, Futreal PA, Chin L. Emerging patterns of somatic mutations in cancer. Nat Rev Genet. 2013;14(10):703-718.
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5): 646-674.
- Jego G, Hazoumé A, Seigneuric R, Garrido C. Targeting heat shock proteins in cancer. Cancer Lett. 2013;332(2):275-285.

- Gobbo J, Marcion G, Cordonnier M, et al. Restoring anticancer immune response by targeting tumor-derived exosomes with a HSP70 peptide aptamer. J Natl Cancer Inst. 2015;108(3).
- Boudesco C, Rattier T, Garrido C, Jego G. Do not stress, just differentiate: role of stress proteins in hematopoiesis. *Cell Death Dis.* 2015;6:e1628.
- Zuo D, Subjeck J, Wang XY. Unfolding the role of large heat shock proteins: New insights and therapeutic implications. *Front Immunol.* 2016;7:75.
- Yamagishi N, Fujii H, Saito Y, Hatayama T. Hsp105beta upregulates hsp70 gene expression through signal transducer and activator of transcription-3. *FEBS J*. 2009;276(20): 5870-5880.
- Berthenet K, Bokhari A, Lagrange A, et al. HSP110 promotes colorectal cancer growth through STAT3 activation. *Oncogene*. 2017; 36(16):2328-2336.
- Berthenet K, Boudesco C, Collura A, et al. Extracellular HSP110 skews macrophage polarization in colorectal cancer. Oncoimmunology. 2016;5(7):e1170264.
- Dorard C, de Thonel A, Collura A, et al. Expression of a mutant HSP110 sensitizes colorectal cancer cells to chemotherapy and improves disease prognosis. *Nat Med.* 2011; 17(10):1283-1289.
- 17. Collura A, Lagrange A, Svrcek M, et al. Patients with colorectal tumors with microsatellite instability and large deletions in HSP110 T17 have improved response to 5-fluorourracil-based chemotherapy. *Gastroenterology*. 2014;146(2):401-11.e1.

Authorship

Contribution: C.B., E.V., L.M., C.C.-C., T.F., A.N.R.W., and G.J. designed the study and experiments; C.B., E.V., L.S., R.M., and O.-O.W. performed the experiments; E.V., L.M., C.C.-C., C.P., T.F., S.R., F.J., and A.N.R.W. provided samples and expertise; C.B., E.V., L.M., C.C.-C., T.F., F.J., A.N.R.W., C.G., and G.J. analyzed the experiments; and G.J. directed the work.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profile: G.J., 0000-0003-0376-9299.

Correspondence: Gaetan Jego, INSERM, UMR 1231, Université Bourgogne Franche-Comté, 7, Boulevard Jeanne d'Arc, 21079 Dijon, France; e-mail: gaetan.jego@u-bourgogne.fr.

Footnotes

Submitted 1 December 2017; accepted 30 May 2018. Prepublished online as *Blood* First Edition paper, 5 June 2018; DOI 10.1182/blood-2017-12-819706.

The online version of this article contains a data supplement.

There is a Blood Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

- Zappasodi R, Bongarzone I, Ghedini GC, et al. Serological identification of HSP105 as a novel non-Hodgkin lymphoma therapeutic target. *Blood*. 2011;118(16):4421-4430.
- Zappasodi R, Ruggiero G, Guarnotta C, et al. HSPH1 inhibition downregulates Bcl-6 and c-Myc and hampers the growth of human aggressive B-cell non-Hodgkin lymphoma. Blood. 2015;125(11):1768-1771.
- Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J Exp Med. 2001;194(12):1861-1874.
- Ngo VN, Young RM, Schmitz R, et al. Oncogenically active MYD88 mutations in human lymphoma. *Nature*. 2011;470(7332): 115-119.
- Davis RE, Ngo VN, Lenz G, et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. *Nature*. 2010; 463(7277):88-92.
- Avbelj M, Wolz OO, Fekonja O, et al. Activation of lymphoma-associated MyD88 mutations via allostery-induced TIR-domain oligomerization. *Blood.* 2014;124(26): 3896-3904.
- Frecha C, Costa C, Lévy C, et al. Efficient and stable transduction of resting B lymphocytes and primary chronic lymphocyte leukemia cells using measles virus gp displaying lentiviral vectors. *Blood*. 2009;114(15):3173-3180.
- 25. Frecha C, Lévy C, Costa C, et al. Measles virus glycoprotein-pseudotyped lentiviral vectormediated gene transfer into quiescent lymphocytes requires binding to both SLAM and

From www.bloodjournal.org at INSERM DISC on August 9, 2019. For personal use only.

CD46 entry receptors. J Virol. 2011;85(12): 5975-5985.

- Girard-Gagnepain A, Amirache F, Costa C, et al. Baboon envelope pseudotyped LVs outperform VSV-G-LVs for gene transfer into early-cytokine-stimulated and resting HSCs. *Blood.* 2014;124(8):1221-1231.
- Bourteele S, Oesterle K, Pleschka S, et al. Constitutive activation of the transcription factor NF-kappaB results in impaired borna disease virus replication. J Virol. 2005;79(10): 6043-6051.
- Lévy C, Amirache F, Girard-Gagnepain A, et al. Measles virus envelope pseudotyped lentiviral vectors transduce quiescent human HSCs at an efficiency without precedent. *Blood Adv.* 2017;1(23):2088-2104.
- Windheim M, Stafford M, Peggie M, Cohen P. Interleukin-1 (IL-1) induces the Lys63-linked polyubiquitination of IL-1 receptor-associated kinase 1 to facilitate NEMO binding and the activation of IkappaBalpha kinase. *Mol Cell Biol.* 2008;28(5):1783-1791.
- Wang C, Chen T, Zhang J, et al. The E3 ubiquitin ligase Nrdp1 'preferentially' promotes TLR-mediated production of type I interferon. Nat Immunol. 2009;10(7):744-752.
- Lee YS, Park JS, Kim JH, et al. Smad6-specific recruitment of Smurf E3 ligases mediates TGF-β1-induced degradation of MyD88 in TLR4 signalling. Nat Commun. 2011;2(1):460.
- 32. Zhao Q, Liang D, Sun R, et al. Kaposi's sarcoma-associated herpesvirus-encoded replication and transcription activator impairs innate immunity via ubiquitin-mediated

degradation of myeloid differentiation factor 88. *J Virol.* 2015;89(1):415-427.

- Moulick K, Ahn JH, Zong H, et al. Affinitybased proteomics reveal cancer-specific networks coordinated by Hsp90. Nat Chem Biol. 2011;7(11):818-826.
- 34. Cerchietti LC, Lopes EC, Yang SN, et al. A purine scaffold Hsp90 inhibitor destabilizes BCL-6 and has specific antitumor activity in BCL-6-dependent B cell lymphomas. Nat Med. 2009;15(12):1369-1376.
- Puente XS, Pinyol M, Quesada V, et al. Wholegenome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature*. 2011;475(7354):101-105.
- 36. Xu L, Hunter ZR, Yang G, et al. MYD88 L265P in Waldenström macroglobulinemia, immunoglobulin M monoclonal gammopathy, and other B-cell lymphoproliferative disorders using conventional and quantitative allelespecific polymerase chain reaction. *Blood*. 2013;121(11):2051-2058.
- Monti S, Savage KJ, Kutok JL, et al. Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response. *Blood.* 2005;105(5):1851-1861.
- Chang KC, Huang GC, Jones D, Lin YH. Distribution patterns of dendritic cells and T cells in diffuse large B-cell lymphomas correlate with prognoses. *Clin Cancer Res.* 2007;13(22 Pt 1):6666-6672.
- Galand C, Donnou S, Molina TJ, Fridman WH, Fisson S, Sautès-Fridman C. Influence of tumor location on the composition of immune

infiltrate and its impact on patient survival. Lessons from DCBCL and animal models. *Front Immunol.* 2012;3:98.

- Wang XY, Kazim L, Repasky EA, Subjeck JR. Characterization of heat shock protein 110 and glucose-regulated protein 170 as cancer vaccines and the effect of fever-range hyperthermia on vaccine activity. J Immunol. 2001;166(1):490-497.
- Hu T, Li D, Zhao Y. Development of the hsp110heparanase vaccine to enhance antitumor immunity using the chaperoning properties of hsp110. *Mol Immunol.* 2009;47(2-3): 298-301.
- Manjili MH, Wang XY, Chen X, et al. HSP110-HER2/neu chaperone complex vaccine induces protective immunity against spontaneous mammary tumors in HER-2/neu transgenic mice. J Immunol. 2003;171(8):4054-4061.
- Wang XY, Li Y, Manjili MH, Repasky EA, Pardoll DM, Subjeck JR. Hsp110 overexpression increases the immunogenicity of the murine CT26 colon tumor. Cancer Immunol Immunother. 2002;51(6):311-319.
- 44. Hussain AR, Uddin S, Ahmed M, Al-Dayel F, Bavi PP, Al-Kuraya KS. Phosphorylated $I\kappa B\alpha$ predicts poor prognosis in activated B-cell lymphoma and its inhibition with thymoquinone induces apoptosis via ROS release. *PLoS One.* 2013;8(3):e60540.
- Lee JH, Jeong H, Choi JW, Oh H, Kim YS. Clinicopathologic significance of MYD88 L265P mutation in diffuse large B-cell lymphoma: a meta-analysis. *Sci Rep.* 2017;7(1):1785.



2018 132: 510-520 doi:10.1182/blood-2017-12-819706 originally published online June 5, 2018

HSP110 sustains chronic NF-κB signaling in activated B-cell diffuse large B-cell lymphoma through MyD88 stabilization

Christophe Boudesco, Els Verhoeyen, Laurent Martin, Catherine Chassagne-Clement, Leila Salmi, Rana Mhaidly, Céline Pangault, Thierry Fest, Selim Ramla, Fabrice Jardin, Olaf-Oliver Wolz, Alexander N. R. Weber, Carmen Garrido and Gaetan Jego

Updated information and services can be found at: http://www.bloodjournal.org/content/132/5/510.full.html

Articles on similar topics can be found in the following Blood collections Lymphoid Neoplasia (3107 articles)

Information about reproducing this article in parts or in its entirety may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at: http://www.bloodjournal.org/site/subscriptions/index.xhtml

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036. Copyright 2011 by The American Society of Hematology; all rights reserved.
Article 6

Baboon envelope LVs efficiently transduced human adult, fetal, and progenitor T cells and corrected SCID-X1 T-cell deficiency

Ornellie Bernadin,^{1,*} Fouzia Amirache,^{1,*} Anais Girard-Gagnepain,^{1,*} Ranjita Devi Moirangthem,^{2,3,*} Camille Lévy,¹ Kuiying Ma,^{2,3} Caroline Costa,¹ Didier Nègre,¹ Christian Reimann,²⁻⁴ David Fenard,⁵ Agata Cieslak,^{6,7} Vahid Asnafi,^{6,7} Hanem Sadek,⁸ Rana Mhaidly,⁹ Marina Cavazzana,^{2,3,8} Chantal Lagresle-Peyrou,⁸ François-Loïc Cosset,^{1,†} Isabelle André,^{2,3,†} and Els Verhoeyen^{1,9,†}

¹Centre International de Recherche en Infectiologie, Université Lyon, Université Claude Bernard Lyon 1, INSERM, U1111, Centre National de la Recherche Scientifique, Unité Mixte de Recherche (UMR) 5308, Ecole Normale Supérieure de Lyon, Lyon, France; ²Laboratory of Human Lymphohematopoiesis, INSERM UMR 1163, and ³Paris Descartes University–Sorbonne Paris Cité, Imagine Institute, Paris, France; ⁴Department of Pediatric Oncology, Lucerne Cantonal Hospital, Lucerne, Switzerland; ⁵Integrare Research Unit, UMR_S951, Généthon, INSERM, University of Evry, Ecole Pratique des Hautes Études, Evry, France; ⁶Université Paris Descartes Sorbonne Cité, Institut Necker-Enfants Malades, INSERM U1151, Paris, France; ⁷Laboratory of Onco-Hematology, Assistance Publique–Hôpitaux de Paris (AP-HP), Hôpital Necker-Enfants Malades, Paris, France; ⁸Biotherapy Clinical Investigation Center, Groupe Hospitalier Universitaire Ouest, AP-HP, INSERM Centres d'Investigation Clinique 1416, Paris, France; and ⁹Université Côte d'Azur, INSERM, Centre Méditerranéen de Médecine Moléculaire, Nice, France

Key Points

- BaEV-LVs efficiently transduce progenitor T cells, providing accelerated T-cell reconstitution in vivo.
- BaEV-LVs efficiently correct human SCID-X1 progenitor T cells.

T cells represent a valuable tool for treating cancers and infectious and inherited diseases; however, they are mainly short-lived in vivo. T-cell therapies would strongly benefit from gene transfer into long-lived persisting naive T cells or T-cell progenitors. Here we demonstrate that baboon envelope glycoprotein pseudotyped lentiviral vectors (BaEV-LVs) far outperformed other LV pseudotypes for transduction of naive adult and fetal interleukin-7-stimulated T cells. Remarkably, BaEV-LVs efficiently transduced thymocytes and T-cell progenitors generated by culture of CD34⁺ cells on Delta-like ligand 4 (Dll4). Upon NOD/SCID γ C^{-/-} engraftment, high transduction levels (80%-90%) were maintained in all T-cell subpopulations. Moreover, T-cell lineage reconstitution was accelerated in NOD/SCID γ C^{-/-} recipients after T-cell progenitor injection compared with hematopoietic stem cell transplantation. Furthermore, γ C-encoding BaEV-LVs very efficiently transduced Dll4-generated T-cell precursors from a patient with X-linked severe combined immunodeficiency (SCID-X1), which fully rescued T-cell development in vitro. These results indicate that BaEV-LVs are valuable tools for the genetic modification of naive T cells, which are important targets for gene therapy. Moreover, they allowed for the generation of gene-corrected T-cell progenitors that rescued SCID-X1 T-cell development in vitro. Ultimately, the coinjection of LV-corrected T-cell progenitors and hematopoietic stem cells might accelerate T-cell reconstitution in immunodeficient patients.

Introduction

Gene transfer into T lymphocytes is a crucial step in the development of therapeutic strategies for the treatment of genetic dysfunctions of the hematopoietic system, such as severe combined immunodeficiency (SCID^{1,2}) as well as cancers^{3,4} and acquired diseases.⁵ More than 15 years ago, children suffering from monogenetic diseases such as adenosine deaminase SCID (ADA-SCID) and SCID-X1 were successfully treated with T-cell gene therapy (ADA-SCID)⁶ or hematopoietic stem cells (HSCs) (SCID-X1^{6,7}; ADA-SCID⁸). T-cell gene therapy might also become an important treatment option for HIV-infected patients because several new combinatorial strategies have been proposed.⁹⁻¹¹ To avoid graft-versus-host

Submitted 23 October 2018; accepted 13 January 2019. DOI 10.1182/ bloodadvances.2018027508.

tF.-L.C., I.A., and E.V. contributed equally to this work as senior authors.

^{*}O.B., F.A., A.G.-G., and R.D.M. contributed equally to this work.

disease in HSC transplantation, retroviral marking of allogenic T cells with a suicide gene is performed, and these T cells can then be eliminated by administering a specific drug.^{3,12-14} A promising anticancer strategy is based on engineered T cells that express a tumor-specific T-cell receptor (TCR) or a chimeric antigen receptor (CAR).¹⁵⁻¹⁷ Ongoing clinical trials have described durable rejection of previously refractory B-cell malignancies in patients after CD19-directed CAR therapy,^{4,18-21} with a complete response rate as high as 86% in patients with leukemia. The successful application of CARs directed to other molecular targets has broadened the indications for this approach to other cancers.²²⁻²⁸

The clinical efficacy of adoptively transferred T lymphocytes is correlated with their ability to persist in vivo,²⁹ which is correlated with a less differentiated T-cell phenotype.^{15,17,30} Naive T cells are especially important as gene therapy target cells because they maintain the capacity to respond to novel antigens and can generate the entire spectrum of immunologic memory.31,32 Indeed, upon infusion, less differentiated naive and central memory T cells display superior proliferation, persistence, and antitumor responses when compared with the effector memory subset.^{15,31,33} Accordingly, naive T cells might constitute the best T-cell target population for gene therapy. Human cord blood (CB) T cells are mostly naive and differ from their adult naive counterparts in that the former represent mainly recent thymocyte emigrants, ^{33,34} which express CD31⁺.^{35,36} Naive CB T cells proliferate significantly more than their adult counterparts in response to interleukin-7 (IL-7).37,38 Interestingly, IL-7 maintains naive CD31⁺ CD4⁺ T cells during adult life.³⁹ These characteristics prompted the development of the first CAR-based CB T-cell strategies for the treatment of cancer and infectious diseases.^{16,40-42}

Other even more immature targets for gene modification are the T-cell progenitors, which are normally found in the thymus and are important for treatment of autoimmune disorders.⁴³⁻⁴⁶ Early T-cell development depends on the interaction between thymocytes and Notch ligand signaling pathways. Delta-like ligand 4 (DII4) has been identified as the essential Notch1 activator in the T-cell engagement of HSCs.⁴⁷⁻⁴⁹ We have previously demonstrated that T-cell progenitors can be generated from CD34⁺ hematopoietic stem and progenitor cells (HSPCs) in a feeder-cell-free culture system based on DII4.^{50,51} They displayed the phenotypic and molecular signatures of immature thymic precursors and were capable of differentiating into T cells and accelerating T-cell reconstitution in vivo compared with HSPCs.⁵⁰

It is now generally accepted that resting T cells cannot readily be transduced by classical vesicular stomatitis virus G (VSV-G) protein pseudotyped lentiviral vectors (LVs)⁵²⁻⁵⁴ because of the absence of the VSV receptor.⁵⁵ The latter is upregulated by TCR stimulation and is correlated with high-level transduction by VSV-G-LVs.⁵⁵ However, TCR stimulation of T cells alters their half-life and immune competence and is associated with the loss of naive T-cell subsets and a skewed TCR repertoire.^{56,57} Exposure of T cells to cytokines such as IL-7 renders them permissive to transduction by VSV-G-LVs, 52,54,58-60 preserving a functional T-cell repertoire without skewing the T-cell populations.^{38,54,58} However, VSV-G-LVs do not allow high-level transduction of IL-7-stimulated naive T cells.^{52,54,59} We previously pseudotyped LVs with measles virus envelope glycoproteins (GPs) H and F (H/F-LVs). They allowed, for the first time, efficient transduction of guiescent memory and naive human T (hT) cells and B cells, with conservation of the T- and B-cell phenotypes.⁶¹⁻⁶³ Furthermore, we engineered LVs that displayed the feline retrovirus GP RD114, which conferred efficient transduction of hT cells upon TCR stimulation.⁶⁴ The baboon envelope retrovirus (BaEV) belongs to the same betaretrovirus family as RD114. Both viruses use the neutral alanine/serine/cysteine amino acid transporter-2 (ASCT-2) present on T cells.^{65,66} Interestingly, BaEV-LVs also bind to ASCT-1⁶⁷ and far outperform VSV-G- and RD114-LVs for human HSC and B-cell transduction.⁶⁸⁻⁷⁰ Therefore, we hypothesized that BaEV-LVs might be excellent candidates for gene delivery into naive hT cells and T-cell progenitors.

Materials and methods

Plasmids

RD114TR, BaEVTR, BaEVRless, and H Δ 24 and F Δ 30 envelope GPs were previously described.^{64,69,71} All chimeric envelope GPs were expressed in the phCMV-G expression plasmid.⁷² The SIN-HIVSFFVGFP vector was described previously.⁷¹

The native IL-2RγC complementary DNA (cDNA) fragment from pRRL.PPT.SF91.IL2RGpre (a gift from A. Shambach, Medizinische Hochschule Hannover, Hannover, Germany) was cloned into the pHEF1-MCS-IRES-GFP vector by ligation of *Bam*HI-*Sal*I digested fragments.

Self-inactivating HIV-1–derived vectors encoding green fluorescent protein (GFP) or γ C under the control of an SFFV or EF1 α promoter, respectively, were generated by transfection of 293T cells as described in detail elsewhere.⁷¹ Titration of LVs^{69,71} is described in detail elsewhere, and isolation of human primary T cells, thymocytes, and CD34⁺ cells is described in the supplemental Methods.

Transduction of primary cells

Peripheral blood (PB) T lymphocytes were prestimulated for 24 hours with anti-human CD3 (hCD3)/anti-hCD28 (1 μ g/mL, BD Pharmingen) in the presence of hIL-2 (1 ng/mL), and CB and PB T cells were prestimulated with recombinant human IL-7 (rhIL-7; 10 ng/mL; BD Biosciences) for 2 and 3 days, respectively.⁵⁴

Next, 5×10^4 CB or PB T cells were seeded in 48-well plates upon TCR stimulation or cytokines and transduced with LVs at the indicated multiplicity of infection (MOI). Cell cultures were replenished with cytokines every 3 days. At 3 and 6 days after transduction, the percentage of GFP⁺ cells was determined by fluorescence-activated cell sorting (FACS). Where indicated, transductions were performed on RetroNectin-coated plates (Takara Bio, Shiga, Japan).

In all, 5×10^5 freshly isolated thymocytes were seeded in RetroNectin-coated 48-well plates in RPMI-1640 and 10% fetal calf serum in the presence of rIL-7 (20 ng/mL) and transduced with LVs at the indicated MOIs. Cell cultures were replenished with cytokines after 3 days. Four days after transduction, the percentage of GFP⁺ cells was determined by FACS.

CB CD34⁺ cells were cultured in 48-well plates coated with a Dll4 Fc fusion protein (Dll4-Fc, 5 μ g/mL; PX'Therapeutics, Grenoble, France⁵⁰). Cultures were initiated at 1 \times 10⁴ CD34⁺ cells per well in X-VIVO-20 medium (Lonza, Basel, Switzerland) and supplemented with 20% defined fetal calf serum (Hyclone; Thermo Fisher Scientific, Illkirch, France) and cytokines hIL-7, human FMS-like tyrosine kinase 3 ligand (hFlt3-L), human stem cell factor (hSCF), and human thrombopoietin (hTPO) (each 100 ng/mL; Miltenyi Biotech). The vectors were pre-incubated with Vectofusin

(12 µg/mL; Miltenyi Biotech) for 10 minutes. After 24 hours of culture, the LVs plus Vectofusin were added to the cells at the indicated MOIs. After 7 days of culture on DII4, cells were transferred to 24-well plates freshly coated with DII4-Fc at a concentration of 1×10^5 cells per well. The percentage of GFP⁺ cells was determined in the thymic subpopulations by FACS.

A total of 1.8×10^5 bone marrow (BM) SCID-X1 CD34⁺ cells were prestimulated for 16 hours in X-VIVO-20 (Lonza) supplemented with 20% fetal bovine serum and 300 ng/mL hSCF, 100 ng/mL hTPO, 300 ng/mL hFlt3-L, and 20 ng/mL hIL-3 on RetroNectin (25 μg/mL) alone or on RetroNectin and Dll4-Fc ligand (5 μg/mL; PX'Therapeutics)-coated wells. In addition, 100 ng/mL of hIL-7 was added for prestimulation on Dll4-coated wells. The prestimulated cells were incubated with BaEV-IL-2R γ C-LVs at an MOI of 10 in the presence of 4 µg/mL protamine sulfate for 6 hours. For cells transduced for 6 hours on Dll4-coated wells, the medium was replaced by Minimum Essential Medium Eagle-Alpha Modification (Gibco, Life Technologies) with 20% fetal bovine serum, 2 mM glutamine (Gibco BRL/Invitrogen), 100 ng/mL hSCF, 100 ng/mL hTPO, 100 ng/mL hFlt3-L, and 100 ng/mL hIL-7. Viability after transduction was determined after exclusion of 7-amino-actinomycin-D⁺ cells analyzed by FACS.

The in vitro T-cell differentiation assay was performed on OP9-hDL1 stroma; 8×10^4 cells per well were cocultured in 6-well plates with OP9-hDL1.⁷³ From week 2, the medium was changed once per week, and cells were analyzed by FACS (see supplemental Data).

qPCR analysis

IL-2RγC messenger RNA (mRNA) expression was assessed by using semi-quantitative real-time polymerase chain reaction (RT-PCR). RT-PCR was performed with equal amounts of cDNA using IL-2RγC and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) TaqMan Gene Expression Assay in an Applied Biosystems ViiA 7 Real-Time PCR System (Applied Biosystems Inc, Foster City, CA). The IL-2RγC mRNA expression was normalized with the GAPDH mRNA expression as endogenous control. The γC mRNA expression was calculated as 2^{-ddCt} .

Quantification of vector copy number and analysis of TCR repertoire

Duplex quantitative RT-PCR was performed with 100 ng genomic DNA using primers and TaqMan probes specifically designed to amplify the HIV and albumin (ALB) sequences in the Applied Biosystems ViiA 7 Real-Time PCR System. Simultaneously, standard amplification curves were generated by serial dilutions of the plasmid pRRL-LTR-PSI-GK-GFP-WPRE-ALB ranging from 10⁷ to 10¹ copies per well. All the reactions were performed in duplicate. The data were normalized to ALB. The vector copy number (VCN) per cell was determined from the linear standard curve. Analysis of the TCR repertoire was performed by multiplex PCR followed by GeneScan analysis as described previously.^{74,75}

Conditioning and reconstitution of NSG mice

NOD/SCID $\gamma C^{-/-}$ (NSG) mice were housed in our animal facility (Plateau de Biologie Expérimentale de la Souris, Lyon, France). Experiments were performed in accordance with the European Union guidelines upon approval of the protocols by the local ethical committee. Two- to 3-day-old newborn NSG mice were irradiated at 1 Gy, and 5 \times 10⁵ transduced progenitor T cells derived from

hCD34⁺ cells or 2×10^5 transduced CD34⁺ cells were injected intrahepatically. Six weeks after reconstitution, the thymocytes of these mice were analyzed by FACS. Statistical analysis was performed using a Student *t* test.

Results

BaEV-LVs permit high-level transduction of both naive and memory hT cells

We previously obtained efficient LV pseudotyping with 2 cytoplasmic tail mutants of the BaEV GP: BaEVTR, in which the cytoplasmic tail has been replaced by that of the murine leukemia retrovirus, and BaEVRless, which lacks the R-peptide.⁶⁹ The new BaEV-LVs¹⁹ were compared with other reported LV pseudotypes that allow CD3⁺ T-cell transduction with differing efficiencies: H/F-LVs,⁶² RD114TR-LVs,⁶⁴ and VSV-G-LVs.⁵⁴

After TCR stimulation, the BaEV-LVs reached 60% to 90% CD3⁺ T-cell gene transfer in the presence of RetroNectin (MOI, 10; Figure 1A). VSV-G-LVs and H/F-LVs reached similar transduction levels⁶² (Figure 1A). Both BaEVTR- and BaEVRless-LVs enabled efficient transduction of rIL-7 prestimulated hT cells and were superior to VSV-G-LVs at an MOI of 10 in the presence of RetroNectin (percentage transduction rates: BaEVTR-LVs, 29%-43%; BaEVRless-LVs, 41%-60%; VSV-G-LVs, 2%-12%) (Figure 1B). They permitted efficient high-level transduction of IL-7–prestimulated naive and memory T cells (Figure 1B). The cell viability was not altered by the transduction process (supplemental Figure 1A-B). H/F-LVs resulted in more than 80% transduction of IL-7–stimulated T cells in the presence of RetroNectin. In this case, the H/F-LVs primarily targeted memory T cells for transduction, confirming previous results⁶² (Figure 1B).

Strict gating on CD45RA-, CD62L-, and CCR7-expressing CD4⁺ and CD8⁺ naive T cells confirmed the highly efficient transduction of these subpopulations by BaEV-LVs (Figure 1C). The RD114TR-LVs resulted in much lower transduction efficiency of rlL-7-prestimulated naive and memory T cells compared with BaEV-LVs (RD114TR-LVs, 5%-12%).

We have previously shown that H/F-LVs allow gene transfer into resting CD3⁺ T cells.^{62,76} The RD114TR- as well as BaEVTR- and BaEVRless-LVs performed poorly on these quiescent target cells (supplemental Figure 1C). Importantly, mRNA levels of both BaEV receptors (ASCT-1 and ASCT-2) were upregulated upon IL-7 stimulation, and the upregulation was even more pronounced after TCR activation of T cells (Figure 1D). This coincided with higher transduction levels obtained in these prestimulated T cells compared with their resting counterparts (Figure 1C vs supplemental Figure 1C). Overall, our results show that the novel BaEVTR- and BaEVRless-LVs are promising gene transfer tools for adult T-cell gene therapy because they can target long-lived naive T cells at low vector doses.

BaEV-LVs GPs allow high-level transduction of recent thymic emigrants

T-cell gene therapy or immunotherapy would benefit from genetically modified naive T cells that are able to persist for the individual's entire life span. Here, we tested the different LV pseudotypes for their ability to efficiently transduce CB CD4⁺ T cells, which are phenotypically similar to recent thymic emigrants (RTEs).



Figure 1, BaEV-LVs allow high-level transduction of naive as well as memory adult human T cells. (A) Freshly isolated peripheral blood T cells were preactivated through the TCR (anti-CD3 + anti-CD28 antibodies + IL-2) for 24 hours and transduced in the absence and presence of RetroNectin with the different LV pseudotypes at indicated MOIs. Six days after transduction, the percentage of GFP⁺CD3⁺ T cells was analyzed by FACS (mean \pm standard deviation [SD]; n = 4). (B-C) Freshly isolated CD3⁺ T cells were prestimulated with IL-7 for 3 days and transduced in the presence of RetroNectin with the different LV pseudotypes at MOI 10 or 100 as indicated. Six days after transduction, the percentage of GFP⁺CD3⁺ T cells was analyzed by FACS for total T cells (anti-CD3) and the naive (CD45RA⁺) or memory (anti-CD45RO⁺) T cells subsets in panel B. (C) More detailed phenotyping of these cells shows the percentage of GFP⁺ naive (CD45RA⁺CD62L⁺CCR7⁺) CD4 and CD8 T cells. (B-C; mean \pm SD; n = 3; VSV-G-LV MOI 10 vs BaEV-LV). (D) ASCT-1 and ASCT-2 expression levels in unstimulated (unstim.) and TCR- or IL-7-activated total CD3⁺ T cells (as in panels A and B, respectively). Relative fold increase in ASCT-1 or ASCT-2 mRNA levels in stimulated (stim.) CD3⁺ T cells compared with unstimulated T cells, for which mRNA levels were normalized to 1 (mean \pm SD; n = 3). *P < .05; **P < .01. ns. nonsignificant

To conserve the naive T-cell phenotype, CB T cells were prestimulated with IL-7 and subsequently transduced with the different LV pseudotypes encoding GFP at MOI 10 (Figure 2A). BaEVTR- and BaEVRless-LVs permitted 40% to 55% transduction of the target cells at MOI 10 and thus outperformed VSV-G-LVs (<20%) and RD114TR-LVs (<10%; Figure 2A). In contrast, RTEs and mature naive CB T cells were poorly permissive for H/F-LVs⁶² (Figure 2A). Importantly, BaEVTR- and BaEVRless-LVs easily reached 55% to 70% transduction when the MOI was doubled to 20 (Figure 2A vs supplemental Figure 2). The RTEs (CD31⁺) were transduced to the same extent as the mature naive CB CD4⁺ T cells (CD31⁻; Figure 2A) without an effect on the distribution of these 2 naive CB T-cell populations (Figure 2B).

BaEV-LVs promote highly efficient transduction of human thymocytes

It would be of major interest to correct T cells or genetically change T-cell functions as early as possible, ideally during thymic

development, which is a tightly regulated process (Figure 3A). We transduced human thymocytes with RD114TR-, BaEVTR-, BaEVRless-, and H/F-LVs (MOI, 10) in the presence of RetroNectin and IL-7. VSV-G-LVs were used at an MOI of 50. Four days later, we determined the transduction levels by FACS in the thymic subsets using CD4/CD8/CD3 staining. VSV-G-LVs transduced only 10% to 20% of the different thymic subpopulations (Figure 3B). RD114TR-, BaEVTR-, and BaEVRless-LVs preferentially transduced early thymocytes (ie, double-negative [DN] and immature single-positive cells; Figure 3A-B). The efficiency of RD114TR-LV transduction of all thymic subpopulations was lower compared with that of BaEV-LVs. Interestingly, H/F-LVs did not show a subpopulation preference and ensured an efficient transduction of all thymic subsets (Figure 3B).

The DN CD4⁻CD8⁻ population can be subdivided into early lymphoid progenitors (ELPs) and early thymocyte progenitors (ETPs), which then differentiate into proT and preT cells (see Figure 3A and supplemental Figure 3 for the gating strategy). BaEV-LVs targeted transduction



Figure 2. BaEV-LV GPs allow efficient transduction of immature naive CB T cells. (A) Freshly isolated total CB CD4⁺ T cells were prestimulated with IL-7 for 48 hours and subsequently transduced in the presence of RetroNectin with VSV-G-, RD114TR-, BaEVTR-, BaEVRless-, and H/F-LV, encoding the GFP reporter at an MOI of 10. Three days after transduction, cells were stained for human surface markers CD45RA, CD62L, and CD31, and the percentage of GFP⁺ cells in recent thymocyte emigrants (CD45RA⁺, CD62L⁺, CD31⁺) and more mature naive CB CD4⁺ T cells (CD45RA⁺, CD62L⁺, CD31⁻) was determined by FACS analysis (mean \pm SD; n = 3; **P < .01). (B) Distribution of the recent thymocyte emigrants (CD45RA⁺, CD62L⁺, CD31⁻) and more mature naive CB (CD45RA⁺, CD62L⁺, CD31⁺) and more mature naive CB (CD45RA⁺, CD62L⁺, CD31⁻) code⁺ T cells subpopulations upon incubation of CB T cells with the different vector pseudotypes as in panel A compared with untransduced T cells (mean \pm SD; n = 4).

to the most immature DN thymocytes; BaEVRless-LVs reached 100% of the ETPs and 90% of proT cells (Figure 3C). VSV-G- and RD114TR-LVs resulted in much lower transduction levels. H/F-LVs mediated equivalent high levels of transduction in ETP, proT, and preT cells (Figure 3C).

Our results indicate that BaEV-LVs are better tools than VSV-G- and RD114TR-LVs for the transduction of ETPs and proT cells without population skewing (supplemental Figure 4). This prompted us to test these vectors for transduction of in vitro–generated thymic progenitors.

BaEV-LVs mediate efficient transduction of hT-cell progenitors generated in vitro

We made use of our new culture system based on immobilized Dll4, as described previously.⁵⁰ CB CD34⁺ cells were cultured on Dll4-coated plates in the presence of cytokines inducing T-cell differentiation. After 24 hours, the different LV pseudotypes were added at an MOI of 10. As observed for freshly isolated thymocytes, BaEV-LVs efficiently transduced ELPs (65%-75%) and ETPs (70%-80%) at high levels when generated in vitro (Figure 3C vs Figure 4A-B). As expected, VSV-G- and RD114TR-LVs permitted only poor transduction of ELPs (15%) and ETPs (10%), whereas H/F-LVs achieved transduction levels of 60%⁵⁰ (Figure 4A-B). CD7⁺CD34⁻ proT cells appeared on day 7 of differentiation (supplemental Figure 5A). BaEV-LVs performed stable high-level gene transfer into both ELPs and ETPs but also into proT cells without skewing these T-cell populations, whereas VSV-G- and RD114TR-LVs were much less efficient (supplemental Figure 5B-C) in agreement with Hübner et al.⁷⁷ In contrast, the H/F- and BaEV-LV transduction levels were equivalent at day 7 (supplemental Figure 5B). On day 14, a subset of CD34⁻CD7⁺⁺ started to express CD5. allowing us to distinguish between proT1 (CD7⁺⁺/CD5⁻) and proT2 (CD7⁺⁺/CD5⁺) T-cell stages followed by CD1a expression (preT-cell stage; supplemental Figure 6A). As expected, the highly efficient transductions obtained with BaEV-LVs persisted at day 14 in the ELPs, ETPs, proT1 and proT2 thymocytes, and also in the preT cells (Figure 4C; supplemental Figure 6A). Importantly, differentiation into ETPs and proT cells was not perturbed by the transduction process (supplemental Figure 6B). Because the original CD34⁺ cells had expanded 50-fold, on average, after 14 days of T-cell differentiation, they went from 5 imes 10^4 CD34⁺ cells to 2.5×10^6 T-cell progenitors (ELP, ETP, proT, and preT); these findings confirmed stable transduction in the different T-cell populations.

BaEV- and H/F-LV-transduced T-cell progenitors seed the thymus and enable accelerated in vivo thymopoiesis compared with HSCs in NSG mice

The in vivo T-cell differentiation potential of LV-transduced progenitor T cells and HSCs was evaluated by transplantation into NSG mice.⁵⁰ CD34⁺ cells were plated on Dll4-coated wells with cytokines and then incubated with BaEV- and H/F-LVs at MOI 10 or without vector in the presence of Vectofusin.⁷⁸ On day 8 of T-cell differentiation, these transduced T-cell progenitors were injected intrahepatically into NSG neonates. In parallel, CD34⁺ cells from the same donor were briefly prestimulated with cytokines and were then transduced with BaEV- and H/F-LVs (MOI, 10) or without vector in the presence of RetroNectin. Twenty-four hours after transduction, these transduced CD34^+ cells were transplanted into a second group of NSG recipients (Figure 5A). At 6 weeks after engraftment, high levels of transduction were detected by FACS in all thymic subpopulations for both NSG groups (Figure 5B). However, intrathymic T-cell development was accelerated in recipients of T-cell progenitors compared with recipients of hCD34⁺ cells, which was confirmed by higher percentages of mature CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells (Figure 5C, left vs right panel). Indeed, the CD4-single-positive (CD4-SP) cells in progenitor T-cell NSG recipients were all CD3⁺ (Figure 5C, left panel). For the mice that received CD34⁺ cells, CD4-SP cells did not express CD3 and still corresponded



Figure 3. BaEV-LV GPs confer high-level transduction of human immature thymocyte populations. (A) Schematic representation of T-cell development in the thymus. Phenotypic surface markers are indicated for each differentiation stage. HSCs (CD34⁺CD1a⁻) are migrating from the BM to the thymus. The first stages are characterized by the lack of expression of CD8 and CD4 markers (CD8⁻CD4⁻ DN stage). The DN cells subsequently gain expression of preT and develop into CD4 immature single-positive (ISP) cells. The appearance of the CD8 surface marker together with CD4 and maturation of the TCR is called the DP stage of thymocyte development. Upon selection in the thymus, DP thymocytes develop into CD8⁺CD4⁻ (SP8) and CD8⁻CD4⁺ (SP4) thymocytes. (B) Freshly isolated human thymocytes were transduced in the presence of IL-7 on RetroNectin-coated culture plates with the indicated LV pseudotypes at an MOI of 10, except for VSV-G LVs for which an MOI of 50 was applied. Four days after transduction, the thymocytes were stained for human surface markers (CD3, CD4, and CD8), and the percentage of GFP⁺ cells in the different thymocyte subpopulations was analyzed by FACS (mean \pm Sp; n = 5; DN, CD4⁻CD8⁻; SP-CD4, CD4⁺CD3⁻CD8⁻; SP-CD8, CD4⁻CD3⁺CD8⁺; DP, CD4⁺CD8⁺; ISP, CD4⁺CD3⁻CD8⁻). For BaEV- and RDTR-LVs, the percentage of GFP⁺, DN, and ISP is significantly higher than the percentage of DP, SP4, and SP8. (C) Freshly isolated human thymocytes were transduced as in panel B. Four days after transduction, the thymocytes were stained for human surface markers (CD3⁺, n = 4). For BaEV-LVs, the percentage of GFP⁺ ETPs and proT cells was significantly higher than that of preT cells. Gating strategies for FACS analysis are shown in supplemental Figure 5A. *P* < .01.

to the earlier immature single-positive CD4 stage (Figure 5C, right dot blots). Overall, mature CD3⁺ T-cell levels in the thymus and the periphery were significantly higher in progenitor T-cell recipients than in CD34⁺-cell recipients, and they expressed TCR $\alpha\beta$ (Figure 5D-F; supplemental Figure 7). Importantly, this was true for both nontransduced and LV-transduced progenitor T cells, which emphasizes that transduction did not affect T-cell potential in vivo (Figure 5E-F). To summarize, the BaEV-and H/F-LV gene-marked T-cell progenitors generated in vitro allowed efficient thymopoiesis and accelerated T-cell reconstitution in vivo.

BaEV-LV transduction combined with DLL4 exposure allowed efficient γ C-correction of BM SCID-X1 HSPC-derived progenitor T-cells

SCID-X1 patients exhibit a severe T-cell defect, which is the result of a blockade in a very early stage of T-cell differentiation caused by

a loss-of-function mutation in the γ C gene. SCID-X1 γ C deficiency can be safely and efficiently corrected by the ex vivo transduction of HSPCs as demonstrated in SCID-X1 patients.⁷⁹ Because both BaEV- and H/F-LVs performed similarly for transduction of T-cell progenitors, we selected BaEV-LVs encoding the γC receptor to correct the γ C deficiency in BM SCID-X1 CD34⁺ cells under conventional conditions or in combination with the already established Dll4-based T-cell progenitor differentiation technique. Therefore, BM SCID-X1 CD34 $^{\rm +}$ cells were cultured in the presence of RetroNectin alone (conventional) or Dll4 and RetroNectin (Dll4 culture; supplemental Figure 8A) and then transduced or not (mock) with BaEV-LVs encoding the vC receptor at an MOI of 10. FACS analysis did not show any major differences in the frequencies of gated lymphocytes between the transduced and mock groups, and 97% of the gated lymphocytes were viable (supplemental Figure 8B). Thus, BaEV-LV transduction had no toxic effect on the cells. After 4 days of culture, T-cell progenitor



Figure 4. BaEV-LV GPs confer efficient gene transfer into in vitro-generated early T-cell progenitors. CB CD34⁺ cells were plated on Dll4-coated culture wells in the presence of a cytokine cocktail (IL-7, TPO, Flt3-L) for 24 hours, and they were subsequently transduced with VSV-G-, RD114TR-, BaEVTR-, BaEVTR-, BaEVTR-S- and H/F-LVs

Shood advances 12 February 2019 • Volume 3, Number 3

differentiation revealed higher frequencies and numbers of CD7⁺ early T-cell progenitors in the Dll4 culture than in conventional cultures (Figure 6A-B), suggesting that the Dll4 cultures were able to generate early T-cell progenitors. We then evaluated the efficiency of gene correction by testing the ability of transduced cells to differentiate into T cells. Transduced and nontransduced cells were cocultured with an OP9-hDL1 stromal cell layer, and their T-cell differentiation status was analyzed (supplemental Figure 8A). The VCN in CD7⁺ cells sorted after 5 weeks of coculture reached 4.04 in BaEV-LV transduced conventional cultures and 5.94 in BaEV-LV transduced Dll4 cultures (Figure 6C). IL-2RγC mRNA levels were 60-fold higher in the transduced groups than in the mock groups in CD45⁺ cells sorted at week 7 and, consistent with the high VCN, approximately threefold higher than in healthy CB CD34⁺ cells under Dll4 culture conditions (Figure 6D). CD4⁺ CD8⁺ (DP) cells and CD3⁺ cells were present as early as week 2 in the transduced Dll4 culture conditions and week 3 in the transduced conventional culture conditions and continued to be detected afterward in higher numbers in the DII4 culture conditions until week 5. This suggested accelerated restoration of T-cell differentiation in the BaEV-LV transduced Dll4 culture conditions (Figure 6E-F; supplemental Figure 8C). Importantly, the CD3⁺ cells were found to express either TCR $\gamma\delta$ or TCR $\alpha\beta$; this was especially true in the DII4 culture conditions (supplemental Figure 8D-E). TCR repertoire analysis of the polyclonality in CD7⁺ cells sorted at week 5 revealed only diverse TCR $\gamma\delta$ rearrangement but not TCR $\alpha\beta$ rearrangement in nontransduced conditions (Figure 6G; supplemental Figure 8F); this was consistent with the appearance of TCR $\gamma\delta^+$ CD3⁺ cells but not TCR $\alpha\beta^+$ CD3⁺ cells in the later weeks (supplemental Figure 8D-E). This was also in line with the leaky mutation in the γC gene found in this particular patient. However, the DP and CD3⁺ cell counts remained very low in these mock groups (Figure 6E-F). Conversely, TCR $\gamma\delta$ and TCR $\alpha\beta$ rearrangements were polyclonal under BaEV-LV transduction conditions regardless of the presence or absence of DII4; this confirmed the polyclonality of differentiated cells and the absence of any deleterious effect of Dll4 on the polyclonality of cells (Figure 6G; supplemental Figure 8F). Our data demonstrate the correction of γC deficiency in BM SCID-X1 CD34⁺ cells under both conventional and Dll4 culture conditions. However, T-cell differentiation was faster and more efficient under DII4 conditions. Collectively, BaEV-LVs allowed efficient correction of the vC defect in SCID-X1 HSCs with high VCN, high levels of vC expression, and de novo T-cell differentiation from SCID-X1 HSPCs.

Discussion

Here we highlighted the exceptional capacity of BaEV-LVs to transduce 60% of IL-7-stimulated naive adult T cells and CB RTEs. In contrast, VSV-G- or RD114TR-LVs performed poorly.

Remarkably, BaEV-LVs transduced up to 80% of early thymocytes such as ETPs, proT-cell, and preT-cell lineages differentiated from CD34⁺ cells using a feeder-cell-free DII4 culture system.⁵⁰ Hence, BaEV-LVs constitute a promising tool for the transduction of naive and early progenitor T cells, whereas H/F-LVs are complementary because they can efficiently transduce memory T cells and late-stage thymocytes: CD4⁺CD8⁺ and CD4-SP or CD8-SP cells. Accordingly, we confirmed the upregulation of the BaEV receptors^{67,80} upon IL-7 and TCR T-cell stimulation coinciding with increased BaEV-LV-mediated gene transfer.

A successful T-cell immunotherapy consists of engineering autologous or allogenic T cells to express CARs specific for either viruses or tumor antigens.^{23,28} Recently, this CAR T-cell technology has been translated to naive cells from CB.⁸¹ Micklethwaite et al¹⁶ transduced CB T cells with a CAR against CD19, which also coexpressed TCRs specific for 3 different viruses that can affect patients after a CB transplant. When compared with adult T cells, naive CB T cells showed a greater tolerance to HLA disparity^{82,83} and thus induced graft-versus-host disease less frequently. Therefore, naive CB T cells represent a source of allogenic lymphocytes for cell therapy of viral infections or/and cancer, particularly when patients do not have enough autologous T cells. Indeed, IL-7-stimulated CBT cells combined with BaEV-LVs resulted in gene delivery levels of 70%. This might constitute an important advantage for CAR therapy because naive CB T-cell subsets show a high capacity to expand and might provide longer-lasting antitumor responses in vivo than adult T cells, allowing protection against relapse after transplant.38,39

Infections and relapse are the main causes of morbidity and mortality after allogenic HSC transplantation. This is undoubtedly related to delayed T-cell immune reconstitution and impaired de novo thymopoiesis.84-86 The same is true for autologous HSC-mediated gene therapy. Complete restoration of a polyclonal T-cell repertoire takes even longer, and patients can be immunocompromised for more than a year.^{87,88} In the case of gene therapy, the administration of autologous gene-corrected T-cell progenitors might be an option. We can genetically modify these T-cell progenitors derived from CD34⁺ cells at high levels using the BaEV- or H/F-LVs. Combination of the transduction with the Dll4 culture of hCD34⁺ cells, resulted in the expansion of gene-marked ETPs and proT1 cells, the most primitive T cells. Moreover, these cells retained their in vivo intrathymic T-cell differentiation capacity and displayed a conventional CD4, CD8, and TCRαβ/TCRγδ repertoire. Furthermore, T-cell reconstitution was faster than in hCD34⁺-cell engrafted NSG mice.50

These data suggest that BaEV- or H/F-LVs are valuable treatment options for SCIDs (eg, SCID-X1) and HIV, in which the thymic environment is not ideal for HSC differentiation into T-cell lineages. We have previously evidenced faster immune reconstitution by

Figure 4. (continued) encoding the GFP reporter at an MOI of 10, except for VSV-G-LVs for which a higher vector dose (MOI of 50) was applied. (A-B) At day 3 of culture, the cells were analyzed by FACS for surface expression of CD34 and CD7. The gating strategy to determine GFP expression in the subpopulations of ELP, ETP, and proT cells are shown for differentiation at day 3 in panel A for a representative experiment. The transduction levels for day 3 of differentiation toward T-cell lineage in these subpopulations are summarized in the histograms in panel B (mean \pm SD; n = 3). (C) At day 14 of culture, the cells were analyzed by FACS for surface expression of CD34, CD7, CD5, and CD1a (for gating strategy, see supplemental Figure 6). GFP expression in the subpopulations ELP, ETP, proT1, proT2, and preT cells at day 14 of differentiation toward T-cell lineage is shown for the different pseudotypes (mean \pm SD; n = 3; VSV-G vs other pseudotypes). ***P* < .01.



Figure 5. LV-transduced progenitor T cells allowed accelerated thymopoiesis in vivo in NSG mice compared with LV-transduced CD34⁺ cells. (A) CD34⁺ cells were plated on DII4-coated wells in the presence of a cytokine cocktail (IL-7, TPO, Flt3-L) for 24 hours, and they were subsequently transduced with BaEV- and H/F-LVs encoding GFP at an MOI of 10 or without vector in the presence of Vectofusin, an agent that facilitates transduction.⁷⁸ At 8 days of T-cell differentiation, these transduced T-cell progenitors were injected intrahepatically into NSG recipient mice. In parallel, CD34⁺ cells from the same donor were prestimulated briefly with cytokines that maintain HSC potential (SCF, TPO, Flt3-L) for 24 hours and were transduced with BaEV- and H/F-LVs (MOI, 10) or without vector in the presence of RetroNectin. At 24 hours after

Solood advances 12 February 2019 · Volume 3, Number 3



Figure 5. (continued) transduction, the transduced CD34⁺ cells were engrafted in a second group of NSG recipients. Human thymocyte reconstitution was assessed 6 weeks after injection into NSG mice by FACS. (B) Percentage of GFP⁺ thymocytes (CD4 SP, CD4⁺CD8⁻; DP, CD4⁺CD8⁺; DN, CD4⁻CD8⁻; CD8 SP, CD8⁺ CD4⁻) for T-cell progenitor engraftment compared with CD34⁺ cell engraftment. (C) Distribution of CD4 SP, DP, DN, and CD8 SP thymic subpopulations for proT cells compared with CD34⁺ cell engraftment. CD4 SP cells were analyzed for CD3 surface expression for proT (left panels) and CD34⁺ (right panels) engrafted cells. ISP CD4, CD4⁺CD3⁻ cells. (D) FACS analysis of the percentage of CD3⁺ T cells per total hCD45⁺ cells in the thymus. (E) The absolute numbers of mature CD3⁺ T cells per total human thymocytes for proT cells compared with CD34⁺ cell engraftment (mean ± SD; n = 3). (F) Percentage of mature CD3⁺ T cells in the blood per total hCD45⁺ cells for proT cells compared with CD34⁺ cell engraftment (mean ± SD; n = 3). **P* < .05.

coinfusion of HSC-derived T-cell progenitors and CB HSPCs in the NSG mouse model.⁵⁰ Infusion of autologous corrected T-cell progenitors together with corrected HSCs might accelerate immune reconstitution and reduce the gap in immune system recovery. In a clinical setting, part of the patient's HSPCs would be gene-corrected with the classical protocol and another part would be gene-corrected with the DII4-based protocol described herein. The 2 products could be transplanted simultaneously. We have shown that T-cell progenitors are able to directly seed the thymus without passing through the BM,⁵⁰ thus overcoming 2 main obstacles of post-HSCT T-cell generation: the long time required for the first BM and thymic phases and the limited thymic supply by BM progenitors. We are currently testing this hypothesis in a clinical trial of cell therapy for SCID patients who received a transplantation in a haplo-identical setting.

This is supported by our BaEV-LV–based correction of γ C-deficient CD34⁺ cells in the presence of DII4, resulting in a large amount of T-cell progenitors. We previously confirmed that BaEV-LVs also allow high-level transduction of prestimulated and unstimulated hHSCs.¹⁹ Remarkably, γ C-encoding BaEV-LVs efficiently rescued SCID-X1 CD34⁺-derived T-cell development in vitro, showing the clinical feasibility of this approach.

In conclusion, H/F-LVs and, in particular, BaEV-LVs represent very useful new tools for applications in T-cell gene therapy and immunotherapy. In addition, injection of BaEV-LV corrected autologous HSCs and T-cell



Figure 6. Efficient T-cell differentiation in vitro of SCID-X1 BM CD34⁺ HSPCs after transduction with BaEV-LVs encoding γ **C receptor.** SCID-X1 BM CD34⁺ HSPCs were transduced (BaEV) or not (mock) with BaEV-LVs encoding IL-2R γ C at an MOI of 10 for 6 hours after 16 hours of prestimulation with cytokines in either RetroNectin-coated wells (see supplemental Figure 6A). The X-SCID mutation is a breakpoint mutation in intron 3 of the IL-2RG gene, which is a γ C leaky mutation. Flow cytometry analyses indicating the percentage (A) and absolute cell number (B) of CD34⁺CD7⁺ and CD34⁻CD7⁺ T-cell progenitors after 4 days of culture in the presence (+ DII4) or absence (-DII4) of DII4. After 4 days of culture, the cells were further cultured with OP9-hDL1 stromal cells to pursue the T-cell differentiation process. Analyses of VCN per cell in the genome by duplex quantitative PCR in sorted CD7⁺ cells at week 5 (C) and IL-2R γ C mRNA expression by semi-quantitative RT-PCR in hCD45⁺ cells at week 7 (D) of differentiation (compared with CB CD34⁺ T-cell differentiation cultures in parallel). During coculture with OP9-hDL1 stromal cells, mock and BaEV-LV transduced cells were analyzed once per week by flow cytometry. Analyses of absolute cell numbers of CD4⁺CD8⁺ (E) and CD3⁺ cells (F) at the indicated weeks after OP9-hDL1 cocultures. (G) TCR repertoire analysis by multiplex PCR followed by GeneScan analysis in CD7⁺ cells sorted at week 5 after OP9-hDL1 cocultures. All the populations were gated on live 7-amino-actinomycin-D⁻ cells.

Shood advances 12 February 2019 · Volume 3, Number 3

progenitors might produce both short-term recovery of T-cell immune response and long-term correction in the patients.

Acknowledgments

The authors thank the staff of the Plateau de Biologie Expérimentale de la Souris animal care facility at the Ecole Normale Supérieure de Lyon, the flow cytometry platform (SFR BioSciences Lyon [UMS3444/US8], Lyon, France, and Imagine Institute, Paris, France), and Gisèle Froment, Didier Nègre, and Caroline Costa from the lentivectors production facility/SFR BioSciences Lyon (UMS3444/US8, Lyon, France).

This work was supported by French grants from AFM, ANRS, ARC, and LABEX and grants from the European Community (FP7-HEALTH-2007-B/222878 "PERSIST" and "GENTHERTHAL-PLUS"), by INSERM, a European Union H2020 grant (SCIDNet 666908), an INCA-Plan Cancer grant (2009-2013), and a public grant overseen by the French National Research Agency (ANR) as part of the Investissements d'Avenir program (reference: ANR-10-IAHU-01). R.M. was supported by a scholarship from the French ministry, and K.M. was supported by a scholarship from the China Scholarship Council.

Authorship

Contribution: E.V. coordinated the project, designed and performed experiments, analyzed and discussed the data, and wrote the manuscript; I.A. designed and performed experiments, analyzed and discussed the data, and wrote the manuscript; O.B., F.A., A.G.-G., C.C., C.L., D.N., and R.M. performed experiments, analyzed the data, and discussed results; R.D.M., H.S., C.L.-P., K.M., and M.C. performed T-cell differentiation of SCID-X1 CD34⁺ cells, analyzed data, discussed results, and corrected the manuscript; V.A. and A.C. provided human thymocytes, critical discussion, and technical advice; C.R. provided technical advice and helped with design of experiments; D.F. provided Vectofusin and technical advice; and F.-L.C. provided critical discussion and read the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests

ORCID profile: C.L.-P., 0000-0002-2216-6453.

Correspondence: Els Verhoeyen, Centre International de Recherche en Infectiologie, Enveloppes Virales, Vecteurs et Immunothérapie, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon, France; e-mail: els.verhoeyen@ens-lyon.fr.

References

- 1. Blaese RM, Culver KW, Miller AD, et al. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science*. 1995; 270(5235):475-480.
- 2. Bordignon C, Notarangelo LD, Nobili N, et al. Gene therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients. *Science*. 1995;270(5235):470-475.
- 3. Bonini C, Ferrari G, Verzeletti S, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science*. 1997; 276(5319):1719-1724.
- 4. Kalos M, Levine BL, Porter DL, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med.* 2011;3(95):95ra73.
- Buchschacher GL Jr, Wong-Staal F. Approaches to gene therapy for human immunodeficiency virus infection. Hum Gene Ther. 2001; 12(9):1013-1019.
- Aiuti A, Vai S, Mortellaro A, et al. Immune reconstitution in ADA-SCID after PBL gene therapy and discontinuation of enzyme replacement. Nat Med. 2002;8(5):423-425.
- Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science. 2000;288(5466):669-672.
- 8. Aiuti A, Slavin S, Aker M, et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science*. 2002; 296(5577):2410-2413.
- 9. Centlivre M, Legrand N, Klamer S, et al. Preclinical in vivo evaluation of the safety of a multi-shRNA-based gene therapy against HIV-1. *Mol Ther Nucleic Acids*. 2013;2:e120.
- Chung J, Zhang J, Li H, Ouellet DL, DiGiusto DL, Rossi JJ. Endogenous MCM7 microRNA cluster as a novel platform to multiplex small interfering and nucleolar RNAs for combinational HIV-1 gene therapy. *Hum Gene Ther.* 2012;23(11):1200-1208.
- 11. Tebas P, Stein D, Tang WW, et al. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N Engl J Med. 2014;370(10):901-910.
- 12. Bondanza A, Valtolina V, Magnani Z, et al. Suicide gene therapy of graft-versus-host disease induced by central memory human T lymphocytes. *Blood.* 2006;107(5):1828-1836.
- Cieri N, Mastaglio S, Oliveira G, Casucci M, Bondanza A, Bonini C. Adoptive immunotherapy with genetically modified lymphocytes in allogeneic stem cell transplantation. *Immunol Rev.* 2014;257(1):165-180.
- 14. Marin V, Cribioli E, Philip B, et al. Comparison of different suicide-gene strategies for the safety improvement of genetically manipulated T cells. Hum Gene Ther Methods. 2012;23(6):376-386.
- 15. Gattinoni L, Klebanoff CA, Palmer DC, et al. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest. 2005;115(6):1616-1626.
- Micklethwaite KP, Savoldo B, Hanley PJ, et al. Derivation of human T lymphocytes from cord blood and peripheral blood with antiviral and antileukemic specificity from a single culture as protection against infection and relapse after stem cell transplantation. *Blood.* 2010;115(13):2695-2703.

- 17. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*. 2006; 314(5796):126-129.
- Brentjens RJ, Davila ML, Riviere I, et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med. 2013;5(177):177ra38.
- 19. Girard-Gagnepain A, Amirache F, Costa C, et al. Baboon envelope pseudotyped LVs outperform VSV-G-LVs for gene transfer into cytokine-stimulated and resting HSCs. *Blood*. 2014;124(8):1221-1231.
- 20. Kochenderfer JN, Wilson WH, Janik JE, et al. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood*. 2010;116(20):4099-4102.
- 21. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med. 2011; 365(8):725-733.
- 22. Brown CE, Alizadeh D, Starr R, et al. Regression of glioblastoma after chimeric antigen receptor T-cell therapy. N Engl J Med. 2016;375(26):2561-2569.
- 23. Haso W, Lee DW, Shah NN, et al. Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia. *Blood.* 2013; 121(7):1165-1174.
- 24. Hegde M, Mukherjee M, Grada Z, et al. Tandem CAR T cells targeting HER2 and IL13Rα2 mitigate tumor antigen escape. J Clin Invest. 2016; 126(8):3036-3052.
- 25. Johnson LA, Scholler J, Ohkuri T, et al. Rational development and characterization of humanized anti-EGFR variant III chimeric antigen receptor T cells for glioblastoma. *Sci Transl Med.* 2015;7(275):275ra22.
- 26. Shaffer DR, Savoldo B, Yi Z, et al. T cells redirected against CD70 for the immunotherapy of CD70-positive malignancies. *Blood.* 2011; 117(16):4304-4314.
- 27. Gargett T, Yu W, Dotti G, et al. GD2-specific CAR T cells undergo potent activation and deletion following antigen encounter but can be protected from activation-induced cell death by PD-1 blockade. *Mol Ther.* 2016;24(6):1135-1149.
- 28. Robbins PF, Morgan RA, Feldman SA, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol. 2011;29(7):917-924.
- 29. Robbins PF, Dudley ME, Wunderlich J, et al. Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. J Immunol. 2004;173(12):7125-7130.
- Rosenberg SA, Yang JC, Sherry RM, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res.* 2011;17(13):4550-4557.
- Klebanoff CA, Gattinoni L, Torabi-Parizi P, et al. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci USA. 2005;102(27):9571-9576.
- 32. Hassan J, Reen DJ. Human recent thymic emigrants-identification, expansion, and survival characteristics. J Immunol. 2001;167(4):1970-1976.
- Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. J Clin Invest. 2008;118(1):294-305.
- 34. Marshall-Clarke S, Reen D, Tasker L, Hassan J. Neonatal immunity: how well has it grown up? Immunol Today. 2000;21(1):35-41.
- Assing K, Nielsen C, Kirchhoff M, Madsen HO, Ryder LP, Fisker N. CD4+ CD31+ recent thymic emigrants in CHD7 haploinsufficiency (CHARGE syndrome): a case. *Hum Immunol.* 2013;74(9):1047-1050.
- Kimmig S, Przybylski GK, Schmidt CA, et al. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. J Exp Med. 2002;195(6):789-794.
- Soares MV, Borthwick NJ, Maini MK, Janossy G, Salmon M, Akbar AN. IL-7-dependent extrathymic expansion of CD45RA+ T cells enables preservation of a naive repertoire. J Immunol. 1998;161(11):5909-5917.
- Swainson L, Verhoeyen E, Cosset FL, Taylor N. IL-7R alpha gene expression is inversely correlated with cell cycle progression in IL-7-stimulated T lymphocytes. J Immunol. 2006;176(11):6702-6708.
- Azevedo RI, Soares MV, Barata JT, et al. IL-7 sustains CD31 expression in human naive CD4+ T cells and preferentially expands the CD31+ subset in a PI3K-dependent manner. *Blood.* 2009;113(13):2999-3007.
- Pegram HJ, Purdon TJ, van Leeuwen DG, et al. IL-12-secreting CD19-targeted cord blood-derived T cells for the immunotherapy of B-cell acute lymphoblastic leukemia. Leukemia. 2015;29(2):415-422.
- 41. Merindol N, Grenier AJ, Caty M, et al. Umbilical cord blood T cells respond against the Melan-A/MART-1 tumor antigen and exhibit reduced alloreactivity as compared with adult blood-derived T cells. J Immunol. 2010;185(2):856-866.
- 42. Serrano LM, Pfeiffer T, Olivares S, et al. Differentiation of naive cord-blood T cells into CD19-specific cytolytic effectors for posttransplantation adoptive immunotherapy. *Blood.* 2006;107(7):2643-2652.
- Khoury SJ, Sayegh MH, Hancock WW, Gallon L, Carpenter CB, Weiner HL. Acquired tolerance to experimental autoimmune encephalomyelitis by intrathymic injection of myelin basic protein or its major encephalitogenic peptide. J Exp Med. 1993;178(2):559-566.
- 44. Marodon G, Fisson S, Levacher B, Fabre M, Salomon BL, Klatzmann D. Induction of antigen-specific tolerance by intrathymic injection of lentiviral vectors. Blood. 2006;108(9):2972-2978.
- 45. Posselt AM, Barker CF, Friedman AL, Naji A. Prevention of autoimmune diabetes in the BB rat by intrathymic islet transplantation at birth. *Science*. 1992;256(5061):1321-1324.

- 46. Adjali O, Marodon G, Steinberg M, et al. In vivo correction of ZAP-70 immunodeficiency by intrathymic gene transfer. J Clin Invest. 2005; 115(8):2287-2295.
- 47. Hozumi K, Mailhos C, Negishi N, et al. Delta-like 4 is indispensable in thymic environment specific for T cell development. J Exp Med. 2008; 205(11):2507-2513.
- 48. Koch U, Fiorini E, Benedito R, et al. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. J Exp Med. 2008;205(11):2515-2523.
- 49. Lefort N, Benne C, Lelièvre JD, et al. Short exposure to Notch ligand delta-4 is sufficient to induce T-cell differentiation program and to increase the T cell potential of primary human CD34+ cells. *Exp Hematol.* 2006;34(12):1720-1729.
- Reimann C, Six E, Dal-Cortivo L, et al. Human T-lymphoid progenitors generated in a feeder-cell-free delta-like-4 culture system promote T-cell reconstitution in NOD/SCID/γc(-/-) mice. Stem Cells. 2012;30(8):1771-1780.
- 51. Simons L, Ma K, de Chappedelaine C, et al. Generation of adult human T-cell progenitors for immunotherapeutic applications. J Allergy Clin Immunol. 2018;141(4):1491-1494.e4.
- Dardalhon V, Jaleco S, Kinet S, et al. IL-7 differentially regulates cell cycle progression and HIV-1-based vector infection in neonatal and adult CD4+ T cells. Proc Natl Acad Sci USA. 2001;98(16):9277-9282.
- 53. Korin YD, Zack JA. Progression to the G1b phase of the cell cycle is required for completion of human immunodeficiency virus type 1 reverse transcription in T cells. J Virol. 1998;72(4):3161-3168.
- 54. Verhoeyen E, Dardalhon V, Ducrey-Rundquist O, Trono D, Taylor N, Cosset FL. IL-7 surface-engineered lentiviral vectors promote survival and efficient gene transfer in resting primary T lymphocytes. *Blood.* 2003;101(6):2167-2174.
- 55. Amirache F, Lévy C, Costa C, et al. Mystery solved: VSV-G-LVs do not allow efficient gene transfer into unstimulated T cells, B cells, and HSCs because they lack the LDL receptor. *Blood.* 2014;123(9):1422-1424.
- 56. Ferrand C, Robinet E, Contassot E, et al. Retrovirus-mediated gene transfer in primary T lymphocytes: influence of the transduction/selection process and of ex vivo expansion on the T cell receptor beta chain hypervariable region repertoire. *Hum Gene Ther.* 2000;11(8):1151-1164.
- 57. Marktel S, Magnani Z, Ciceri F, et al. Immunologic potential of donor lymphocytes expressing a suicide gene for early immune reconstitution after hematopoietic T-cell-depleted stem cell transplantation. *Blood.* 2003;101(4):1290-1298.
- Cavalieri S, Cazzaniga S, Geuna M, et al. Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. *Blood.* 2003;102(2):497-505.
- Ducrey-Rundquist O, Guyader M, Trono D. Modalities of interleukin-7-induced human immunodeficiency virus permissiveness in quiescent T lymphocytes. J Virol. 2002;76(18):9103-9111.
- 60. Unutmaz D, KewalRamani VN, Marmon S, Littman DR. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. J Exp Med. 1999;189(11):1735-1746.
- 61. Frecha C, Costa C, Lévy C, et al. Efficient and stable transduction of resting B lymphocytes and primary chronic lymphocyte leukemia cells using measles virus gp displaying lentiviral vectors. *Blood.* 2009;114(15):3173-3180.
- 62. Frecha C, Costa C, Nègre D, et al. Stable transduction of quiescent T cells without induction of cycle progression by a novel lentiviral vector pseudotyped with measles virus glycoproteins. *Blood.* 2008;112(13):4843-4852.
- 63. Frecha C, Lévy C, Cosset FL, Verhoeyen E. Advances in the field of lentivector-based transduction of T and B lymphocytes for gene therapy. *Mol Ther.* 2010;18(10):1748-1757.
- 64. Sandrin V, Boson B, Salmon P, et al. Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and nonhuman primates. *Blood.* 2002;100(3):823-832.
- 65. Rasko JE, Battini JL, Gottschalk RJ, Mazo I, Miller AD. The RD114/simian type D retrovirus receptor is a neutral amino acid transporter. Proc Natl Acad Sci USA. 1999;96(5):2129-2134.
- Tailor CS, Marin M, Nouri A, Kavanaugh MP, Kabat D. Truncated forms of the dual function human ASCT2 neutral amino acid transporter/retroviral receptor are translationally initiated at multiple alternative CUG and GUG codons. J Biol Chem. 2001;276(29):27221-27230.
- 67. Marin M, Tailor CS, Nouri A, Kabat D. Sodium-dependent neutral amino acid transporter type 1 is an auxiliary receptor for baboon endogenous retrovirus. *J Virol.* 2000;74(17):8085-8093.
- Fusil F, Calattini S, Amirache F, et al. A lentiviral vector allowing physiologically regulated membrane-anchored and secreted antibody expression depending on B-cell maturation status. *Mol Ther.* 2015;23(11):1734-1747.
- 69. Girard-Gagnepain A, Amirache F, Costa C, et al. Baboon envelope pseudotyped LVs outperform VSV-G-LVs for gene transfer into early-cytokinestimulated and resting HSCs. *Blood.* 2014;124(8):1221-1231.
- Levy C, Fusil F, Amirache F, et al. Baboon envelope pseudotyped lentiviral vectors efficiently transduce human B cells and allow active factor IX B cell secretion in vivo in NOD/SCIDyc^{-/-} mice. J Thromb Haemost. 2016;14(12):2478-2492.
- 71. Lévy C, Amirache F, Girard-Gagnepain A, et al. Measles virus envelope pseudotyped lentiviral vectors transduce quiescent human HSCs at an efficiency without precedent. *Blood Adv.* 2017;1(23):2088-2104.
- Maurice M, Verhoeyen E, Salmon P, Trono D, Russell SJ, Cosset FL. Efficient gene transfer into human primary blood lymphocytes by surface-engineered lentiviral vectors that display a T cell-activating polypeptide. *Blood*. 2002;99(7):2342-2350.
- 73. Six EM, Benjelloun F, Garrigue A, et al. Cytokines and culture medium have a major impact on human in vitro T-cell differentiation. *Blood Cells Mol Dis.* 2011;47(1):72-78.

- 74. van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17(12):2257-2317.
- 75. Villarese P, Lours C, Trinquand A, et al. TCRα rearrangements identify a subgroup of NKL-deregulated adult T-ALLs associated with favorable outcome. *Leukemia*. 2018;32(1):61-71.
- Frecha C, Lévy C, Costa C, et al. Measles virus glycoprotein-pseudotyped lentiviral vector-mediated gene transfer into quiescent lymphocytes requires binding to both SLAM and CD46 entry receptors. J Virol. 2011;85(12):5975-5985.
- 77. Hübner J, Hoseini SS, Suerth JD, et al. Generation of genetically engineered precursor T-cells from human umbilical cord blood using an optimized alpharetroviral vector platform. *Mol Ther.* 2016;24(7):1216-1226.
- Majdoul S, Seye AK, Kichler A, et al. Molecular determinants of vectofusin-1 and its derivatives for the enhancement of lentivirally mediated gene transfer into hematopoietic stem/progenitor cells. J Biol Chem. 2016;291(5):2161-2169.
- Hacein-Bey-Abina S, Pai SY, Gaspar HB, et al. A modified γ-retrovirus vector for X-linked severe combined immunodeficiency. N Engl J Med. 2014; 371(15):1407-1417.
- Marin M, Lavillette D, Kelly SM, Kabat D. N-linked glycosylation and sequence changes in a critical negative control region of the ASCT1 and ASCT2 neutral amino acid transporters determine their retroviral receptor functions. J Virol. 2003;77(5):2936-2945.
- Frumento G, Zheng Y, Aubert G, et al. Cord blood T cells retain early differentiation phenotype suitable for immunotherapy after TCR gene transfer to confer EBV specificity. Am J Transplant. 2013;13(1):45-55.
- Laughlin MJ, Barker J, Bambach B, et al. Hematopoietic engraftment and survival in adult recipients of umbilical-cord blood from unrelated donors. N Engl J Med. 2001;344(24):1815-1822.
- Rocha V, Labopin M, Sanz G, et al; Eurocord-Netcord Registry. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. N Engl J Med. 2004;351(22):2276-2285.
- Admiraal R, de Koning CCH, Lindemans CA, et al. Viral reactivations and associated outcomes in the context of immune reconstitution after pediatric hematopoietic cell transplantation. J Allergy Clin Immunol. 2017;140(6):1643-1650.e9.
- Heimall J, Logan BR, Cowan MJ, et al. Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. *Blood.* 2017;130(25):2718-2727.
- Storek J, Geddes M, Khan F, et al. Reconstitution of the immune system after hematopoietic stem cell transplantation in humans. Semin Immunopathol. 2008;30(4):425-437.
- 87. Komanduri KV, St John LS, de Lima M, et al. Delayed immune reconstitution after cord blood transplantation is characterized by impaired thymopoiesis and late memory T-cell skewing. *Blood*. 2007;110(13):4543-4551.
- 88. Parkman R, Weinberg KI. Immunological reconstitution following bone marrow transplantation. Immunol Rev. 1997;157(1):73-78.

Article 7

Cell Metabolism

GAPDH Expression Predicts the Response to R-CHOP, the Tumor Metabolic Status, and the Response of DLBCL Patients to Metabolic Inhibitors

Graphical Abstract



Highlights

- GAPDH is a clinical biomarker of DLBCL response to R-CHOP
- GAPDH expression predicts B cell lymphoma metabolic status
- GAPDH^{low} B cell lymphomas are sensitive to mitochondrial metabolism inhibitors
- DLBCL-GAPDH^{/ow} R-CHOP refractory patients respond to specific metabolic inhibitors

Authors

Johanna Chiche, Julie Reverso-Meinietti, Annabelle Mouchotte, ..., Nicolas Mounier, Catherine Thieblemont, Jean-Ehrland Ricci

Correspondence

catherine.thieblemont@sls.aphp.fr (C.T.), ricci@unice.fr (J.-E.R.)

In Brief

About 40% of diffuse large B cell lymphoma (DLBCL) patients are refractory to the combined immunochemotherapy (R-CHOP) standard of care. DLBCL is metabolically heterogeneous, and Chiche et al. show that low levels of GAPDH, which predict poor R-CHOP response, are associated with OxPhos metabolism, mTORC1 signaling, and glutaminolysis. Disruptors of these pathways improved GAPDH^{low} DLBCL response in mice and patients.



Chiche et al., 2019, Cell Metabolism 29, 1243–1257 June 4, 2019 © 2019 Elsevier Inc. https://doi.org/10.1016/j.cmet.2019.02.002



GAPDH Expression Predicts the Response to R-CHOP, the Tumor Metabolic Status, and the Response of DLBCL Patients to Metabolic Inhibitors

Johanna Chiche,¹ Julie Reverso-Meinietti,^{1,2} Annabelle Mouchotte,¹ Camila Rubio-Patiño,¹ Rana Mhaidly,¹ Elodie Villa,¹ Jozef P. Bossowski,¹ Emma Proics,¹ Manuel Grima-Reyes,¹ Agnès Paquet,³ Konstantina Fragaki,^{4,5,6} Sandrine Marchetti,¹ Josette Briere,⁷ Damien Ambrosetti,^{2,5} Jean-François Michiels,^{2,4} Thierry Jo Molina,⁸ Christiane Copie-Bergman,⁹ Jacqueline Lehmann-Che,¹⁰ Isabelle Peyrottes,¹¹ Frederic Peyrade,¹¹ Eric de Kerviler,¹² Bruno Taillan,¹³ Georges Garnier,¹³ Els Verhoeyen,¹ Véronique Paquis-Flucklinger,^{4,5,6} Laetitia Shintu,¹⁴ Vincent Delwail,¹⁵ Celine Delpech-Debiais,¹⁶ Richard Delarue,¹⁷ André Bosly,¹⁸ Tony Petrella,¹⁹ Gabriel Brisou,²⁰ Bertrand Nadel,²⁰ Pascal Barbry,³ Nicolas Mounier,²¹ Catherine Thieblemont,^{7,22,*} and Jean-Ehrland Ricci^{1,23,*} ¹Université Côte d'Azur, INSERM, C3M, Nice, France ²Laboratoire Central d'AnatomocytoPathologie (LCAP), CHU de Nice, Nice, France

³Université Côte d'Azur, CNRS, Institut de Pharmacologie Moléculaire et Cellulaire, 06560 Sophia Antipolis, France ⁴Université Côte d'Azur, Nice, France

⁵IRCAN, UMR CNRS 7284/INSERM U1081/UNS, School of Medicine, Nice Sophia-Antipolis University, Nice, France ⁶Department of Medical Genetics, National Centre for Mitochondrial Diseases, Nice Teaching Hospital, Nice, France

⁷APHP Hopital Saint-Iouis, Hemato-oncologie – Université Paris Diderot, Sorbonne Paris Cité, Paris, France

⁸Département de Pathologie, Hôpital Necker, AP-HP, EA 7324, Université Paris Descartes, Sorbonne Paris Cité, Paris, France ⁹Department of Pathology, Henri Mondor Hospital, APHP, INSERM U955, Paris-Est Créteil University, Créteil, France

¹⁰AP-HP, Hôpital Saint Louis, Unité d'oncologie moléculaire, Université Paris Diderot, Sorbonne Paris Cité, 75010 Paris, France ¹¹Centre Antoine-Lacassagne, Nice, France

¹²APHP, Hopital Saint-Louis, Service de Radiologie, Université Paris Diderot, Sorbonne Paris Cité, Paris 75010, France ¹³Centre Hospitalier Princesse Grace de Monaco, Monaco, Monaco

¹⁴Aix Marseille University, CNRS, Centrale Marseille, Institut des Sciences Moléculaires de Marseille (ISM2), Marseille, France 15 Service d'Oncologie Hématologique et de Thérapie Cellulaire, CHU de Poitiers, INSERM, CIC 1402, Centre d'Investigation Clinique,

Université de Poitiers, Poitiers, France

¹⁶Department of Pathology, CHU/Université de Poitiers, Poitiers, France

¹⁷Department of Hematology, AP-HP Hôpital Necker, Paris, France

¹⁸CHU Dinant Godinne, UcL Namur, Yvoir, Belgium

¹⁹Department of Pathology, Hôpital Maisonneuve-Rosemont, Montréal, QC, Canada

²⁰Aix Marseille University, CNRS, INSERM, CIML, Centre d'Immunologie de Marseille-Luminy, Marseille, France

²¹Centre Hospitalier Universitaire de Nice, Département d'Onco-Hématologie, Nice, France

²²NF-kappaB, Différenciation et Cancer, Université Paris Descartes, Paris EA7324, France

²³Lead Contact

*Correspondence: catherine.thieblemont@sls.aphp.fr (C.T.), ricci@unice.fr (J.-E.R.)

https://doi.org/10.1016/j.cmet.2019.02.002

SUMMARY

Diffuse large B cell lymphoma (DLBCL) is a heterogeneous disease treated with anti-CD20-based immuno-chemotherapy (R-CHOP). We identified that low levels of GAPDH predict a poor response to R-CHOP treatment. Importantly, we demonstrated that GAPDH^{low} lymphomas use OxPhos metabolism and rely on mTORC1 signaling and glutaminolysis. Consistently, disruptors of OxPhos metabolism (phenformin) or glutaminolysis (L-asparaginase) induce cytotoxic responses in GAPDH^{low} B cells and improve GAPDH^{low} B cell-lymphoma-bearing mice survival, while they are low or not efficient on GAPDH high B cell lymphomas. Ultimately, we selected four GAPDH^{low} DLBCL patients, who were refractory to all anti-CD20-based therapies, and targeted DLBCL metabolism using L-asparaginase (K), mTOR inhibitor (T), and metformin (M) (called KTM therapy). Three out of the four patients presented a complete response upon one cycle of KTM. These findings establish that the GAPDH expression level predicts DLBCL patients' response to R-CHOP treatment and their sensitivity to specific metabolic inhibitors.

INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) is a genetically heterogeneous group of tumors and the most common type of lymphoid malignancies (Swerdlow et al., 2016). DLBCLs represent highly metabolically active tumors previously treated with a polychemotherapy-cyclophosphamide, hydroxydaunorubicin, Oncovin, and prednisone (CHOP) (Fisher et al., 1993). Since 1998, immunotherapy anti-CD20 (rituximab) combined with CHOP (referred as R-CHOP) became the standard treatment for this disease, as it significantly improved the survival of patients





Figure 1. DLBCLs Expressing Low Levels of GAPDH Are Associated with a Poor Response to R-CHOP Treatment and with OxPhos Metabolism

(A) Distribution of *gapdh* expression values (log₂ mRNA intensity) in newly diagnosed DLBCLs characterized as DLBCLs-*gapdh*^{low} (n = 125) and DLBCLs-*gapdh*^{low} (n = 108) (analysis from Lenz et al., 2008). p value from Wilcoxon rank-sum test.

1244 Cell Metabolism 29, 1243–1257, June 4, 2019

(legend continued on next page)

(Coiffier et al., 2002). However, approximately 40% of patients with DLBCL still experience therapeutic failure with R-CHOP (Sehn and Gascoyne, 2015). The molecular heterogeneity of DLBCLs is considered a major factor influencing the response to R-CHOP therapy (Lenz and Staudt, 2010; Lenz et al., 2008). The cell-of-origin (COO) classification delineates DLBCL subsets into distinct transcriptional profiles: the germinal center B cell (GCB)-, the activated B cell (ABC)-, and the unclassified-DLBCL subtypes (Alizadeh et al., 2000). Unlike GCB-DLBCLs, ABC-DLBCLs are associated with a poor outcome with R-CHOP treatment (Lenz et al., 2008). Comparison of other gene signatures highlighted three other DLBCL clusters that are recapitulated in the consensus cluster classification (CCC): (1) the B cell receptor (BCR)/proliferation (BCR-DLBCL) upregulates genes encoding BCR signaling components; (2) the oxidative phosphorylation (OxPhos) cluster (OxPhos-DLBCL) is enriched in genes involved in electron transport chain (ETC) complexes, OxPhos metabolism, and other mitochondrial functions; and (3) the host response (HR) cluster is characterized by a host inflammatory infiltrate (Monti et al., 2005). Importantly, there is no link between the COO and the CCC classifications (Caro et al., 2012; Monti et al., 2005). Further characterization demonstrated that OxPhos-DLBCL cell lines lack the BCR and are insensitive to the inhibition of the BCR signaling pathway (Chen et al., 2008). In contrast, BCR-DLBCL cell lines express a functional BCR and primarily rely on glycolysis to produce energy (Caro et al., 2012; Chen et al., 2008). Altogether those studies have identified, for the first time, the metabolic heterogeneity within the same tumor entity (Caro et al., 2012; Monti et al., 2005; Norberg et al., 2017). However, whether distinct metabolic fingerprints influence DLBCL response to R-CHOP remains unknown. Moreover, it is unclear whether targeting DLBCL metabolic specificity might be a valuable therapeutic approach in the clinic, in particular for R-CHOP low responder patients. In this study, we addressed two unmet clinical needs: (1) can we identify, at the diagnosis, which DLCBL patients are likely to be low responders to R-CHOP? (2) Can we foretell if those patients will be likely to respond to specific metabolic inhibitors according to the metabolic status of their DLBCL? Using unbiased analysis, we identified the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the only glycolytic enzyme able to predict the overall survival (OS) of patients with DLBCL treated with R-CHOP. Moreover, we established in vivo as well as in

the clinic, that GAPDH holds potential as a marker of DLBCL metabolic status and of patients' response to specific metabolic inhibitors.

RESULTS

GAPDH Expression Predicts Overall Survival of Patients with DLBCL Treated with R-CHOP and DLBCL Metabolic Status

Unbiased analysis of expression profile dataset from 233 newly diagnosed DLBCLs (Lenz et al., 2008) allowed us to identify 587 probesets (p < 0.05), corresponding to 524 unique genes associated with differences in OS upon R-CHOP treatment (Table S1; Figure S1A). Analysis of molecular functions associated with OS predicting genes identified classical pathways involved in resistance to chemotherapies (e.g., cell survival, proliferation, and DNA repair) and highlighted the mitochondrial energetic function (Table S1; Figure S1B). In contrast, only one gene involved in glycolysis, glyceraldehyde-3-phosphate dehydrogenase (gapdh), predicts a favorable outcome when highly expressed (3 independent probesets: HR = 0.53, 0.53, and 0.54 and corresponding p value = 0.01573, 0.01771, and 0.01929; Table S1). Of note, we also observed that lactate dehydrogenase b subunit (Idhb) is significantly associated with a poor outcome (Table S1). Though LDH-B converts lactate to pyruvate, a source for OxPhos metabolism or for gluconeogenesis (Doherty and Cleveland, 2013), we focused our attention on gapdh.

When subgrouping DLBCLs according to *gapdh* mRNA levels (high or low; Figure 1A), patients with DLBCL-*gapdh*^{low} have a lower OS upon R-CHOP than those with DLBCL-*gapdh*^{low} (Figure 1B). To further determine whether GAPDH protein expression predicts differences in OS upon R-CHOP, we set up an automated immunohistochemical (IHC) staining of GAPDH (Figure 1C) and a scoring system to quantify its expression in 43 newly diagnosed DLBCL (training cohort; Table S2). As for *gapdh* mRNA (Figure 1B), DLBCLs expressing low levels of GAPDH protein (GAPDH^{low}, score \leq 150) are associated with a poorer OS upon R-CHOP than DLBCLs expressing high levels of GAPDH (GAPDH^{high}, score > 150) (Figures 1D and 1E; see also STAR Methods for determination of GAPDH IHC score and cutoff of 150). This finding was confirmed in a larger cohort of 294 *de novo* DLBCLs treated with R-CHOP (validation cohort; Table S2)

⁽B) Overall survival (OS) of R-CHOP-treated DLBCL patients, according to *gapdh*^{low} (n = 125) and *gapdh*^{high} (n = 108) mRNA levels in DLBCL biopsies at diagnosis (analysis from Lenz et al., 2008). p value from log-rank test.

⁽C) Illustration of IHC staining of GAPDH in germinal center (GC) B cells of a reactive lymph node (LN) (score = 80) and in *de novo* DLBCL expressing low (score = 80) or high (score = 260) levels of GAPDH protein. Magnification 20× (100 μm).

⁽D) Distribution of GAPDH protein expression scored by IHC in GC B cells from human paraffin-embedded reactive LNs (n = 5) or newly diagnosed DLBCLs expressing low (score \leq 150; n = 15) or high (score > 150; n = 28) GAPDH protein (training cohort). p value from Wilcoxon rank-sum test.

⁽E) OS of R-CHOP-treated DLBCL patients according to the levels of GAPDH expression determined by IHC in newly diagnosed DLBCLs (n = 15 GAPDH^{low} and n = 28 GAPDH^{high}, training cohort). p value from log-rank test.

⁽F) Distribution of GAPDH protein expression scored by IHC staining of GAPDH in tissue-microarray corresponding to 294 paraffin-embedded *de novo* DLBCL biopsies. 204 DLBCLs-GAPDH^{low} and 90 DLBCLs-GAPDH^{bigh} are identified (validation cohort). p value from Wilcoxon rank-sum test.

⁽G) OS of 294 R-CHOP-treated patients with DLBCLs grouped on the basis of GAPDH expression levels presented in (F). p value from log-rank test.

⁽H) Transcript abundance (probe intensity) of *gapdh* mRNA according to the CCC classification (n = 82 BCR-DLBCL and n = 71 OxPhos-DLBCL) identified by genomic clustering (Monti et al., 2005); analysis from Lenz et al. (2008). p value from Wilcoxon rank-sum test.

⁽I) Correlation between GAPDH expression levels scored by IHC and glycolytic ATP in malignant B cells harvested from patients with newly diagnosed DLBCLs. Blue dots represent DLBCLs-GAPDH^{low} (n = 3), and red dots represent DLBCL-GAPDH^{high} (n = 8). R, Pearson correlation coefficient. p value from correlation test. See also Tables S1, S2, and S3 and Figures S1 and S2.

Table 1. GAPDH Expression Levels Are Not Associated with Other Biological Prognostic Factors

		Training Cohort (n = 43)					Validation Cohort (n = 294)					
			DH ^{low}	GAPDH high			GAPDH low		GAPDH high			
DLBCL		(n = 1	5)	(n = 28)			(n = 204)		(n = 90)			
Parameters		No.	%	No.	%	p Value	No.	%	No.	%	p Value	
Bcl2	<70%	1	6.7	10	35.7	0.0648	66 <i>(a)</i>	37.7	35 <i>(b)</i>	40.2	0.7876	
	≥70%	14	93.3	18	64.3	-	109 <i>(a</i>)	62.3	52 <i>(b)</i>	59.8	-	
Bcl6	≤25%	6	40	18	64.3	0.1982	63 <i>(c)</i>	36.2	33 <i>(d)</i>	39.3	0.6808	
	>25%	9	60	10	35.7	-	111 <i>(c)</i>	63.8	51 <i>(d)</i>	60.7	-	
Мус	<40%	5	33.3	23 <i>(e)</i>	85.2	0.0014	98 <i>(f)</i>	62.4	48 <i>(g)</i>	72.7	0.1657	
	\geq 40%	10	66.7	4 <i>(e)</i>	14.8	-	59 <i>(f)</i>	37.6	18 <i>(g)</i>	27.3	-	
DE (Myc-Bcl2)	Myc < 40%; Bcl2 < 70%	6	40	26 <i>(e)</i>	85.2	9.38 e-05	115 <i>(h)</i>	73.3	51 <i>(i)</i>	78.5	0.4990	
	Myc \geq 40%; Bcl2 \geq 70%	9	60	1 <i>(e)</i>	14.8	-	41 <i>(h)</i>	26.3	14 <i>(i)</i>	21.5	-	
Double Hit Myc/Bcl2	No	4 <i>(j)</i>	100	11 <i>(k)</i>	91.67	1.0000	148 <i>(l)</i>	92.5	60 <i>(m)</i>	95.2	0.5652	
	Yes	0 <i>(j)</i>	0	1 <i>(k)</i>	8.33	-	12 <i>(l</i>)	7.5	3 (m)	4.8	-	
Triple Hit Myc/Bcl2/Bcl6	No	4 <i>(j)</i>	100	12 <i>(k)</i>	100	n.a.	158 <i>(l)</i>	98.7	62 <i>(m)</i>	98.4	1.0000	
	Yes	0 <i>(j)</i>	0	0 <i>(k)</i>	0	-	2 (1)	1.3	1 <i>(m)</i>	1.6	-	

 $BCL2 \ge 70\%$ means that 70% (or more) of malignant B cells were stained for BCL2 protein by IHC (Green et al., 2012; Petrella et al., 2017); BCL6 > 25% means up to 25% of malignant B cells were stained for BCL6 by IHC (Horn et al., 2013); MYC $\ge 40\%$ (Johnson et al., 2012) means that 40% (or more) of malignant B cells were stained for MYC protein by IHC. Double hit (*MYC/BCL2*) and triple hit (*MYC/BCL2/BCL6*) were obtained by fluorescence *in situ* hybridization (FISH). p value from Fisher's test. Number of samples that could not be analyzed: (a) 29; (b) 3; (c) 30; (d) 6; (e) 1; (f) 47; (g) 24; (h) 48; (i) 25; (j) 11; (k) 16; (l) 44; (m) 27. DE, double expressors (Myc-Bcl2); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; R-CHOP, rituximab with cyclophosphamide, doxorubicin, Oncovin, and prednisone; n.a., not applicable. See also Tables S2 and S3.

(Delarue et al., 2013) by staining and scoring GAPDH from a paraffin-embedded tissue microarray (TMA) using the previously established IHC score cutoff (Figures 1F and 1G).

Interestingly, GAPDH expression is not associated with clinical factors: age-adjusted International Prognostic Index (aaIPI), or COO or Hans classification (Hans et al., 2004), or with expression of already established bad prognosis markers (Bcl2, Bcl6, and Myc) and translocations (double hit MYC/BCL2 and triple hit MYC/BCL2/BCL6) (Horn et al., 2013; Johnson et al., 2012) (Table 1). Of note, there is no difference in OS noted on the basis of BCL6, or MYC or MYC-BCL2 expressions in the validation cohort. However, BCL2 expression significantly impacts the OS (Petrella et al., 2017). Multivariate analysis showed that GAPDH^{high} expression remained a significant marker predicting improved OS (HR = 0.603, p = 0.0371), independently of BCL2 (HR = 1.686, p = 0.0213). When compared to the aaIPI, multivariate analysis showed that GAPDH expression also remained a significant marker predicting improved OS in the training and validation cohorts (Table S3).

Altogether, these results demonstrate that the predictive value of GAPDH expression levels is independent of known biomarkers/factors of poor prognosis.

According to the CCC classification, we observed that 69% of DLBCL-*gapdh*^{low} (n = 49 out of 71) are OxPhos-DLBCLs, while 61% of DLBCL-*gapdh*^{high} (n = 51 out of 82) belong to the BCR-DLBCL relying on glycolysis (two-sided p = 0.00019; analysis from Lenz et al., 2008 cohort). OxPhos-DLBCLs significantly express lower levels of *gapdh* mRNA than BCR-DLBCLs (Figure 1H). In addition, we did not observe an association between GAPDH expression levels and specific COO subtypes in the training and validation cohorts (Table S2), consistent with the absence of correlation between the CCC and the COO classification (Caro et al., 2012; Monti et al., 2005). Moreover, pa-

tients with BCR- or OxPhos-DLBCL display the same outcome with CHOP treatment (Monti et al., 2005). Accordingly, distinct *gapdh* mRNA levels display similar OS when DLBCLs are treated with the polychemotherapy CHOP only (Figure S1C).

Arguments from the literature suggest that OxPhos-DLBCL might be less sensitive than BCR-DLBCL to rituximab because of the lack of Syk (spleen tyrosine kinase)-dependent BCR signaling (Kheirallah et al., 2010). Compared with BCR-DLBCL cell lines (SU-DHL-4 and SU-DHL-6), i.e., glycolytic lymphoma cells expressing a functional BCR (IgM or IgG) (Chen et al., 2008), OxPhos-DLBCL cell lines (Toledo and Karpas 422) express lower levels of GAPDH, secrete less lactate, express neither IgM nor IgG, and are less sensitive to rituximab-induced death (Figures S2A-S2D). Of note, GAPDH expression (mRNA and protein) is not influenced upon BCR activation with anti-IgM in SU-DHL-6 (Figures S2E-S2G). In another approach, we investigated the energetic status of malignant B cells harvested from fresh micro-biopsies of primary DLBCL tumors, according to the level of GAPDH. DLBCL-GAPDH^{low} cells produce less glycolytic ATP than DLBCL-GAPDH^{high} cells, and GAPDH expression significantly correlates with the percentage ATP generated from glycolysis (Figure 1I).

We concluded that DLBCL-GAPDH^{low} are low responders to R-CHOP treatment and mainly rely on OxPhos metabolism to fulfill their energetic demand.

Mouse Primary B Cell Lymphomas Expressing Low Levels of GAPDH Are Sensitive to the Inhibition of Mitochondrial ETC Complex I

Identification of a novel biomarker predicting DLBCL sensitivity to current treatment is only of clinical interest if we can propose new therapeutic options. Since R-CHOP low responders, i.e., DLBCL-GAPDH^{low}, primarily rely on OxPhos metabolism



Figure 2. Mouse Primary B Cell Lymphomas Expressing Low Levels of GAPDH Rely on OxPhos Metabolism and Are Sensitive to Inhibition of the Electron Transport Chain Complex I Activity

(A) Quantification of intracellular ATP levels produced in Eμ-Myc-GAPDH^{low} and in Eμ-Myc-GAPDH^{high} cells (n = 5 independent lymphomas per group). Results are expressed as relative luminescence units normalized by live cell number.

(B) Glycolytic ATP production measured as the percentage of total ATP produced by E_{μ} -Myc-GAPDH^{low} and E_{μ} -Myc-GAPDH^{high} cells (n = 5 independent lymphomas per group). Data are expressed as mean \pm SD (n = 3 independent experiments).

(C) Lactate secretion was determined in the supernatant of Eµ-Myc-GAPDH^{low} and of Eµ-Myc-GAPDH^{high} cells (n = 5 independent lymphomas per group), 24 h after seeding (mean ± SD; n = 3 independent experiments).

(D) Baseline oxygen consumption rate (OCR) of E μ -Myc-GAPDH^{low} and of E μ -Myc-GAPDH^{high} cells was determined with Clarck electrode 24 h after seeding (n = 5 independent lymphomas per group). Data are expressed as mean ± SD (n = 3 independent experiments).

(E) Eµ-Myc-GAPDH^{low} and Eµ-Myc-GAPDH^{high} cells (n = 3 independent lymphomas per group) were seeded in the presence or absence (Ctl, DMSO) of phenformin (Phen, 300 µM) for 24 h. Cell death was determined by DAPI staining and analyzed by flow cytometry. Data are expressed as mean ± SD (n = 4 independent experiments).

(F and G) Kaplan–Meier curves for the survival of syngeneic C57BL/6 mice intravenously injected with primary Eµ-Myc-GAPDH^{low} (F) or Eµ-Myc-GAPDH^{high} (G) cells and treated 6 days later, with either vehicle (H₂O) or 100 mg/kg/day of phenformin, orally and daily during 3 weeks (n = 10 mice per group, p value from log-rank test, *** p< 0.001).

(A)–(E), ***p < 0.001 indicated the significance of the observed differences. ns, not significant. See also Figures S3 and S4.

(Figures 1H and 1I), we investigated their sensitivity to inhibitors of mitochondrial energetics.

For that matter, we used the pre-clinical Eµ-*Myc* mouse model that spontaneously developed non-Hodgkin (NH) B cell lymphomas (Adams et al., 1985), and that we previously discriminated into GAPDH^{low} or GAPDH^{high} B cell lymphomas, according to GAPDH expression levels (mRNA and protein) (Chiche et al., 2015; Figures S3A–S3C). GAPDH^{low} or GAPDH^{high} B cell lymphomas express an equivalent amount of other glycolytic enzymes, and there is no link between GAPDH and LDH (total and LDH-B) expression (Figure S3A). While the exact nature of GAPDH regulation remains to be found, *gapdh* mRNA was more stable in GAPDH^{high} compared to GAPDH^{low} cells (Figure S3D). Cells from Eµ-*Myc*-GAPDH^{low} and Eµ-*Myc*-GAPDH^{high} lymphomas produce an equivalent amount of ATP (Figure 2A). However, Eµ-*Myc*-GAPDH^{low} cells produce less glycolytic ATP (Figure 2B), as observed in human DLBCL cells (Figure 1I) and secrete less lactate than Eµ-*Myc*-GAPDH^{high} cells (Figure 2C). Accordingly, Eµ-*Myc*-GAPDH^{low} cells consume more O₂ (Figure 2D), while glucose transporters' expression (Figure S3E) and the mitochondrial mass (Figure S3F) are similar between the two groups.

Cell Metabolism 29, 1243–1257, June 4, 2019 1247



Figure 3. Active mTORC1 Signaling in B Cell Lymphomas Expressing Low Levels of GAPDH Is Implicated in OxPhos Metabolism

(A) Whole-cell lysates prepared from Eμ-Myc cells harvested from independent Eμ-Myc lymphomas were analyzed by immunoblot with the indicated antibodies. Each lane represents an independent lymphoma. Erk2 is used as a loading control.

(B) Representative immunohistochemistry slides of *de novo* DLBCL biopsies stained for GAPDH and P-p70S6K (T389) proteins. Magnification $20 \times (100 \,\mu\text{m})$. The table represents the summary of the GAPDH and P-p70S6K (T389) stained slides for 18 *de novo* DLBCL biopsies. p value from Fisher's test. (C) E μ -*Myc*-GAPDH^{low} cells (lymphomas #F) were seeded in the presence or absence (Ctl, DMSO) of 10 and 20 nM of rapamycin for 15 h. Whole-cell lysates were then analyzed for the indicated proteins. Erk2 was used as a loading control.

1248 Cell Metabolism 29, 1243–1257, June 4, 2019

(legend continued on next page)

In vitro, the mitochondrial complex I inhibitor phenformin (Wheaton et al., 2014) reduces baseline O_2 consumption driven by complex I and induces cytotoxic responses in Eµ-*Myc*-GAPDH^{low} cells, but not in Eµ-*Myc*-GAPDH^{high} cells (Figures 2E and S3G–S3I). Importantly, *gapdh* silencing in Eµ-*Myc*-GAPDH^{high} cells decreases lactate production and increases cell sensitivity to phenformin-induced death (Figures S4A–S4D). *In vivo*, phenformin treatment increases the survival of Eµ-*Myc*-GAPDH^{ligh} lymphoma-bearing mice, but not that of Eµ-*Myc*-GAPDH^{high} lymphoma-bearing mice (Figures 2F and 2G), which demonstrates the causal correlation between the low expression of GAPDH and phenformin sensitivity. We confirmed that phenformin was indeed active at the dose administrated *in vivo* (Figures S3J and S3K).

Overall, targeting mitochondrial metabolism of E_{μ} -*Myc*-GAPDH^{low} lymphomas might represent a potential therapeutic strategy for patients with DLCBL-GAPDH^{low} who are low responders to R-CHOP treatment.

Active mTORC1 Signaling in GAPDH^{low} Lymphomas Is Implicated in OxPhos Metabolism

GAPDH was shown to modulate mTOR signaling through its binding to Rheb (Lee et al., 2009). We therefore investigated mTORC1 signaling in Eµ-*Myc* lymphoma cells according to GAPDH expression levels. We observed a high mTORC1 activity in Eµ-*Myc*-GAPDH^{low} lymphomas, as shown by p70-S6K phosphorylation on threonine 389 (Figure 3A). This was confirmed in human DLBCL biopsies, for which 57% of DLBCL-GAPDH^{low} stained positive for p70-S6K phosphorylation (indicating high mTORC1 activity), while 91% of DLBCL-GAPDH^{low} for express this form (indicating low mTORC1 activity) (Figure 3B).

Inhibition of mTORC1 signaling with rapamycin in E μ -*Myc*-GAPDH^{low} cells decreases basal O₂ consumption rate, maximal respiratory capacities, and ATP-coupled oxygen consumption rate (OCR) along with an increase in glycolytic ATP (Figures 3C–3G). Of note, the total amount of intracellular ATP was not modulated upon rapamycin treatment (Figure S5A). More specifically, rapamycin decreases O₂ consumption driven by complex I, but not complex II (Figures S5B and S5C), because of the inhibition of complex I activity, but not of complex II activity (Figure S5D). The exact reason for such an effect remains to be determined. Similar results were obtained using an independent

Eμ-*Myc*-GAPDH^{low} lymphoma (Figures S5E–S5G). As previously reported in several cancer cell lines (Fruman and Rommel, 2014), rapamycin does not induce cytotoxic responses (Figure 3H) but rather cytostatic effects, as we observed (Figure S5H). Finally, *in vivo* inhibition of mTORC1 activity with temsirolimus (Dancey, 2010) significantly increases the survival of OxPhos Eμ-*Myc*-GAPDH^{low}-bearing mice (Figures 3I and 3J).

These results suggest that mTORC1 signaling is involved in the control of GAPDH^{iow} B cell lymphoma metabolism.

B Cell Lymphomas Expressing Low Levels of GAPDH Rely on Glutamine Metabolism and Are Sensitive to the Hydrolysis of Extracellular Glutamine and Asparagine with *E. coli* L-Asparaginase

mTORC1 is a central sensor of amino acids, including Gln (Durán et al., 2012). Eµ-Myc-GAPDH^{low} lymphomas display active mTORC1 signaling (Figure 3A) and consume more Gln than the Eµ-Myc-GAPDH^{high} cells (Figure 4A) because of a specific increase in Gln transport rates (Figures 4B, S6A, and S6B). We next profiled metabolic differences between Eµ-Myc-GAPDH^{low} and Eµ-Myc-GAPDH^{high} lymphoma cells using liquid chromatography-mass spectrometry (LC-MS)-based metabolomics with the intent to further characterize the intracellular fate of Gln (Figure 4C). The relative intracellular levels of metabolites involved in glycolysis (G6P, G3P, and lactate) and in the non-oxidative arm of the pentose phosphate pathway (PPP) (sedoheptulose-7-phosphate) are significantly decreased in Eµ-Myc-GAPDH^{low} cells (Figure 4C). Glutamine is metabolized through glutaminolysis to produce glutamate and α-ketoglutarate, with the latter being used to replenish the tricarboxylic acid (TCA) cycle. In favor of an active glutaminolysis, glutamate and $\alpha\text{-ketoglutarate levels}$ are higher in Eµ-Myc-GAPDH low cells (Figure 4C), consistent with an increased expression of glutaminase (GLS), the mitochondrial enzyme catalyzing the conversion of glutamine into glutamate and ammonia (Figure 4D). While fewer metabolites could be detected by ¹H HRMAS NMR spectroscopy, a significant increase in intracellular glutamate levels was also confirmed in Eµ-Myc-GAPDH^{low} cells (Figures S6C and S6D). Extracellular sources of GIn are required for Eµ-Myc-GAPDH^{low} cells to sustain basal O₂ consumption, maximal respiratory capacities, and ATP-coupled OCR (Figures S6E–S6G). Pharmacologic hydrolysis of extracellular sources of

See also Figure S5.

⁽D–F) Baseline OCR (D), maximal respiration (E), and ATP-coupled OCR (F) of Eµ-Myc-GAPDH^{low} cells (lymphoma #F) pre-treated or not (Ctl, DMSO) with indicated doses of rapamycin for 15 h were determined with Seahorse XF96 Extracellular Flux Analyzer. Maximal respiration (E) is immediately determined after an injection of 0.5 µM FCCP. ATP-coupled OCR (F) represents oligomycin-sensitive respiration. Data are expressed as mean ± SD (n = 3 independent experiments, in quadruplicates).

⁽G) Glycolytic ATP production measured as the percentage of total ATP in E_{μ} -*Myc*-GAPDH^{low} cells (lymphoma #F) incubated in the presence or absence (Ctl, DMSO) of 10 and 20 nM of rapamycin for 15 h, was quantified using a luciferin/luciferase-based assay. The presented data are expressed as mean \pm SD (n = 2 independent experiments).

⁽H) Eµ-*Myc*-GAPDH^{low} and Eµ-*Myc*-GAPDH^{high} cells (n = 3 independent lymphomas per group) were seeded in the presence or absence (Ctl, DMSO) of indicated doses of rapamycin for 15, 24, and 48 h. Cell death was determined by DAPI staining and analyzed by flow cytometry. Data are expressed as mean ± SD (n = 2 independent experiments).

⁽I) Survival of syngeneic C57BL/6 mice intravenously injected with primary Eµ-Myc-GAPDH^{low} cells (isolated from lymphoma #F) and treated 6 days later with either vehicle (NaCl 0.9%-5%Tween-5% PEG-200-1% EtOH) or temsirolimus (10 mg/kg) intraperitoneally two times per week (n = 6 mice per group, p value from log-rank test, ***p < 0.001).

⁽J) Whole-cell lysates prepared from axillary lymph node tumors of vehicle or temsirolimus (10 mg/kg)-treated E μ -Myc-GAPDH^{low}-bearing mice were analyzed by immunoblot for the indicated antibodies. Each lane represents an independent mouse. *n.s* means non-specific band. Erk2 is used as a loading control. ***p < 0.001 indicated significance of the observed differences.

CellPress





(A) Glutamine consumption was determined in the supernatant of Eμ-*Myc*-GAPDH^{low} and Eμ-*Myc*-GAPDH^{high} cells (n = 5 independent lymphomas per group), 24 h after seeding. Data are expressed as mean ± SD (n = 2 independent experiments).

(B) Glutamine transport rates (in cpm) are measured in 5.10⁶ E μ -*Myc*-GAPDH^{low} or E μ -*Myc*-GAPDH^{high} cells (n = 4 independent lymphomas per group) incubated for 30 min in an amino-acid-free, 20 mM glucose-containing DMEM supplemented with [¹⁴C]-Glutamine. Data are expressed as mean \pm SD (n = 2 independent experiments in triplicate).

(C) Heatmap depicting significantly changed glycolytic/non-oxidative pentose phosphate pathway (PPP) and glutaminolysis/tricarboxylic acid (TCA) cycle metabolite levels in Eµ-*Myc*-GAPDH^{low} and and an Eµ-*Myc*-GAPDH^{low} an Eµ-*Myc*-GAPDH^{low} an Eµ-*Myc*-GAPDH^{low} an Eµ-*Myc*-GAPDH^{low} an Eµ-*Myc*-GAPDH^{low} an Eµ-*Myc*-GAPDH^{low} an Eµ-*Myc*-*Myc*-*Myc*-*Myc*-*Myc*-*Myc*-*Myc*-*Myc*-*Myc*-

1250 Cell Metabolism 29, 1243–1257, June 4, 2019

(legend continued on next page)

L-glutamine and L-asparagine with *E. coli* L-asparaginase (L-ase; Figure 4E) reduces mTORC1 activity (Figure S6H; Willems et al., 2013) and induces cytotoxic responses in Eµ-Myc-GAPDH^{low} lymphoma cells (Figure 4F). In addition, *gapdh*-silenced Eµ-Myc-GAPDH^{high} lymphoma cells are more sensitive than control cells to *E. coli* L-ase-induced death (Figure S4E). *In vivo*, plasma sources of Gln were depleted in *E. coli* L-ase-treated mice, regardless of the Eµ-Myc-GAPDH^{low}-bearing mice was enhanced upon *E. coli* L-ase treatment (Figures 4H and 4I). Of note, *E. coli* L-ase treatment inhibited mTORC1 signaling *in vivo* (Figures S6I and S6J).

Specific Overexpression of GAPDH Reduces mTORC1 Signaling and B Cell Lymphomas' Sensitivity to Phenformin and L-Asparaginase *In Vivo*

To further support our conclusions, we overexpressed GAPDH in E_{μ} -*Myc*-GAPDH^{low} cells (Figure 5A). GAPDH overexpression was sufficient to reduce mTORC1 signaling (Figure 5A) and to induce a metabolic switch toward glycolysis, as judged by the increase in glycolytic ATP, lactate secretion along with the reduction in baseline OCR and Gln consumption (Figures 5B–5F). Equivalent observations were obtained upon GAPDH expression in the OxPhos-DLBCL cell line Karpas 422 (Figures S7A–S7C). Importantly, the metabolic switch induced by GAPDH overexpression prevented most of the beneficial effects brought by either phenformin or *E. coli* L-asparaginase *in vivo* (Figures 5G–5I).

Together we confirmed that GAPDH expression influences the metabolic state and the sensitivity of B cell lymphomas to phenformin or *E. coli* L-asparaginase.

Treatment of DLBCL-GAPDH^{low} with Specific Metabolic Inhibitors Demonstrates a Significant Benefit for Patients

We demonstrated that GAPDH^{Iow} B cell lymphomas preferentially use OxPhos metabolism to produce energy and are sensitive to cell death induced by phenformin or *E. coli* L-asparaginase. To ultimately validate our findings, we designed a clinical therapeutic intervention (called KTM), to interfere with DLBCL-GAPDH^{Iow} metabolism (Figure 6A). KTM therapy consists of a 4-week cycle of treatment including *E. coli* L-asparaginase (K, Kidrolase), mTOR inhibitor temsirolimus (T, Torisel) and metformin (Figure 6B). In the first 2 weeks, patients receive *E. coli* L-asparaginase on days 1, 3, 5, 7, 9, 11, and 13 and temsirolimus on days 1, 7, and 14. This combination was not administered longer than 14 days since *E. coli* L-asparaginase is not well tolerated in adults (Boissel and Sender, 2015). Instead, in the last 2 weeks of each KTM cycle, patients received metformin to sustain a therapeutic pressure on mitochondrial metabolism (Figure 6B). It is important to consider that prior to KTM, patients were refractory to all R-based therapies, and only supportive care was proposed.

Figure 6C illustrates patient #1, a 24-year-old man who presented a DLBCL in therapeutic failure. At diagnosis, the clinical presentation was a cervical bulky mass corresponding to a Myc-translocated GC-DLBCL (diameter 240 × 100 mm) with bone marrow and blood infiltration (Table S4). Following diagnosis, the patient received four regimens of immuno-chemotherapy. Early tumor progression was systematically observed before initiating each new cycle of rituximab-based therapy. Verification of GAPDH expression at diagnosis by IHC showed a low GAPDH IHC score (of 35). As we could not offer any other standard therapeutic option, patient #1 was selected for KTM treatment. Seven and 15 days after the beginning of KTM therapy, the tumor mass significantly decreased (Figure 6C). After 30 days of KTM treatment, patient #1 had an 83% reduction of the tumor mass (Figure 6D) and was negative at positron emission tomography (PET) scan analysis. He had a normal life for 4 months and then died upon local and central nervous system relapse of the lymphoma. To demonstrate that KTM efficacy is not limited to one single patient presenting a DLBCL-GAPDH^{low}, three other patients with identical eligible criteria (Myc⁺-DLBCL-GAPDH^{low} at diagnosis and treatment-refractory disease) were treated with KTM (Figure 6E). Three out of four patients had a complete response after two cycles of treatment, and one patient had a progressive disease due to early adverse events during the first cycle of treatment, and KTM treatment had to be discontinued (Figure 6F). Median duration of response was 6 months (4-6 months).

DISCUSSION

Several metabolic inhibitors were developed with the intent to kill or to sensitize cancer cells to chemotherapies (Galluzzi et al., 2013). So far, more than 100 clinical trials using metabolic inhibitors are ongoing in the field of cancer (Meynet and Ricci, 2014). Unfortunately, to date, most of those trials have failed to

(A)–(G), **p < 0.01, **p < 0.001 indicated the significance of the observed differences. ns, not significant. See also Figure S6.

⁽D) Whole-cell lysates prepared from Eµ-Myc-GAPDH high and low cells were analyzed by immunoblot for mitochondrial glutaminase (GLS). Erk2 is used as a loading control.

⁽E) The concentration of glutamine (left panel) and of asparagine (right panel) in the supernatant of Eμ-Myc-GAPDH high (in red) and low (in blue) cells (n = 4 independent lymphomas per group) treated or not (Ctl) with *E.coli* L-asparaginase (L-ase) at indicated doses for 4 h. The presented data are expressed as mean ± SD (n = 3 independent experiments).

⁽F) Eµ-*Myc*-GAPDH^{low} and Eµ-*Myc*-GAPDH^{high} cells (n = 3 independent lymphomas per group) were seeded in the presence or absence (Ctl) of *E. coli* L-asparaginase (L-ase, at indicated doses) for 15 h. Cell death was determined by DAPI staining and analyzed by flow cytometry. Data are expressed as mean ± SD (n = 3 independent experiments).

⁽G) Plasma glutamine concentration 4 h after the last intraperitoneal (i.p.) administration of NaCl 0.9% (Ctl) or *E. coli* L-asparaginase (L-ase, 2,500 IU/kg) in Eµ-Myc-GAPDH^{low}- and in Eµ-Myc-GAPDH^{high}-bearing mice (n = 4 mice per group).

⁽H and I) Survival of syngeneic C57BL/6 mice intravenously injected with primary E μ -Myc-GAPDH^{low} (H) or E μ -Myc-GAPDH^{high} cells (I) and treated 6 days later with either vehicle (NaCl 0.9%) or 2,500 IU/kg of *E. coli* L-asparaginase (L-ase) intraperitoneally five times per week the first week and three times per week the following weeks (n = 6 mice per group, p value from log-rank test, ***p < 0.001).





(A) Total cell extracts from mouse primary Eµ-Myc-GAPDH^{low} cells (lymphoma #F) stably transduced with control (pMIG) or GAPDH-V5-encoding pMIG vectors were immunoblotted for the indicated proteins. Erk2 is used as a loading control.

(B) Intracellular ATP levels produced in E μ -Myc-GAPDH^{low} cells stably overexpressing GAPDH-V5 or control vector (pMIG) were quantified using a luciferin/ luciferase-based assay, and results were expressed as relative luminescence units normalized by cell number. Data are expressed as mean \pm SD (n = 3 independent experiments).

(C) Glycolytic ATP production was measured as the percentage of total ATP in cells presented in (A). Data are presented as means ± SD (n = 3 independent experiments).

(D) Lactate production was determined in the supernatant of mouse primary E_{μ} -Myc-GAPDH^{low} cells (lymphoma #F) stably transduced with control (pMIG) or GAPDH-V5-encoding pMIG vectors, 24 h after seeding. Data are presented as mean \pm SD (n = 3 independent experiments).

(E) Baseline OCR of cells presented in (A) was determined with Clarck electrode 24 h after cell seeding. Data are expressed as mean ± SD (n = 3 independent experiments).

(legend continued on next page)

improve the outcome of patients (Kordes et al., 2015). This may not be linked to the inefficacy of the compound to inhibit its target but rather to a subset of tumors with a distinct metabolic dependence from the targeted metabolic pathway. Altogether, our study highlighted that tumor heterogeneity within the same tumor entity has to be taken into consideration for ongoing clinical trials using metabolic inhibitors.

While more than 150,000 claimed biomarkers have been described in the literature, less than 100 are routinely used in the clinic (Poste, 2011), and we still lack a marker of the tumor metabolic status. Here, we identified GAPDH as a clinically compatible biomarker of (1) DLBCL response to the conventional R-CHOP treatment and (2) DLCBL metabolic status, which help to further predict the efficacy of mitochondrial metabolism disruptors in DLCBL-GAPDH^{low} that are associated with a poor outcome of patients treated with R-CHOP.

GAPDH is a multifunctional protein harboring glycolytic and non-glycolytic functions (Colell et al., 2009). It is highly regulated at the transcriptional, translational, and post-translational levels. Endogenous gapdh mRNA and protein are highly abundant in all mammalian cells, and increased expression levels of gapdh by 30% or more significantly impacts cell survival and growth (Colell et al., 2009), particularly in B cell lymphomas (Chiche et al., 2015). We observed that human DLBCL expressed heterogeneous levels of gapdh mRNA and protein, defining patients with DLBCL-GAPDH^{low} or DLBCL-GAPDH^{high}. As expected, when comparing healthy tissues (GCB cells from non-cancerous reactive lymph nodes) to cancer samples, DLBCL-GAPDH^{low} expresses significantly higher levels of GAPDH (Figure 1D). Moreover, when compared to DLBCL-GAPDH^{low}, DLBCL-GAPDH^{high} expresses approximatively two times more GAPDH, which also highlights GAPDH as a marker of tumor heterogeneity within the same tumor entity. Interestingly, gapdh mRNA stability was higher in Eµ-Myc-GAPDH^{high} cells (Figure S3D). As GAPDH is an RNA-binding protein (Colell et al., 2009), we could speculate that it could bind and stabilize its own mRNA. However, the exact nature of gapdh regulation in DLBCL is still unclear and will be investigated in further studies.

Mechanistically, we observed a central role of GAPDH-dependent regulation of mTORC1 signaling in the control of DLBCL metabolic status. It was previously reported that *gapdh*-silenced HEK293 cells increase mTORC1 signaling by preventing GAPDH-Rheb binding (Lee et al., 2009). Using the E μ -*Myc* model and newly diagnosed DLBCL samples, we also observed an increase in mTORC1 signaling, glutamine uptake, glutaminolysis, and sensitivity to L-asparaginase-induced death in GAPDH^{low} B cell lymphomas (Figures 3 and 4). Altogether, our findings are consistent with the role of functional mTORC1 on glutamine uptake (Csibi et al., 2014) and the feedback role of glutaminolysis on the activation of Rag-mTORC1 signaling (Durán et al., 2012). How does GAPDH-dependent control of mTORC1 regulate metabolism? mTORC1 is a known regulator of HIF-1 α and c-Myc translation, which in turn induce the expression of genes involved in glucose uptake and glycolysis (Gordan et al., 2007). In primary Eµ-Myc lymphomas, we did not observe differences in glucose transporters' expression (Figure S3E) according to GAPDH expression levels, despite differences in mTORC1 signaling. In contrast, inhibition of mTORC1 reduces O2 consumption of Eµ-Myc GAPDH^{low} cells, in agreement with previous studies (Cunningham et al., 2007; Morita et al., 2013). It seems therefore possible that E_{μ} -Myc lymphomas and human DLBCLs do not follow the classical Warburg scheme in which mTORC1 activates glycolysis. Instead, mTORC1 seems implicated in the regulation of OxPhos metabolism in this biological model (Figure 3). This notion is further supported by the increase in glutamine uptake, along with an increase in the relative intracellular levels of glutamate and α-ketoglutarate and L-asparaginase efficacy on reduced mTORC1 signaling in vitro and in vivo (Willems et al., 2013) (Figures 4 and S6).

Since IHC detection of GAPDH seemed to help for the determination of the tumor metabolic status, we selected four patients with Myc+-DLBCL-GAPDH^{low}. All patients were refractory to R-based therapies. In the face of a therapeutic dead end, we ultimately target DLBCLs-GAPDH^{low} metabolism with already available (Food and Drug Administration-approved) metabolic disruptors (KTM therapy) (Figure 6). Based on the observed cytostatic effect of temsirolimus in vitro and in vivo (Figures 3I and S5H), we decided still to administer this mTOR inhibitor either with L-asparaginase (D1 and D7 of a KTM cycle) or with metformin (D14 of a KTM cycle) to maximize the potential beneficial effect of the co-treatment. However, knowing that rapalogs alone or in combination with R-CHOP did not provide significant benefits in randomized phase 2 and phase 3 trials for patients with NH B cell lymphoma (Ricci and Chiche, 2018), it is unlikely that the rapid and complete responses obtained after 1 week of KTM treatment can be attributed to temsirolimus. According to the beneficial effect observed in Eµ-Myc-GAPDH^{low} lymphomas upon E. coli L-ase (Kidrolase) and to the early reduction of the tumor burden observed from D7 in patient #1 (Figure 6C), it is likely that most of the treatment efficacy can be attributed to the L-ase activity. Unfortunately, in the clinic, E. coli L-ase cannot be administrated longer than 2 weeks in a row in adults, as it may provoke major toxicities (Boissel and Sender, 2015). We therefore sustained inhibition of mitochondrial energetics with metformin and mTOR inhibitor for the last 2 weeks of KTM cycle of treatment (Figure 6B). Despite the drastic reduction of the tumor mass, all patients finally relapsed, which very likely reflects

⁽F) Glutamine consumption was determined 24 h after cell seeding in the supernatant of $E\mu$ -Myc-GAPDH^{low} cells (lymphoma #F) stably transduced with control (pMIG) or GAPDH-V5-encoding pMIG vectors. Data are expressed as mean \pm SD (n = 3 independent experiments).

⁽G) Weight of inguinal (left) and axillary (right) lymphomas harvested from wild-type syngeneic C57BL/6 mice 20 days after intravenous injection of primary E_{μ} -*Myc*-GAPDH^{low} cells stably over-expressing or not (pMIG) GAPDH-V5. Phenformin (100 mg/kg/day) or vehicle (H₂O) were administrated every day for the last 13 days (pMIG-Vehicle, n = 8; pMIG-Phenformin, n = 9; GAPDH-V5-Vehicle, n = 10; GAPDH-V5-Phenformin, n = 7).

⁽H and I) Survival of syngeneic C57BL/6 mice intravenously injected with primary E_{μ} -Myc-GAPDH^{low} cells stably transduced with control pMIG vector (H) or GAPDH-V5-encoding pMIG vectors (I) and treated 6 days later with either vehicle (NaCl 0.9%) or 2,500 IU/kg of *E. coli* L-asparaginase (L-ase) intraperitoneally five times per week the first week and three times per week the following weeks (n = 10 mice per group, p value from log-rank test, **p < 0.01; ***p < 0.001). (A)–(G), *p < 0.05, **p < 0.01, ***p < 0.001 indicated significance of the observed differences. ns, not significant. See also Figure S7.

DLBCL-GAPDH low $DLBCL \longrightarrow R-CHOP$ 40% 40%KTM treatment

B KTM cycle

Α

К К К К К К <u>Мetformin</u> Т Т Т Т	D1	D3	D5	D7	D9	D11	D13	D14		D28
т т т	к	к	к	к	к	к	к		Metformin	
	Т			т				Т		



GAPDH score= 35



GAPDH score= 40

Duration (weeks)

8 12 16 20 24 28 32 36 40 44 48 52 56 60 64

l

4

Figure 6. Patients with DLBCL-GAPDH^{low} Are Sensitive to Specific Inhibitors of Mitochondrial Metabolism (KTM Treatment)

(A) Schematic representation of conventional therapeutic protocol for patients with DLBCL. Once diagnosed, patients with DLBCL receive immunochemotherapy, R-CHOP. Approximately 40% of the patients will experience therapeutic failure (refractory or relapse). Other anti-CD20-based therapies such as R-DHAP—rituximab, dexamethasone, high-dose Ara-C (cytarabine)-Platinol (cisplatin)—or R-ICE—rituximab, ifosfamide-carboplatin-etoposide—are then proposed. Another set of 40% of patients will not respond to those treatments and will be eligible for high-dose therapy with autologous stem cell transplantation (HDT/ASCT). In the case of *de novo* DLBCL-GAPDH^{low} (low responders to R-CHOP), we propose a clinical protocol called Kidrolase-Torisel-metformin (KTM), to interfere with tumor metabolism upon relapse of all anti-CD20-based therapies.

(B) Schematic representation of one cycle of KTM therapy. Four-week cycle of KTM consisted of the combination of *E. coli* L-asparaginase (K, Kidrolase 6,000 IU/m²) on days 1, 3, 5, 7, 9, 11, and 13 and mTOR inhibitor temsirolimus (T, Torisel 75 mg on days 1, 7, and 14) and metformin (1,000 mg/day) from day 14 to day 28. Four patients with DLBCL-GAPDH^{Iow} at diagnosis and refractory to all anti-CD20-based therapies were eligible for KTM therapy.

the metabolic flexibility and adaptation of the remaining malignant B cells during or after KTM therapy.

Limitations of Study

A major limitation in the field of DLBCL is a lack of robust preclinical models (cell lines or in vivo mouse models) that recapitulate the major aspects of the human disease. As human cancer cell lines were isolated decades ago and since grown in high glucose, high oxygenated media, it is possible that their metabolism does not reflect the metabolic status of the initial tumor cells. We therefore decided to characterize several independent murine primary NH B cell lymphomas harvested from the $E\mu$ -Myc mice model (Figures 2, 3, 4, and 5). While this model represents highly aggressive tumors (Ki67 100%) and is largely used as a robust model to test new therapeutic options for NH B cell lymphomas, they lack key features of human DLBCL such as marker of mature B cells. We therefore validated our findings in humans, using malignant B cells immediately isolated from "fresh" DLBCL micro-biopsies and ultimately by treating a few patients suffering from the refractory disease with specific metabolic inhibitors (Figures 1, 3, and 6). Another limitation of our work is the low number of patients included in the KTM therapy. However, our study established the proof of principle that patients with DLBCL-GAPDH^{low} are low responders to immunochemotherapy R-CHOP but are potential candidates for KTM therapy. Finally, while GAPDH levels represent the first clinically compatible marker of DLBCL metabolic heterogeneity, it remains to be determined if this marker can be extended to other hematological malignancies and solid tumors.

STAR*METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - O Mice and In Vivo Studies Using Eμ-Myc Cells
 - Cell Lines and Culture Conditions
 - O Microarray Data Analysis
 - O DLBCL Patients: Training & Validation Cohorts
 - Clinical Protocol KTM (Kidrolase-Torisel-Metformin)
- METHOD DETAILS
 - COO Classification
 - GAPDH Immunohistochemical (IHC) Staining and Scoring
 - Immunohistochemistry and Fluorescence In Situ Hybridization
 - ATP Analysis
 - Lactate Measurement

- Glutamine Consumption
- *L*-[¹⁴C]-Glutamine Uptake
- O 2-NBDG Uptake
- Measurement of the Mitochondrial Mass
- O Oxygen Consumption
- Mitochondrial Respiratory Complex Activities
- Cell Viability Assays
- Activation of the B-Cell Receptor
- Isolation of Human Tumor Cells from DLBCL Core-Needle Biopsies
- Plasmids and Generation of GAPDH-Overexpressing Cells and gapdh-Silenced Cells
- O RNA Extraction and Real-Time Quantitative PCR
- Western Blot Analysis
- Flow Cytometry Analysis
- Targeted LC-MS Metabolites Analyses
 ¹H HRMAS NMR Spectroscopy
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY
 - Code Availability

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j. cmet.2019.02.002.

ACKNOWLEDGMENTS

We gratefully acknowledge the C3M animal facility, the Tumorothèque of Hôpital Saint-Louis, and the Lymphoma Study Association (LYSA). We thank the technician of the Laboratoire Central d'AnatomocytoPathologie (LCAP), CHU de Nice, S. Destrée, and A. Borderie. We thank I. Nemazanyy from the Platform for Metabolic Analyses (Neckert-Enfants Malades Institute, Paris). We thank A. Vandenberghe, N. Alet, N. Jourdan-Desrayaud, F. Larbret, F. Reinier, N. Nottet, M. Baia, N. Vailhen, S. Roulland, M. Zala, and I. Ben-Sahra for reagents and input. This work has been supported by the Fondation ARC pour la Recherche sur le Cancer (PGA1 RC-20170205463), the Agence Nationale de la Recherche (LABEX SIGNALIFE ANR-11-LABX-0028-01), the Fondation de France, Centre Scientifique de Monaco, the Cancéropôle PACA, and INSERM transfert. This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 766214 (Meta-Can), C.T. is funded by the Nella and Amadeus Barletta foundation (FNAB), B.N. is funded by Cancéropole PACA, la Fondation ARC (PGA120150202381), and Plan cancer 2015 ITMO cancer (C15077AS). The IPMC functional genomics platform is a member of the "France Génomique" consortium (ANR-10-Infra-01), with financial support from the Cancéropôle PACA. E. Villa is supported by La Ligue Contre le Cancer, J.C. is supported by foundation Tourre and France Lymphoma Espoir, G.B. is supported by la Fondation pour la Recherche Médicale (FRM, FDM20150734500), and J.R.-M. is supported by INCa.

AUTHOR CONTRIBUTIONS

J.C. performed the majority of research described herein and was assisted by A.M., C.R.-P., R.M., E. Villa, J.P.B., E.P., M.G.-R., K.F., S.M., E. Verhoeyen,

(C) Progressive regression of the patient #1's DLBCL (CD10⁺, bcl2⁺, Mum1⁻, Ki67⁺ 80%, MYC⁺, Ann Arbor stage 4) upon KTM treatment.

(D) 3D model of computed tomography (CT) scan of patient #1 before (T = 0) and after (T = 30 days) two cycles of KTM treatment.

(E) Illustration of GAPDH expression (and respective GAPDH IHC score) in DLBCL biopsies at diagnosis, from the four patients enrolled in the KTM study. (F) Duration of treatment and therapeutic response (by CT or PET) to KTM for the four individual patients presenting DLBCL-GAPDH^{low} at diagnosis (E). KTM, Kidrolase, Torisel, and metformin; CR, complete response; PR, partial response; PD, progressive disease; PAC, patient alive at last consultation; AE, adverse event.

See also Table S4.

V.P.-F., L.S., G.B., and B.N.; J.R.-M., J.B., D.A., J.-F.M., T.J.M., C.C.-B., J.L.-C., I.P., F.P., E.D.K., B.T., G.G., V.D., C.D.-D., R.D., A.B., T.P., N.M., and C.T. contributed to clinical study and patient samples. Bioinformatic analysis was conducted by A.P. and P.B.; J.-E.R. secured funding and supervised the study; C.T. supervised the clinical study; and J.C. and J.-E.R. designed the research and wrote the manuscript.

DECLARATION OF INTERESTS

C.T. is on the scientific board for Jazz Pharmaceuticals, Celgene, Roche, Abb-Vie, and Gilead. The remaining authors declare no competing interests. Related patents are as follows: WO2015/132163: A method for predicting the responsiveness a patient to a treatment with an anti-CD20 antibody; WO2017/055484: Methods for determining the metabolic status of lymphomas; EP17305294.5: A method for predicting the responsiveness of a patient to a treatment with mTOR inhibitors.

Received: September 21, 2018 Revised: December 21, 2018 Accepted: February 6, 2019 Published: February 28, 2019

REFERENCES

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D., and Brinster, R.L. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature *318*, 533–538.

Alizadeh, A.A., Eisen, M.B., Davis, R.E., Ma, C., Lossos, I.S., Rosenwald, A., Boldrick, J.C., Sabet, H., Tran, T., Yu, X., et al. (2000). Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature *403*, 503–511.

Boissel, N., and Sender, L.S. (2015). Best practices in adolescent and young adult patients with acute lymphoblastic leukemia: a focus on asparaginase. J. Adolesc. Young Adult Oncol *4*, 118–128.

Caro, P., Kishan, A.U., Norberg, E., Stanley, I.A., Chapuy, B., Ficarro, S.B., Polak, K., Tondera, D., Gounarides, J., Yin, H., et al. (2012). Metabolic signatures uncover distinct targets in molecular subsets of diffuse large B cell lymphoma. Cancer Cell *22*, 547–560.

Chen, L., Monti, S., Juszczynski, P., Daley, J., Chen, W., Witzig, T.E., Habermann, T.M., Kutok, J.L., and Shipp, M.A. (2008). SYK-dependent tonic B-cell receptor signaling is a rational treatment target in diffuse large B-cell lymphoma. Blood *111*, 2230–2237.

Chiche, J., Pommier, S., Beneteau, M., Mondragón, L., Meynet, O., Zunino, B., Mouchotte, A., Verhoeyen, E., Guyot, M., Pagès, G., et al. (2015). GAPDH enhances the aggressiveness and the vascularization of non-Hodgkin's B lymphomas via NF- κ B-dependent induction of HIF-1alpha. Leukemia *29*, 1163–1176.

Coiffier, B., Lepage, E., Briere, J., Herbrecht, R., Tilly, H., Bouabdallah, R., Morel, P., Van Den Neste, E., Salles, G., Gaulard, P., et al. (2002). CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N. Engl. J. Med *346*, 235–242.

Colell, A., Green, D.R., and Ricci, J.E. (2009). Novel roles for GAPDH in cell death and carcinogenesis. Cell Death Differ *16*, 1573–1581.

Copie-Bergman, C., Gaulard, P., Leroy, K., Briere, J., Baia, M., Jais, J.P., Salles, G.A., Berger, F., Haioun, C., Tilly, H., et al. (2009). Immuno-fluorescence in situ hybridization index predicts survival in patients with diffuse large B-cell lymphoma treated with R-CHOP: a GELA study. J. Clin. Oncol *27*, 5573–5579.

Csibi, A., Lee, G., Yoon, S.O., Tong, H., Ilter, D., Elia, I., Fendt, S.M., Roberts, T.M., and Blenis, J. (2014). The mTORC1/S6K1 pathway regulates glutamine metabolism through the eIF4B-dependent control of c-Myc translation. Curr. Biol. *24*, 2274–2280.

Cunningham, J.T., Rodgers, J.T., Arlow, D.H., Vazquez, F., Mootha, V.K., and Puigserver, P. (2007). mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature *450*, 736–740.

1256 Cell Metabolism 29, 1243–1257, June 4, 2019

Dancey, J. (2010). mTOR signaling and drug development in cancer. Nat. Rev. Clin. Oncol 7, 209–219.

Delarue, R., Tilly, H., Mounier, N., Petrella, T., Salles, G., Thieblemont, C., Bologna, S., Ghesquières, H., Hacini, M., Fruchart, C., et al. (2013). Dosedense rituximab-CHOP compared with standard rituximab-CHOP in elderly patients with diffuse large B-cell lymphoma (the LNH03-6B study): a randomised phase 3 trial. Lancet. Oncol *14*, 525–533.

Doherty, J.R., and Cleveland, J.L. (2013). Targeting lactate metabolism for cancer therapeutics. J. Clin. Invest *123*, 3685–3692.

Dubois, S., Viailly, P.J., Bohers, E., Bertrand, P., Ruminy, P., Marchand, V., Maingonnat, C., Mareschal, S., Picquenot, J.M., Penther, D., et al. (2017). Biological and clinical relevance of associated genomic alterations in MYD88 L265P and non-L265P-mutated diffuse large b-cell lymphoma: analysis of 361 cases. Clin. Cancer Res. 23, 2232–2244.

Durán, R.V., Oppliger, W., Robitaille, A.M., Heiserich, L., Skendaj, R., Gottlieb, E., and Hall, M.N. (2012). Glutaminolysis activates Rag-mTORC1 signaling. Mol. Cell *47*, 349–358.

Fisher, R.I., Gaynor, E.R., Dahlberg, S., Oken, M.M., Grogan, T.M., Mize, E.M., Glick, J.H., Coltman, C.A., Jr., and Miller, T.P. (1993). Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. N. Engl. J. Med *328*, 1002–1006.

Frecha, C., Fusil, F., Cosset, F.L., and Verhoeyen, E. (2011). In vivo gene delivery into hCD34+ cells in a humanized mouse model. Methods Mol. Biol. 737, 367–390.

Fruman, D.A., and Rommel, C. (2014). PI3K and cancer: lessons, challenges and opportunities. Nat. Rev. Drug Discov 13, 140–156.

Galluzzi, L., Kepp, O., Vander Heiden, M.G., and Kroemer, G. (2013). Metabolic targets for cancer therapy. Nat. Rev. Drug Discov *12*, 829–846.

Gautier, L., Cope, L., Bolstad, B.M., and Irizarry, R.A. (2004). Affy–analysis of Affymetrix GeneChip data at the probe level. Bioinformatics *20*, 307–315.

Gordan, J.D., Thompson, C.B., and Simon, M.C. (2007). HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. Cancer Cell *12*, 108–113.

Green, T.M., Young, K.H., Visco, C., Xu-Monette, Z.Y., Orazi, A., Go, R.S., Nielsen, O., Gadeberg, O.V., Mourits-Andersen, T., Frederiksen, M., et al. (2012). Immunohistochemical double-hit score is a strong predictor of outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J. Clin. Oncol 30, 3460–3467.

Hans, C.P., Weisenburger, D.D., Greiner, T.C., Gascoyne, R.D., Delabie, J., Ott, G., Müller-Hermelink, H.K., Campo, E., Braziel, R.M., Jaffe, E.S., et al. (2004). Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood *103*, 275–282.

Haralambieva, E., Kleiverda, K., Mason, D.Y., Schuuring, E., and Kluin, P.M. (2002). Detection of three common translocation breakpoints in non-Hodgkin's lymphomas by fluorescence in situ hybridization on routine paraffin-embedded tissue sections. J. Pathol *198*, 163–170.

Horn, H., Ziepert, M., Becher, C., Barth, T.F., Bernd, H.W., Feller, A.C., Klapper, W., Hummel, M., Stein, H., Hansmann, M.L., et al. (2013). MYC status in concert with BCL2 and BCL6 expression predicts outcome in diffuse large B-cell lymphoma. Blood *121*, 2253–2263.

Johnson, N.A., Slack, G.W., Savage, K.J., Connors, J.M., Ben-Neriah, S., Rogic, S., Scott, D.W., Tan, K.L., Steidl, C., Sehn, L.H., et al. (2012). Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J. Clin. Oncol *30*, 3452–3459.

Kheirallah, S., Caron, P., Gross, E., Quillet-Mary, A., Bertrand-Michel, J., Fournié, J.J., Laurent, G., and Bezombes, C. (2010). Rituximab inhibits B-cell receptor signaling. Blood *115*, 985–994.

Kordes, S., Pollak, M.N., Zwinderman, A.H., Mathôt, R.A., Weterman, M.J., Beeker, A., Punt, C.J., Richel, D.J., and Wilmink, J.W. (2015). Metformin in patients with advanced pancreatic cancer: a double-blind, randomised, placebo-controlled phase 2 trial. Lancet. Oncol *16*, 839–847.

Lee, M.N., Ha, S.H., Kim, J., Koh, A., Lee, C.S., Kim, J.H., Jeon, H., Kim, D.H., Suh, P.G., and Ryu, S.H. (2009). Glycolytic flux signals to mTOR through glyceraldehyde-3-phosphate dehydrogenase-mediated regulation of Rheb. Mol. Cell. Biol. *29*, 3991–4001.

Lenz, G., and Staudt, L.M. (2010). Aggressive lymphomas. N. Engl. J. Med *362*, 1417–1429.

Lenz, G., Wright, G., Dave, S.S., Xiao, W., Powell, J., Zhao, H., Xu, W., Tan, B., Goldschmidt, N., Iqbal, J., et al. (2008). Stromal gene signatures in large-B-cell lymphomas. N. Engl. J. Med *359*, 2313–2323.

Lipton, A., Köstler, W.J., Leitzel, K., Ali, S.M., Sperinde, J., Weidler, J., Paquet, A., Sherwood, T., Huang, W., Bates, M., et al. (2010). Quantitative HER2 protein levels predict outcome in fluorescence in situ hybridization-positive patients with metastatic breast cancer treated with trastuzumab. Cancer *116*, 5168–5178.

Mackay, G.M., Zheng, L., van den Broek, N.J., and Gottlieb, E. (2015). Analysis of cell metabolism using LC-MS and isotope tracers. Methods Enzymol *561*, 171–196.

Mareschal, S., Ruminy, P., Bagacean, C., Marchand, V., Cornic, M., Jais, J.P., Figeac, M., Picquenot, J.M., Molina, T.J., Fest, T., et al. (2015). Accurate classification of germinal center B-cell-like/activated B-cell-like diffuse large B-cell lymphoma using a simple and rapid reverse transcriptase-multiplex ligation-dependent probe amplification assay: a CALYM study. J. Mol. Diagn.

Meynet, O., and Ricci, J.E. (2014). Caloric restriction and cancer: molecular mechanisms and clinical implications. Trends Mol. Med 20, 419–427.

Molina, T.J., Canioni, D., Copie-Bergman, C., Recher, C., Brière, J., Haioun, C., Berger, F., Fermé, C., Copin, M.C., Casasnovas, O., et al. (2014). Young patients with non-germinal center B-cell-like diffuse large B-cell lymphoma benefit from intensified chemotherapy with ACVBP plus rituximab compared with CHOP plus rituximab: analysis of data from the Groupe d'Etudes des Lymphomes de l'Adulte/lymphoma study association phase III trial LNH 03-2B. J. Clin. Oncol *32*, 3996–4003.

Monti, S., Savage, K.J., Kutok, J.L., Feuerhake, F., Kurtin, P., Mihm, M., Wu, B., Pasqualucci, L., Neuberg, D., Aguiar, R.C., et al. (2005). Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response. Blood *105*, 1851–1861.

Morita, M., Gravel, S.P., Chénard, V., Sikström, K., Zheng, L., Alain, T., Gandin, V., Avizonis, D., Arguello, M., Zakaria, C., et al. (2013). mTORC1 controls mito-

chondrial activity and biogenesis through 4E-BP-dependent translational regulation. Cell Metab 18, 698–711.

Nicholson, J.K., Foxall, P.J., Spraul, M., Farrant, R.D., and Lindon, J.C. (1995). 750 MHz 1H and 1H-13C NMR spectroscopy of human blood plasma. Anal. Chem. 67, 793–811.

Norberg, E., Lako, A., Chen, P.H., Stanley, I.A., Zhou, F., Ficarro, S.B., Chapuy, B., Chen, L., Rodig, S., Shin, D., et al. (2017). Differential contribution of the mitochondrial translation pathway to the survival of diffuse large B-cell lymphoma subsets. Cell Death Differ 24, 251–262.

Petrella, T., Copie-Bergman, C., Brière, J., Delarue, R., Jardin, F., Ruminy, P., Thieblemont, C., Figeac, M., Canioni, D., Feugier, P., et al. (2017). BCL2 expression but not MYC and BCL2 coexpression predicts survival in elderly patients with diffuse large B-cell lymphoma independently of cell of origin in the phase 3 LNH03-6B trial. Ann. Oncol *28*, 1042–1049.

Poste, G. (2011). Bring on the biomarkers. Nature 469, 156-157.

Ricci, J.E., and Chiche, J. (2018). Metabolic reprogramming of non-Hodgkin's B-cell lymphomas and potential therapeutic strategies. Front. Oncol *8*, 556.

Rustin, P., Chretien, D., Bourgeron, T., Gérard, B., Rötig, A., Saudubray, J.M., and Munnich, A. (1994). Biochemical and molecular investigations in respiratory chain deficiencies. Clin. Chim. Acta *228*, 35–51.

Sehn, L.H., and Gascoyne, R.D. (2015). Diffuse large B-cell lymphoma: optimizing outcome in the context of clinical and biologic heterogeneity. Blood *125*, 22–32.

Swerdlow, S.H., Campo, E., Pileri, S.A., Harris, N.L., Stein, H., Siebert, R., Advani, R., Ghielmini, M., Salles, G.A., Zelenetz, A.D., et al. (2016). The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood *127*, 2375–2390.

Wheaton, W.W., Weinberg, S.E., Hamanaka, R.B., Soberanes, S., Sullivan, L.B., Anso, E., Glasauer, A., Dufour, E., Mutlu, G.M., Budigner, G.S., et al. (2014). Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. ELife 3, e02242.

Willems, L., Jacque, N., Jacquel, A., Neveux, N., Maciel, T.T., Lambert, M., Schmitt, A., Poulain, L., Green, A.S., Uzunov, M., et al. (2013). Inhibiting glutamine uptake represents an attractive new strategy for treating acute myeloid leukemia. Blood *122*, 3521–3532.

Wishart, D.S., Jewison, T., Guo, A.C., Wilson, M., Knox, C., Liu, Y., Djoumbou, Y., Mandal, R., Aziat, F., Dong, E., et al. (2013). HMDB 3.0-the human metabolome database in 2013. Nucleic Acids Res. *41*, D801–D807.
STAR***METHODS**

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies	000.02	
Rabbit polyclonal anti-human GAPDH antibody	Sigma-Aldrich	HPA040067; RRID: AB_10965903
Mouse monoclonal anti-human BCL6	Agilent (Dako)	Réf M7211; RRID: AB_2063451
Mouse monoclonal anti-human BCL2	Agilent (Dako)	Réf M0887; RRID: AB_2064429
Mouse monoclonal anti-human CD10	Agilent (Dako)	GA64861-2; RRID: N/A
Mouse monoclonal anti-human MUM1	Agilent (Dako)	M7259; RRID: AB_2127157
Mouse monoclonal anti-human CD79A	Agilent (Dako)	M7050; RRID: AB_2244527
Mouse monoclonal anti-human CD20	Agilent (Dako)	M0755; RRID: AB_2282030
Mouse monoclonal anti-CD5	Agilent (Dako)	M3641; RRID: N/A
Rabbit monoclonal anti-c-Myc	Abcam	ab32072; RRID: AB_731658
Mouse monoclonal anti-V5	Invitrogen	R96025; RRID: AB_159313
Rabbit polyclonal anti-GAPDH	Abcam	ab9485; RRID: AB_307275
Rabbit monoclonal anti-LDH	Abcam	ab52488; RRID: AB_2134961
Mouse monoclonal anti-LDH-B	Abcam	ab85319; RRID: AB_1860766
Rabbit polyclonal anti-GLS	Abcam	ab93434; RRID: AB_10561964
Mouse monoclonal anti-Erk2	Santa Cruz	sc-1647; RRID: AB_627547
Rabbit polyclonal anti-HKII	Cell Signaling Technology	2106; RRID: AB_823520
Rabbit polyclonal anti-ENO1	Cell Signaling Technology	3810; RRID: AB_2246524
Rabbit polyclonal anti-PKM2	Cell Signaling Technology	3198; RRID: AB_225325
Mouse monoclonal anti-p53	Cell Signaling Technology	2524; RRID: AB_331743
Rabbit monoclonal anti-phospho-acetylCoA carboxylase (ACC) (Ser79)	Cell Signaling Technology	11818; RRID: AB_2687505
Rabbit monoclonal anti-acetylCoA carboxylase (ACC)	Cell Signaling Technology	3676; RRID: AB_2219397
Rabbit monoclonal anti-phospho-p70S6K (T389)	Cell Signaling Technology	9234; RRID: AB_2269803
Rabbit polyclonal anti-p70S6K	Cell Signaling Technology	9202; RRID: AB_331676
Rabbit monoclonal anti-phospho-SYK (Tyr 525/526)	Cell Signaling Technology	2710; RRID: AB_2197222
Rabbit polycolonal anti-SYK	Cell Signaling Technology	2712; RRID: AB_2197223
Anti-mouse IgG HRP-linked antibody	Cell Signaling Technology	7076; RRID: AB_330924
Anti-rabbit IgG HRP-linked antibody	Cell Signaling Technology	7074; RRID: AB_2099233
FITC anti-IgG (human)	Miltenyi Biotec	130-093-192; RRID: AB1036185
APC anti-IgM (human)	Miltenyi Biotec	130-093-076; RRID: AB_1036084
AffiniPure F(ab')2 Fragment Goat Anti-Human IgM	Jackson ImmunoResearch	109-006-129; RRID: AB_2337553
Monoclonal anti-CD20 (Rituximab, Mabthera)	Roche	9197719
Biological Samples		
All human DLBCL samples analyzed by IHC	This manuscript & GELA-LNH03-6B	N/A
Human tumor cells from DLBCL core-needle biopsies	This manuscript	N/A
Chemicals, Peptides, and Recombinant Proteins		
2-mercaptoethanol	Thermo Fisher Scientific	31350010
D-(+)-Glucose	Sigma-Aldrich	G6152
L-glutamine	Thermo Fisher Scientific	25030081
L-asparagine	Sigma-Aldrich	A0884
L-glutamate	Sigma-Aldrich	G1626
Malate	Sigma-Aldrich	M9138
Succinate	Sigma-Aldrich	S2378
Adenosine 5'-diphosphate disodium salt (ADP)	Sigma-Aldrich	A2754
Adenosine 5'-triphosphate disodium salt (ATP)	Sigma-Aldrich	A7699

e1 Cell Metabolism 29, 1243–1257.e1–e10, June 4, 2019

(Continued on next page)

Construction Construction EAGENT or RESOURCE SOURCE DENTIFIER EAGENT or RESOURCE Sigma-Aldrich S2127 Sodium purvate Thermo Fisher Scientific 11300083 HEPES Thermo Fisher Scientific 11300083 HEPES Thermo Fisher Scientific 11300083 Iodoacetate Sigma-Aldrich 19148 Coloacetate Sigma-Aldrich 04376 Cathoryl cyanide 4 (trifluoromethoxylphenylhydrazone (FCCP) Sigma-Aldrich R8875 Cathoryl cyanide 4 (trifluoromethoxylphenylhydrazone (FCCP) Sigma-Aldrich R8875 Artimych A Sigma-Aldrich R8875 Artimych A Sigma-Aldrich R8875 VIIIma Gold PerkinElmer NEC4510SOUC Uitma Gold PerkinElmer NEC4510SOUC Uitma Gold PerkinElmer 03321 Mitotracker Green Invitrogen M7514 Digitonin Sigma-Aldrich 09542 Typan blue Sigma-Aldrich 125001 Catadaeute-Pyruvate transaminase (GPT) Sigma-Aldri	Continued		
BCBCL 00 PLCSORCE DC4/III. BLIMINE CONCEL DC4/III. Boldium proveste Sigma-Akdrich S2250 Sodium proveste Thermo Fisher Scientific 11360088 BFPES Thermo Fisher Scientific 15630055 brikotinamide adenine dinucleotide hydrate (i-NAD') Sigma-Akdrich N1848 Digomycin Sigma-Akdrich 19148 Digomycin Sigma-Akdrich 04876 Carbonyl cyanide 4-(trifluoromethoxylphenylhydrazone (FCCP) Sigma-Akdrich A8674 Carbonyl cyanide 4-(trifluorinotethoxylphenylhydrazone (FCCP) Sigma-Akdrich A8674 Chelonyl Carbonyl cyanide 4-(trifluorinotethoxylphenylhydrazone (FCCP) Sigma-Akdrich B0528 Chelonyl Carbonyl Carbonyl cyanide 4-(trifluorinotethoxylphenylhydrazone (FCCP) Sigma-Akdrich		SOLIDCE	IDENTIEIED
Landari Dignar Addrich ELCO Sodium carbonate Sigma-Addrich S2127 Sodium pryuvate Thermo Fisher Scientific 11500088 HEPES Thermo Fisher Scientific 15630056 Nationaride Sigma-Addrich N1538 Joganycin Sigma-Addrich N1538 Digonycin Sigma-Addrich N1538 Carbonyl cyanide 4 (trifluoromethoxylphenylhydrazone (FCCP) Sigma-Addrich A8874 Schanone Sigma-Addrich R8874 Arithrycin A Sigma-Addrich M23002-v Arithrycin A Sigma-Addrich M23002-v Arithrycin A Sigma-Addrich M23002-v Arithrycin A Sigma-Addrich M251050UC Ultima Gold PerkinElmer 6013321 Mitoracker Green Invitrogen M7514 Digitanin Digima-Addrich D5628 DAPI Sigma-Addrich D6542 Carbonatese (from Clostridium histolyticum) Sigma-Addrich D4627 Carbonatese (from Robit muscle) Sigma-Addrich		Sigma-Aldrich	12250
Optime Mathemic Science Sigma Addrich Defail HEPES Themo Fisher Scientific 11360083 B-Nicotinamide adenine dinucleotide hydrate (β-NAD') Sigma Addrich N1636 Oldoaceetate Sigma Addrich 19148 Olgomycin Sigma Addrich Q3902 Carbonyl cyanide 4-(trifluoromethoxylphenylthydrazone (FCP) Sigma Addrich R8975 Artimycin A Sigma Addrich M2833 Cyfdryll-Giutamine PerkinElmer MC541050UC Ultima Gold PerkinElmer MC541050UC Digtonin Sigma-Addrich D9542 Digtonin Sigma-Addrich D9542 Digtonin Sigma-Addrich D9542 Collagenase (from Clostridum histolyticum) Sigma-Addrich D9542 Collagenase (from rabbit muscle) Sigma-Addrich D4827 Collagenase (from rabbi	Sodium carbonate	Sigma-Aldrich	S2127
Docksin Protection Formo Field Scientific 16630056 b-Nicotinamide adenine dinucleotide hydrate (if-NAD') Sigma-Aldrich N1636 jodoacetate Sigma-Aldrich O4876 Carboryl cyanide 4-(trifluoromethoxylphenylhydrazone (FCCP) Sigma-Aldrich C8890 Rotenone Sigma-Aldrich A8871 Antimycin A A8674 23002-v 2-NBDG Sigma-Aldrich A8874 2-NBDG Sigma-Aldrich A8871 2-NBDG Sigma-Aldrich X333 2-NBDG Sigma-Aldrich M2331 2-NIBDG Sigma-Aldrich D5628 Digitorin Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 Dapitorin Sigma-Aldrich D5628 Dapitorin Sigma-Aldrich D6528 Dapitorin Sigma-Aldrich D6528 Dapitorin Sigma-Aldrich D4627 Dapitor	Sodium pyruvate	Thermo Fisher Scientific	11360088
Induction Induction Induction Histochamide adenine dinuclectide hydrate (Ir-NAD') Sigma-Aldrich N1636 Iodoacetate Sigma-Aldrich 1918 Oligomycin Sigma-Aldrich O4876 Cathonyl cyanide 4 (trifluoromethoxylphenylhydrazone (FCCP) Sigma-Aldrich C2920 Ratenone Sigma-Aldrich A8874 Antimycin A Sigma-Aldrich A8874 Antimycin A Sigma-Aldrich A8874 ArkHolp Jaminojlsobutyric acid (MeAIB) Sigma-Aldrich M2383 L(¹⁴ C(U))-Glutamine PerkinElmer 6013321 Wiltbracker Green Invitrogen M7514 Ultima Gold PerkinElmer 05522 DaPl Sigma-Aldrich D5542 Oligonase (Iron rabbit muscle) Sigma-Aldrich D5542 Dapl Sigma-Aldrich C3991 Deoxyribonuclease I (DNase) Sigma-Aldrich C3991 Deoxyribonuclease I (DNase) Sigma-Aldrich A9415 Cellaensee (from rabbit muscle) Sigma-Aldrich P4920 Cellaensee	HEPES	Thermo Fisher Scientific	15630056
Notional information of particle (No. 19) Sigma Addrich Instantion Oligomycin Sigma-Aldrich Ol4876 Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) Sigma-Aldrich R8875 Antimycin A Sigma-Aldrich R8875 Antimycin A Sigma-Aldrich R8875 Antimycin A Sigma-Aldrich M2333 2-MBDG Peptide Institute 2002-v 2-MBDG Sigma-Aldrich M2333 2-MBDG Sigma-Aldrich M2333 2-MBDG PerkinElmer NEC451050UC Ultima Gold PerkinElmer 013321 Mitotracker Green Invitrogen M7514 Digtorin Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 DAPI Sigma-Aldrich D4627 Catabomycin D Roche 10737127001 Collagenase (from Clostridium histolyticum) Sigma-Aldrich L250061 Sigma-Aldrich L25001 Sigma-Aldrich L25001 Colagenase (from cabbit muscle) Sigma-Aldrich	β-Nicotinamide adenine dinucleotide hydrate (β-NAD ⁺)	Sigma-Aldrich	N1636
Bigman Aldrich Order Digomycin Sigma Aldrich O4876 Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) Sigma Aldrich R8875 Rotenone Sigma Aldrich A8874 Antimycin A Sigma Aldrich A8874 2-NBDG Peptide Institute 23002-v 2-NBDG PerkinElmer 6013321 Ultima Gold PerkinElmer 601321 Ultima Gold PerkinElmer 601321 Ultima Gold Sigma-Aldrich D9562 Digitonin Sigma-Aldrich D9562 Digitonin Sigma-Aldrich D9562 Digitonin Sigma-Aldrich D9562 Collagenase (from Clostridium histolyticum) Sigma-Aldrich D9562 Digitonin Sigma-Aldrich D4627 Collagenase (from Clostridium histolyticum) Sigma-Aldrich D4627 Collagenase (from Clostridium histolyticum) Sigma-Aldrich D4627 Lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich D4627 Lactic dehydrogenase (from rabbit muscle) S	Indoacetate	Sigma-Aldrich	19148
Califording Sigma-Aldrich C2920 Cathonic yanide 4-(triflucromethoxy)phenylhydrazone (FCCP) Sigma-Aldrich R8875 Rotenone Sigma-Aldrich C2920 Rotenone Sigma-Aldrich R8875 Antimyoin A Sigma-Aldrich A8674 2-NBDG Peptide Institute 23002-v z-Wethylamino)isobutyric acid (MeAIB) Sigma-Aldrich M2383 z-Uff-CQU)-Glutamine PerkinElmer NEC451050UC Ultima Gold PerkinElmer NEC451050UC Ultima Gold Sigma-Aldrich D5628 Dapti Sigma-Aldrich D5628 DAPI Sigma-Aldrich D9542 Typan blue Thermo Fisher Scientific 15550061 Glutamate-Pyruvate Transaminase (GPT) Roche 10737127001 Collagenase (from Clostridium histolyticum) Sigma-Aldrich D4627 L-lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich D4627 L-lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich P1045 Rapamycin Gaesta-Aldrich D4627	Oligomycin	Sigma-Aldrich	04876
Base Base Antimycin Y glanta Yndyr Garlan (Sarb) Sigma-Aldrich Relation Antimycin A Sigma-Aldrich A6674 2-NBDG Peptide Institute 2002-v ar.(Methylaino)isobutyric acid (MeAIB) Sigma-Aldrich M2383 L-[1 ⁴ C(U)]-Glutamine PerkinElmer NEC451050UC Utima Gold PerkinElmer 6013321 Mitotracker Green Invitrogen M7514 Digitorin Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 DAPI Sigma-Aldrich C828061 Glutamate-Pyruvate Transaminase (GPT) Roche 10737127001 Collagenase (from Clostridium histolyticum) Sigma-Aldrich C8290 Deoxyribonuclease I (DNase) Sigma-Aldrich 2500 Actionorycin D Sigma-Aldrich 2490 Call Tack Thermo Fisher Scientific 1317081 Pherofermin hydrochloride Sigma-Aldrich 29020 Temsirolimus Yords 29020 Temsirolimus (Torisel) Mator NA	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (ECCP)	Sigma-Aldrich	C2920
Naturation Signar-Aldrich Aedro Antimycin A Sigma-Aldrich Aedro 2-NBDG Peptide Institute 23002-v 2-NBDG Sigma-Aldrich M2383 2-NEDG PerkinElmer 6013321 Ultima Gold PerkinElmer 6013321 Mitotracker Green Invitrogen M7514 Digitonin Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 Collagenase (from Costridium histolyticum) Sigma-Aldrich D5629 Deoxyribonuclease I (DNase) Sigma-Aldrich C3600 Lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich D4627 Lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich P3112 Lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich P2002 Lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich P2020 Tensiolimus Sigma-Aldrich	Botenone	Sigma-Aldrich	B8875
Addition Fight Function Function 2-NBDG Peptide Institute 23002-v ar-(Methylamino)isobutyric acid (MeAIB) Sigma-Aldrich M2383 2-(1 ^{+C} (U))-Guttamine PerkinElmer NEC451050UC Ultima Gold PerkinElmer 6013321 Miktoracker Green Invitrogen M7514 Digitonin Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 Guttamate-Pyruvate Transaminase (GPT) Roche 10737127001 Collagenase (from Clostridium histolyticum) Sigma-Aldrich C9891 Deoxyribonuclease (ICNAse) Sigma-Aldrich D4627 Cell Tack Thermo Fisher Scientific 10317081 Cell Tack Thermo Fisher Scientific 10317081 Penetomin hydrochloride Sigma-Aldrich P20020 Temsirolimus Gone-techne 1292 Temsirolimus Sigma-Aldrich P20020 Temsirolimus (Torisel) Alzz Pharmaceutical N/A E-Col-L-asparaginase (Kidrolase)	Antimycin A	Sigma-Aldrich	A8674
Avdetby/amino)isobutyric acid (MeAIB) Sigma-Aldrich M2383 -(Methy/amino)isobutyric acid (MeAIB) Sigma-Aldrich M2383 L-[1 ⁴ C(U)]-Glutamine PerkinElmer NEC451050UC Ultima Gold PerkinElmer 6013321 Mitotracker Green Invitrogen M7514 Digitorin Sigma-Aldrich D5628 DAPI Sigma-Aldrich D5628 Collagenase (from Clostrichum histolyticum) Sigma-Aldrich D491 Collagenase (from Clostrichum histolyticum) Sigma-Aldrich D4627 Collagenase (from rabbit muscle) Sigma-Aldrich D4627 L-lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich D4627 L-lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich D4627 L-lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich P7045 Rapamycin Bio-techne 10317081 Performin hydrochloride Sigma-Aldrich P7045 Rapamycin Bio-techne 1292 Temsicilmus Sigma-Aldrich P20020 Temsicilmus (Torisei) Priz	2-NBDG	Pentide Institute	23002-v
Artesting Organization Description [1 ⁴ C(U)]-Glutaminie Organization NEC451050UC Uttima Gold PerkinElmer 6013321 Mitotracker Green Invitrogen M7514 Digitoriin Sigma-Aldrich D628 DAPI Sigma-Aldrich D9542 Trypan blue Thermo Fisher Scientific 15250061 Glutamate-Pyruvate Transaminase (GPT) Roche 10737127001 Collagenase (from Clostridium histolyticum) Sigma-Aldrich C9891 Deoxyfionuclease I (DNase) Sigma-Aldrich D427 Collagenase (from rabbit muscle) Sigma-Aldrich A9415 Cell Tack Thermo Fisher Scientific 10317081 Cell Tack Thermo Fisher Scientific 10317081 Phenformin hydrochloride Sigma-Aldrich P20020 Rapamycin Bio-techne 1292 Temsirolimus (Torisel) Piter N/A ErColi-L-asparaginase (Kidrolase) Jazz Pharmaceutical N/A ErColi-Lasparaginase (Kidrolase) Jazz Pharmaceutical N/A <tr< td=""><td>a-(Methylamino)isobutyric acid (MeAIB)</td><td>Sigma-Aldrich</td><td>M2383</td></tr<>	a-(Methylamino)isobutyric acid (MeAIB)	Sigma-Aldrich	M2383
I octaminationFormulationRecorrectionI octaminationPerkinElmer6013321Mitotracker GreenInvitrogenM7514DigitoninSigma-AldrichD5628DAPISigma-AldrichD9542Trypan blueThermo Fisher Scientific15250061Glutamate-Pyruvate Transaminase (GPT)Roche10737127001Collagenase (from Clostridium histolyticum)Sigma-AldrichC9891Deoxytibonuclease I (DNase)Sigma-AldrichL2500Actiomycin DSigma-AldrichL2500Actiomycin DSigma-AldrichL2500Cell TackThermo Fisher Scientific10317081Cell TackThermo Fisher Scientific1292Temsirolimus (Torisel)PfizerN/AE-Col/L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce ECL Western Blotting substrateThermo Fisher Scientific23226Bio-Rad Tortein Assay Dye Reagent concentrateBio-Rad500-006Bio-Rad Scientific321632163216Coll Cotein Assay Dye Reagent concentrateBio-Rad500-006Reasy mini kit		PerkinElmer	NEC451050LIC
Number Formulation Formulation Witotracker Green Invitrogen M7514 Digitonin Sigma-Aldrich D5628 DAPI Sigma-Aldrich D9542 Trypan blue Thermo Fisher Scientific 15250061 Glutamate-Pyruvate Transaminase (GPT) Roche 10737127001 Collagenase (from Clostridium histolyticum) Sigma-Aldrich D4827 Deoxyribonuclease I (DNase) Sigma-Aldrich D4527 L-lactic dehydrogenase (from rabbit muscle) Sigma-Aldrich D4937 Cell Tack Thermo Fisher Scientific 1317081 Phenormin hydrochloride Sigma-Aldrich P4045 Rapamycin Bio-techne 1292 Temsirolimus (Torisel) Pfizer N/A E-Col/L-asparaginase (Kidrolase) Jazz Pharmaceutical N/A Verter N/A Sigma-Aldrich 2325 Pierce ECA Protein Assay Kit Thermo Fisher Scientific 23216 E-Col/L-asparaginase (Kidrolase) Jazz Pharmaceutical N/A Pierce ECA Western Blotting substrate Thermo Fish		PerkinElmer	6013321
MinistrationMinistrationDigitorinSigma-AldrichD5628DAPISigma-AldrichD9542Trypan blueThermo Fisher Scientific15250061Glutamate-Pyruvate Transaminase (GPT)Roche10737127001Collagenase (from Clostridium histolyticum)Sigma-AldrichC9891Deoxyribonuclease I (DNase)Sigma-AldrichD4627L-lactic dehydrogenase (from rabbit muscle)Sigma-AldrichA9415Cell TackThermo Fisher Scientific10317081Phenformin hydrochlorideSigma-AldrichP7045RapamycinBio-techne1292TemsirolimusSigma-AldrichP20020TemsirolimusSigma-AldrichP20020Temsirolimus (Torisel)PfizerN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce ECA Protein Assay KitThermo Fisher Scientific23225Pierce ECA Protein Assay Dye Reagent concentrateBio-Rad500-006Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Orniscript RT kitQiagen74104Orniscript RT kitDi Biosciences55491Cell Titer GloPromega67570BD Pharm LyseBD Bioscience555899	Mitotracker Green	Invitrogen	M7514
DarpiDignet MithenDescriptionDAPISigma-AkirichD9542Trypan blueThermo Fisher Scientific15250061Glutamate-Pyruvate Transaminase (GPT)Roche10737127001Collagenase (from Clostridium histolyticum)Sigma-AkirichC9891Deoxyribonuclease I (DNase)Sigma-AkirichD4627L-lactic dehydrogenase (from rabbit muscle)Sigma-AkirichA9415Cell TackThermo Fisher Scientific10317081Phenformin hydrochlorideSigma-AkirichP7045RapamycinBio-techne1292TemsirolimusSigma-AkirichP20020Temsirolimus (Torisel)PfizerN/AE-Coli-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminWylanN/ACritical Commercial AssaysThermo Fisher Scientific32106Pierce ECL Western Blotting substrateThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen205113Orniscript RT kitQiagen205113Christer Scientific4366072205113Cyftix/Cytoperm kitBD Biosciences55714Cell Titer GloPromega67570BD Pharm LyseBD Bioscience55899	Digitonin	Sigma-Aldrich	D5628
DescriptionDescriptionTrypan blueThermo Fisher Scientific15250061Glutamate-Pyruvate Transaminase (GPT)Roche10737127001Collagenase (from Clostridium histolyticum)Sigma-AldrichC9891Deoxyribonuclease I (DNase)Sigma-AldrichD4627L-lactic dehydrogenase (from rabbit muscle)Sigma-AldrichL2500Actinomycin DSigma-AldrichA9415Cell TackThermo Fisher Scientific10317081Phenformin hydrochlorideSigma-AldrichP7045RapamycinBio-techne1292TemsirolimusSigma-AldrichP20020Temsirolimus (Torisel)PfizerN/AE-Coli-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific32106Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-103Reagen pini kitQiagen205113Tagman Fast Universal PCR Master MixFisher Scientific366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharn LyseBD Bioscience55889		Sigma-Aldrich	D9542
TypeTypeTypeTypeType1000000000000000000000000000000000000	Trypan blue	Thermo Fisher Scientific	15250061
Collagenase (from Clostridium histolyticum)Sigma-AldrichC9891Collagenase (from Clostridium histolyticum)Sigma-AldrichD4627L-lactic dehydrogenase (from rabbit muscle)Sigma-AldrichL2500Actinomycin DSigma-AldrichA9415Cell TackThermo Fisher Scientific10317081Phenformin hydrochlorideSigma-AldrichP7045RapamycinBio-techne1292TemsirolimusSigma-AldrichPZ0020Temsirolimus (Torisel)PfizerN/AE-Coli-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen74104Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience55889	Glutamate-Pyruvate Transaminase (GPT)	Boche	10737127001
Debayring Debayring Debayring DebayringSigma-AldrichDebayring Debayring DebayringDebayring Debayring Lactic dehydrogenase (from rabbit muscle)Sigma-AldrichL2500Actinomycin DSigma-AldrichA9415Cell TackThermo Fisher Scientific10317081Phenformin hydrochlorideSigma-AldrichP7045RapamycinBio-techne1292TemsirolimusSigma-AldrichP20020TemsirolimusFizerN/AE-Coll-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific354714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience55889	Collagenase (from Clostridium histolyticum)	Sigma-Aldrich	C9891
Decory monitorical decory monitoriesDiginal ArtanianiaDecoryL-lactic dehydrogenase (from rabbit muscle)Sigma-AldrichL2500Actinomycin DSigma-AldrichA9415Cell TackThermo Fisher Scientific10317081Phenformin hydrochlorideSigma-AldrichP7045RapamycinBio-techne1292Temsirolimus (Torisel)PfizerN/AE-Col/-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience55899		Sigma-Aldrich	D4627
Label ControlSigma AldrichEconomicActinomycin DSigma AldrichA9415Cell TackThermo Fisher Scientific10317081Phenformin hydrochlorideSigma AldrichP7045RapamycinBio-techne1292TemsirolimusSigma AldrichP20020Temsirolimus (Torisel)PfizerN/AE-Coll-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006Bio-Rad Protein Assay Dye Reagent concentrateGiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific356072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience555899	L-lactic dehydrogenase (from rabbit muscle)	Sigma-Aldrich	1 2500
NationOppint ProductionOppint ProductionCell TackThermo Fisher Scientific10317081Phenformin hydrochlorideSigma-AldrichP7045RapamycinBio-techne1292Temsirolimus (Torisel)Sigma-AldrichPZ0020Temsirolimus (Torisel)PfizerN/AE-Coli-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific23225Pierce ECL Western Blotting substrateBio-Rad500-006Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific360072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience55899		Sigma-Aldrich	A9415
ConstructionInformite ConstructionFor HorizonPhenformin hydrochlorideSigma-AldrichP7045RapamycinBio-techne1292TemsirolimusSigma-AldrichP20020Temsirolimus (Torisel)PfizerN/AE-Coli-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific36072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience555899	Cell Tack	Thermo Fisher Scientific	10317081
RanamycinFirstRapamycinBio-techne1292TemsirolimusSigma-AldrichPZ0020Temsirolimus (Torisel)PfizerN/AE-Coli-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific3366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience55899	Phenformin hydrochloride	Sigma-Aldrich	P7045
TensirolinusElectronicTensiTensirolinusSigma-AldrichPZ0020Tensirolinus (Torisel)PfizerN/AE-Coli-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific354714Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience555899	Banamycin	Bio-techne	1292
TomoroundedOrginal FlaterionFlaterFlaterTemsirolimus (Torisel)PfizerN/AE-Coli-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience55899	Temsirolimus	Sigma-Aldrich	P70020
E-Coli-L-asparaginase (Kidrolase)Jazz PharmaceuticalN/AMetforminMylanN/ACritical Commercial AssaysPierce BCA Protein Assay KitThermo Fisher Scientific23225Pierce ECL Western Blotting substrateThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseDB Bioscience55899	Temsirolimus (Torisel)	Pfizer	N/A
DefinitionMylanN/AMetforminMylanN/ACritical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience55899	F-Coli-L-asparaginase (Kidrolase)	Jazz Pharmaceutical	N/A
Critical Commercial AssaysThermo Fisher Scientific23225Pierce BCA Protein Assay KitThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Plarm LyseBD Bioscience55899	Metformin	Mylan	N/A
Pierce BCA Protein Assay KitThermo Fisher Scientific23225Pierce ECL Western Blotting substrateThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience55899	Critical Commercial Assays		
Pierce ECL Western Blotting substrateThermo Fisher Scientific32106Bio-Rad Protein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience55899	Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23225
Bio-RadProtein Assay Dye Reagent concentrateBio-Rad500-006RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience55899	Pierce ECI. Western Blotting substrate	Thermo Fisher Scientific	32106
RNeasy mini kitQiagen74104Omniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Plasscience55899	Rio-Bad Protein Assay Dye Reagent concentrate	Bio-Bad	500-006
AndersynthickAndersynthickAndersynthickOmniscript RT kitQiagen205113Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Plarm LyseBD Bioscience555899	RNeasy mini kit	Oiagen	74104
Taqman Fast Universal PCR Master MixFisher Scientific4366072Cytofix/Cytoperm kitBD Biosciences554714Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience555899	Omniscrint BT kit	Qiagen	205113
Cytoir/Cytoperm kit BD Biosciences 554714 Cell Titer Glo Promega G7570 BD Pioscience 555899	Tagman Fast Universal PCB Master Mix	Fisher Scientific	4366072
Cell Titer GloPromegaG7570BD Pharm LyseBD Bioscience555899	Cvtofix/Cvtoperm kit	BD Biosciences	554714
BD Pharm Lyse BD Bioscience 555899	Cell Titer Glo	Promega	G7570
	BD Pharm I vse	BD Bioscience	555899
Seaborse XFe96 extracellular Flux Assav Kit Agilent 102416-100	Seahorse XEe96 extracellular Elux Assav Kit	Agilent	102416-100
EnVision FLEX, High pH, (Link) immunohistochemistry Agilent (Dako) K8000 detection system Kit	EnVision FLEX, High pH, (Link) immunohistochemistry detection system Kit	Agilent (Dako)	K8000
EnVision FLEX Target Retrieval Solution, High pH (50x) Agilent (Dako) K8004	EnVision FLEX Target Retrieval Solution, High pH (50x)	Agilent (Dako)	K8004
EnVision FLEX Target Retrieval Solution, Low pH (50x) Agilent (Dako) K8005	EnVision FLEX Target Retrieval Solution, Low pH (50x)	Agilent (Dako)	K8005
EnVision FLEX Antibody Diluent Agilent (Dako) K8006	EnVision FLEX Antibody Diluent	Agilent (Dako)	K8006
EnVision FLEX Hematoxylin, (Link) Agilent (Dako) SK308	EnVision FLEX Hematoxylin, (Link)	Agilent (Dako)	SK308
Histology FISH Accessory Kit Agilent (Dako) K5799	Histology FISH Accessory Kit	Agilent (Dako)	K5799

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
R code	Agnes Paquet	https://doi.org/10.17632/f4f3wbszmd.1
Experimental Models: Cell Lines		
Toledo	ATCC	ATCC CRL-2631
Karpas 422	ECACC	06101702
SU-DHL-4	DSMZ	ACC 495
SU-DHL-6	DSMZ	ACC 572
293T	ATCC	CBL-1573
Experimental Models: Organisms/Strains		
Mouse: Eu-Mvc	The Jackson laboratory	002728
Mouse: C57BL/6JOlaHsd	Envigo	N/A
Mouse gapdh	Thermo Fisher Scientific	Mm99999915 a1
Mouse m18s	Thermo Fisher Scientific	Mm03028990 g1
Human gandh	Thermo Fisher Scientific	Hs02758991 a1
	Thermo Fisher Scientific	Hc0000002 m1
		N/A
(pSUPER vector): forward 5'-GATCCCCGTATGATGACATC AAGAAGTTCAAGAGACTTCTTGATGTCATCATACTTTTA-3'	mismanuscript	N/A
Oligonucleotide for mouse <i>gapdh</i> targeting by shRNA (pSUPER vector): reverse 5'-AGCTTAAAAAGTATGATGACA TCAAGAAGTCTCTTGAACTTCTTGATGTCATCATACGGG-3'	This manuscript	N/A
Recombinant DNA		
FISH DNA probe for c-MYC/8q24	Dako	Y5410
FISH DNA probe for BCL2/18q21	Dako	Y5407
FISH DNA probe for BCL6/3q27	Dako	Y5408
Human gapdh coding sequence (M33197)	This manuscript	N/A
pCDNA3.1/V5-His TOPO TA plasmid	Invitrogen	K480001
pMIG vector	Addgene	9044
pSUPER.retro.neo+gfp	Oligoengine	VEC-PRT-0006
phCMV-VSV-G	Els Verhoeyen	N/A
MLV-Gag-Pol	Els Verhoeyen	N/A
Software and Algorithms		
Macsquantify Version 2.11	Miltenyi	N/A
Multigauge software	Fuji Film	N/A
Image J	NIH	https://imagej.nih.gov/ij/
Prism 7	Graphpad	https://www.graphpad.com/ scientificsoftware/prism/
Metaboanalyst		https://www.metaboanalyst.ca/
R project	The R foundation	https://www.r-project.org/
Ingenuity Pathway Analysis software	QIAGEN	http://www.ingenuity.com/
SlidePath Digital Image	Leica Biosystems	N/A
Hans algorithm	Hans et al. (2004).	N/A
Other		
DMEM GlutamaX	Thermofisher Scientific	31966047
DMEM, high glucose, no glutamine	Thermofisher Scientific	11960044
DMEM no glucose	Thermofisher Scientific	11966025
RPMI medium 1640 GlutamaX	Thermofisher Scientific	61870044
DMEM Low Glucose, w/o Amino Acids. Pvruvic Acid	US Biological	D9800-13
XF Base Medium Minimal DMEM	Agilent	102353-100

e3 Cell Metabolism 29, 1243–1257.e1–e10, June 4, 2019

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jean-Ehrland Ricci (ricci@unice.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice and In Vivo Studies Using Eµ-Myc Cells

C57BL/6 Eµ-Myc transgenic mice were purchased from the Jackson Laboratory (#002728) and housed in our local animal facility (C3M, Nice, France).

The lymphoma transfer of E μ -*Myc* cells was performed into syngeneic, nontransgenic, 6-weeks-old C57BL/6JOlaHsd females (Envigo) by intravenous injection (iv) of 0.1 × 10⁶ viable E μ -*Myc* lymphoma cells per recipient mouse (in 150 µl of sterile PBS). Six days after cell injection, mice received treatments. 100 mg/kg/day of Phenformin (P7045, Sigma-Aldrich) or vehicle (H₂O) (n=10 mice/group) were administrated by gavage (*p.o*) every day for three weeks. 2500 Ul/kg/day of *E*-*Coli* L-asparaginase (Kidrolase, L-ase, Jazz Pharmaceutical) or vehicle (NaCl 0.9%) was injected intraperitoneally for five days the first week and three times per week the following weeks, for a total of three weeks of treatment (n=6 mice/group). 10 mg/kg of Temsirolimus (PZ0020, Sigma-Aldrich) or vehicle (NaCl 0.9%-5% Tween-5% PEG-200-1% EtOH) were intraperitoneally injected two times per week for three weeks (n=6 mice per group). Tumor onset was determined by inguinal lymph node palpation. Lymphoma-bearing animals were sacrificed by cervical dislocation. Phenformin-treated mice were sacrificed two hours after the last gavage of phenformin to analyze effects of the compound on lymphoma cell signaling. *E*-*Coli* L-asparaginase (L-ase) treated mice were sacrificed four hours after the last *ip* injection of L-ase and plasma was immediately isolated to analyze plasma glutamine concentrations. Temsirolimus treated mice were sacrificed 24 hours after the last treatment to analyze *in vivo* efficacy of this compound within the lymphomas.

All mice were maintained in specific pathogen-free conditions and all experimental procedures were approved by the national Animal Care and Use Committee and the regional ethics committee (05111.02 from Direction Générale de la Recherche et de l'Innovation). Survival was determined as the time between the *i.v.* injection of the cells and the time when mice had to be sacrificed, as soon as they exhibit systemic signs of illness (apathy, hair loss, breathing problems, precipitous weight loss, and limited ability to reach food or water), in agreement with the guidelines of the Institutional Animal Care and Use Committee. Food was given *ad libitum*. Experiments using the Eµ-*Myc* cells transplantation in syngeneic C57BL/6 mice have shown statistical significance starting from 6 mice/group. No randomization was performed. Endpoint was evaluated by blinding.

Cell Lines and Culture Conditions

Mouse primary Eµ-*Myc* lymphomas cells were isolated from different C57BL/6 Eµ-*Myc* transgenic mice as previously described (Chiche et al., 2015) and maintained in DMEM-GlutamaX (#31966047, Thermo Fisher Scientific) supplemented with 10% FBS, 50 µM of 2-mercaptoethanol (#31350010, Thermo Fisher Scientific), 0.37 mM of L-asparagine (A0884, Sigma-Aldrich) and 10 mM of HEPES pH 7.4 (#15630056, Thermo Fisher Scientific).

The human DLBCL cell lines Toledo, Karpas 422, SU-DHL-4 and SU-DHL-6 were tested mycoplasma-free. Cell line authentication was performed by DSMZ by DNA profiling using 8 different and highly polymorphic short tandem repeat (STR) loci. There was also no presence of mitochondrial DNA sequences from rodent cells as mouse, rat, Chinese and Syrian hamster. The Karpas 422 cell line was from the ECACC (European Collection of Authenticated Cell cultures), the Toledo cell line was from ATCC (American Type Culture Collection), the SU-DHL-4 and SUD-HL-6 cell lines were from DSMZ (German Collection of Microorganisms and Cell Culture). Toledo, Karpas 422 and SU-DHL-4 cells were maintained in RPMI-GlutamaX (#61870044, Thermo Fisher Scientific), supplemented with 10% FBS. SU-DHL-6 cells were maintained in RPMI-GlutamaX supplemented with 20% FBS.

Microarray Data Analysis

Raw.CEL files and clinical information from the study of Lenz et al. (2008). were downloaded from the Gene Expression Omnibus database (GEO: GSE10846). The total number of samples included in this study was 414, 181 treated with CHOP and 233 with R-CHOP. Data were summarized, normalized and log_2 transformed using the justRMA function from the Bioconductor package affy (version 1.40.0; Gautier et al., 2004). Follow-up time was limited to 5 years. Association between gene expression levels and overall survival was performed on samples from the R-CHOP arm only. Samples with low expression levels were filtered out (log_2 intensity < 8). For each remaining probeset, samples were split into two groups (below and above the median) and difference in survival was assessed using Cox proportional hazards models. 587 candidate probesets were selected based on nominal p-value < 0.05 and an $abs(log_2 HR) > 0.75$.

Association of expression levels with OS was first tested by categorizing samples into high/low expression samples using median values. Then for *gapdh*, cutoff was optimized using positional scanning (Lipton et al., 2010). Survival curves were plotted following the Kaplan and Meier method, and statistical significance was assessed using the log-rank test. Survival analysis was performed using functions from the R package survival.

OxPhos and BCR classification were obtained from the supplemental data of Caro et al. (2012). Statistical significance was assessed using Wilcoxon rank sum test. Enrichment in biological themes (Molecular function and canonical pathways) were performed using Ingenuity Pathway Analysis software.

DLBCL Patients: Training & Validation Cohorts

DLBCL tumors were required to be *de novo* and treated with Rituximab-CHOP (R-CHOP). All cases were diagnosed and reviewed by experienced hematopathologists and patient clinical information were obtained from the Hopital Saint Louis, Centre Hospitalier Princesse Grace and Centre Antoine Lacassagne institutions. DLBCLs were classified according to the WHO classification. Clinical data including gender, age, and age-adjusted International Prognosis Index (IPlaa) (scored (0-1, 2 and 3) based on i) the Ann Arbor stage (II-II vs III-IV), ii) the level of lactate dehydrogenase in the serum (LDH \leq normal vs \geq normal) and iii) the Performance status (PS 0-1 vs 2-4)) were collected in all cases and are summarized in Table S2. These cases were anonymized and processed in accordance with a protocol approved by scientific committee of the LYSA (The Lymphoma Study Association). All patients gave written non-opposition consent. Patients samples were allocated to GAPDH high and low groups according to their respective expression (see GAPDH immunohistochemical (IHC) staining and scoring).

Training Cohort

A total of 43 patients aged >18 years (aged between 24-85 years) who had been diagnosed with *de novo* DLBCL and then treated with R-CHOP between 2008 and 2014 at Onco-Hematology Department of Nice CHU (France) and Saint-Louis Hospital, APHP, Paris (France) were included in this training set. The samples were prepared from the paraffin blocks of lymph nodes that were recovered from the archives of the Pathology departments.

Validation Cohort

All cases analyzed in the validation cohort were enrolled in the prospectively randomized phase 3 multi-center GELA-LNH03-6B trial (Delarue et al., 2013) in which 602 patients with untreated DLBCL and at least one adverse prognostic factor aged between 60-80 years had been randomly assigned to receive eight cycles of R-CHOP-14 or eight cycles of R-CHOP-21. They did not show any difference in efficacy endpoints between dose-dense and standard regimens of R-CHOP. 294 biopsies specimens at diagnosis were available for the tissue microarray (TMA). TMA contains three representative 0.6-mm cores of routinely processed tissues (Beecher Instruments, Silver Spring, MD).

Clinical Protocol KTM (Kidrolase-Torisel-Metformin)

Four patients harboring *de novo Myc*-translocated-DLBCLs and selected for KTM treatment presented i) a retrospective GAPDH^{low} levels (scored by IHC in diagnosed biopsies), ii) a therapeutic failure after a median of 2 prior lines of Rituximab-based therapies and iii) adequate performance status and organ functions. KTM treatment consisted in L-asparaginase (K, Kidrolase, Jazz Pharama-ceutical, 6000 UI/m²) on days 1, 3, 5, 7, 9, 11, and 13, mTOR inhibitor (T, Torisel, Pfizer, 75 mg) on days 1, 7 and 14, and Metformin (Mylan, 1000 mg/day) on day 14 to day 28. The study was approved on Oct 16th, 2012 by the Scientific Committee of the LYSA (The Lymphoma Study Association, reference: REMtor). All patients gave a written informed consent to receive the treatment. Consent to publish patients' images was obtained. There was no clinical trial registration number because the therapeutic protocol was conducted on a local cohort.

METHOD DETAILS

COO Classification

Frozen tissues were analyzed using GeneChip Human Genome HGU133 Plus 2.0 arrays (Affymetrix, Santa Clara) as previously reported (Dubois et al., 2017). From the LNH03-6B TMA (validation cohort), sixty DLBCLs had both COO molecular classification.

Reverse transcription-multiplex ligation-dependent probe amplification (RT-MLPA) was performed to determine the COO on thirty-five formalin-fixed paraffin-embedded (FFPE) DLBCLs (training cohort), using a targeted molecular gene expression approach as recently described (Mareschal et al., 2015).

GAPDH Immunohistochemical (IHC) Staining and Scoring

Sections (4 µm) of formalin-fixed, paraffin-embedded DLBCL biopsies (training cohort) or tissue-microarray (TMA) from the LYSA group (validation cohort) were treated for deparaffinization, rehydration, and antigen retrieval using standard procedures (EnVision FLEX reagents, Agilent, Dako). Before immunohistochemistry, antigen retrieval was performed at pH 9.0, for 20 minutes at 97°C, according to the manufacturer's instructions (EnVision FLEX reagents, Agilent, Dako). After washing in diluted EnVision FLEX Wash Buffer, slides were incubated in EnVision FLEX Peroxidase-Blocking Reagent solution for 5 min to quench endogenous peroxidase. Slides were then washed, stained at room temperature on an automated system (Autostainer link 48, Dako) with diluted (EnVision FLEX antibody diluent) anti-GAPDH (rabbit polyclonal anti-human GAPDH, HPA40067, Sigma-Aldrich; dilution 1:500) for 20 minutes, washed, incubated 20 minutes with Dako EnVision FLEX /HRP), washed, incubated 8 minutes with EnVision FLEX Substrate Working solution and counter-stained with Hematoxylin (EnVision FLEX Hematoxylin). Sections were then dehydrated and mounted with pertex (Histolab).

Cytoplasmic GAPDH IHC staining was blindly scored by two pathologists from the LCAP from Nice CHU (Dr D. Ambrosetti and Dr. J. Reverso-Meinietti) based on two parameters: the GAPDH IHC staining intensity in tumor cells (scored 0 for absence of staining, 1 for a weak staining, 2 for a medium staining and 3 for a strong staining) and the proportion of tumor cells displaying each intensity. Then GAPDH IHC score was obtained by multiplying each GAPDH staining intensity with the corresponding percentage of tumor cells stained (example: GAPDH IHC score= 0 (non-stained) x % tumor cells + 1 (weak intensity) x % tumor cells stained + 2 (medium intensity) x % tumor cells stained + 3 (strong intensity) x % tumor cells stained). For the training cohort, GAPDH IHC

staining was scored on the whole slide. For the TMA, GAPDH IHC score represents the mean of three different tissue spots from the same tumor. GAPDH IHC cut off of 150 was determined using positional scanning (Lipton et al., 2010). Illustrations of GAPDH IHC staining were performed using SlidePath Digital Image software (Leica Biosystems).

Immunohistochemistry and Fluorescence In Situ Hybridization

Sections (4 µm) of formalin-fixed, paraffin-embedded DLBCL biopsies (training cohort) were treated for deparaffinization, rehydration, and antigen retrieval using standard procedures (EnVision FLEX reagents, Agilent, Dako). Antibodies against CD5 (M3641, Agilent, Dako), CD79A (M7050, Agilent, Dako), CD20 (M0755, Agilent, Dako), CD10 (GA64861-2, Agilent, Dako), BCL2 (M0887, Agilent, Dako), BCL6 (M7211, Agilent, Dako), MUM1 (M7259, Agilent, Dako), c-MYC (ab32072, Abcam) and phospho-p70S6K (T389) (rabbit monoclonal anti-phospho-p70S6K (T389), #9234, Cell Signaling Technology) were used for immunostainings performed on an automated system (Autostainer link 48, Dako). Antigen retrieval was performed at specific pH prior incubation with indicated antibodies: pH 6.0 prior anti-CD79A, anti-CD20, anti-MUM1 and pH 9.0 prior anti-CD5, anti-CD10, anti-BCL2, anti-BCL6, anti-c-Myc and anti-phospho-p70S6K (T389). Primary antibodies were diluted in EnVision FLEX antibody diluent (anti-CD5, anti-CD10, anti-CD20, anti-BCL2 are prediluted; anti-BCL6, dilution 1:50; anti-phospho-p70S6K (T389) 1:50; anti-CD79A, dilution 1:100; anti-MUM1, dilution 1:100; anti-c-Myc, dilution 1:100) (see IHC procedure in GAPDH immunohistochemical (IHC) staining and scoring section).

Tissue-microarrays (TMA) from the LYSA group (validation cohort) were treated and immunoassayed for the same proteins using standard procedures of the LYSA platform as previously described (Molina et al., 2014). GCB vs non-GCB scoring according to the Hans algorithm (Hans et al., 2004), as well as MYC and BCL2 were evaluated (Petrella et al., 2017). In 30 out of the 294 cases, COO classification by IHC (Hans algorithm) could not be assessed. The thresholds employed were 40% for MYC and 70% for BCL2 as previously reported (Green et al., 2012; Johnson et al., 2012).

FISH analyses were performed on 3 μm tissue sections using split signal FISH DNA probes for cMYC/8q24 (Y5410, Dako), BCL2/ 18q21 (Y5407, Dako), BCL6/3q27 (Y5408, Dako) and slides were analyzed as previously described (Copie-Bergman et al., 2009). Scoring of the hybridization signals were performed according to the algorithm published by Haralambieva et al. (2002).

ATP Analysis

ATP was measured using the Cell Titer Glo kit (G7570, Promega). Briefly, 20,000 cells were resuspended in 80 μ L of corresponding medium supplemented with 10% FBS and distributed in a 96 well plate. Cells were then treated in triplicates for one hour with PBS (control), or sodium iodoacetate (100 μ M) to inhibit glycolysis, or a combination of 100 μ M of iodoacetate (I9148, Sigma-Aldrich) and 10 μ g/ml of oligomycin (O4876, Sigma-Aldrich) to obtain the residual amount of ATP. Short time treatment (1hour) was chosen to avoid a metabolic switch upon inhibition of glycolysis and to avoid cell death upon inhibition of both metabolic pathways. 100 μ L of Cell Titer Glo reaction mix was then added to each well for a final volume of 200 μ L. Plates were analyzed for luminescence with a Luminoscan (Berthold Technologies). We verified that ATP measurements were in the linear range of the detection. The difference between total ATP production and the ATP produced under iodoacetate treatment results in glycolytic ATP contribution. Glycolytic ATP is represented by percentage of total ATP produced by the cells.

Lactate Measurement

Lactate concentration in the supernatant was determined by an enzyme-based assay using 900 μ M β -NAD⁺ (N1636, Sigma-Aldrich), 175 μ g/ml L-lactic dehydrogenase (L2500, Sigma-Aldrich) and 100 μ g/ml glutamate-pyruvate transaminase (#10737127001, Roche) diluted in a 620 mM sodium carbonate (S2127, Sigma-Aldrich)-79 mM L-glutamate (G1626, Sigma-Aldrich) buffer adjusted to pH 10. Lithium lactate (L2250, Sigma-Aldrich) was used as a standard. Measurements were recorded from a microplate reader (excitation 355 nm, emission 612 nm) after 30 min incubation at 37°C. Lactate secretion was expressed as mM and normalized to viable cell numbers (using Trypan blue exclusion method).

Lactate concentration in the supernatant was also determined electro-enzymatically using the YSI 2950 Biochemistry Analyzer (Yellow Springs Instruments) and normalized to viable cell number.

Glutamine Consumption

Cells were grown in DMEM high glucose, no glutamine (#11960044, Thermo Fisher Scientific) supplemented with 2 mM of L-Glutamine (#25030081, Thermo Fisher Scientific), 10% FBS, 50 µM of 2-mercaptoethanol (#31350010, Thermo Fisher Scientific), 0.37 mM of L-asparagine (A0884, Sigma-Aldrich) and 10 mM of HEPES pH 7.4 (#15630056, Thermo Fisher Scientific) for 24 hours. The concentrations of L-Glutamine (mg/L) from cultured-cell supernatants were determined electro-enzymatically using the YSI 2950 Biochemistry Analyzer (Yellow Springs Instruments) and normalized to viable cell number.

L-[¹⁴C]-Glutamine Uptake

Cells (5x10⁶, in triplicate for each lymphoma) were washed with PBS and incubated 5 min at 37°C in 80 μ l of pre-warmed DMEM low glucose (5.5 mM), amino acid-, pyruvic acid- HCO₃⁻ -free (US Biological, D9800-13) supplemented to a final concentration of 15 mM D-glucose (G6152, Sigma-Aldrich), 20 mM HEPES (#15630056, Thermo Fisher Scientific) and adjusted to pH 7.4, prior addition of 1.25 μ Ci/ml of L-[¹⁴C]-Glutamine (NEC451050UC, PerkinElmer,) for 30 min at 37°C in a CO₂-free incubator. Subsequently, cells were washed twice with PBS and lysed with 120 μ l of 0.1N NaOH and mixed with 3 ml of Ultima Gold (#6013321, PerkinElmer).

Radioactivity was measured using a β -scintillation counter. Inhibition experiments were performed in the presence of 10 mM of the system A transporter inhibitor α -(Methylamino)isobutyric acid (MeAIB, M2383, Sigma-Aldrich).

2-NBDG Uptake

200.000 cells were incubated in glucose-free DMEM medium (#11966025, Thermo Fisher Scientific) containing 40 μM 2-NBDG (#23002-v, Peptide Institute) for 2 hours and analyzed by flow cytometry (Miltenyi Biotec) in the presence of 0.5 μg/ml of DAPI (D9542, Sigma-Aldrich) to represent glucose uptake (Mean Fluorescence intensity) in viable cells.

Measurement of the Mitochondrial Mass

The mitochondrial mass was measured by flow cytometry (Miltenyi Biotec) in viable (DAPI negative) cells), after 30 min incubation of cells at 37°C in the presence of 150 nM of Mitotracker green probe (M7514, Invitrogen).

Oxygen Consumption

Polarographic Studies

Intact cell respiration and mitochondrial substrates oxidation on 0.004% digitonin (D5628, Sigma-Aldrich)-permeabilized cells were carried out as previously described (Rustin et al., 1994). Proteins were measured according to Bradford microassay (#500-006, Bio-Rad). Results were expressed in nanomols of oxygen per minute per milligram of proteins or per cell number.

XF Seahorse Analyzer

Oxygen consumption rate (OCR) was measured in real time using the XF96 extracellular flux analyzer (Agilent). $E\mu$ -Myc cells were seeded on Cell-Tak (#10317081, Thermo Fisher Scientific)- coated XF96 plates at 0.12x10⁶ cells/well in 180 µl of XF base medium minimal DMEM media (#102353-100, Agilent) supplemented with 20 mM D-glucose (G6152, Sigma-Aldrich), 1 mM sodium pyruvate (#11360088, Thermo Fisher Scientific), 2 mM L-glutamine (#25030081, Thermo Fisher Scientific) and adjusted to pH 7.4. The plates were spun at 200 g (breaks off) and incubated at 37°C for 20 min to ensure cell attachment. Measurements were obtained under basal conditions and in response to mitochondrial inhibitors, 1 μ M oligomycin (O4876, Sigma-Aldrich), 0.5 μ M of Carbonyl cyanide 4-(trifluoromethoxy)phenylhudrazone (FCCP, C2920, Sigma-Aldrich), and 1 μ M rotenone (R8875, Sigma-Aldrich) combined with 2 μ M antimycin A (A8674, Sigma-Aldrich).

Mitochondrial Respiratory Complex Activities

Enzymatic spectrophotometric measurements of the OxPhos respiratory chain complex I, complex II and citrate synthase were performed at 37°C on cells according to standard procedures (Rustin et al., 1994). Proteins were measured according to Bradford microassay (#500-006, Bio-Rad). Results are expressed as activity ratios compared to the activity of citrate synthase.

Cell Viability Assays

Cells (0.3×10^6) were seeded in 96-well plates in the presence or absence (Ctl, DMSO or NaCl 0.9%) of phenformin (P7045, Sigma-Aldrich), *E-Coli* L-asparaginase (Kidrolase, Jazz Pharmaceutical), Rapamycin (#1292, Bio-techne) or anti-CD20 antibody Rituximab (Mabthera, #9197719, Roche) at indicated concentrations and period of time. Cells were then labeled with 0.5 µg/ml of DAPI (D9542, Sigma-Aldrich) and analyzed immediately by flow cytometry (Miltenyi Biotec).

Activation of the B-Cell Receptor

SU-DHL-6 cells (1.10^6 cells/ml) were incubated in the presence of 10 μ g/ml of anti-IgM antibody (#109-006-129, Jackson ImmunoResearch) for 10 min and 18hours.

Isolation of Human Tumor Cells from DLBCL Core-Needle Biopsies

'Fresh' image-guided core-needle biopsies (non-frozen tumor biopsies) were harvested in the radiology departments of St Louis hospital in Paris (APHP, France), Centre Hospitalier Princesse Grace (CHPG, Monaco) and the Centre Antoine Lacassagne (CAL, Nice, France), anonymized and immediately sent to our laboratory (Nice, France) in accordance with the GlyPhome protocol approved by institutional review boards of APHP, CHPG and CAL and Commission Nationale de l'Informatique et des Libertés (CNIL) reference 912657 V1. All patients gave written non-opposition consent. Immediately upon receipt, tumor cells were dissociated in RPMI medium (#61870044, Thermo Fisher Scientific) supplemented with 2% of FBS, 10 μg/ml of DNAse (D4627, Sigma-Aldrich) and 0.1 mg/ml of collagenase (C9891, Sigma-Aldrich) 5 min at 37°C. Red blood cells were lyzed (BD Pharm Lyse, #555899, BD Bioscience) 1 min at room temperature. After PBS washing, cells were counted (by flow cytometry, using DAPI staining) and analyzed for glycolytic ATP production in RPMI medium (#61870044, Thermo Fisher Scientific) supplemented with 10% FBS. Diagnosis of the biopsies was provided by histopathology analyses.

Plasmids and Generation of GAPDH-Overexpressing Cells and gapdh-Silenced Cells

The coding sequence of human *gapdh* (M33197) was first cloned by PCR into a pcDNA3.1/V5-His TOPO TA expression plasmid (K480001, Invitrogen) following classical methods. *gapdh* was then subcloned into a pMIG vector (9044, Addgene) for retroviral infection.

e7 Cell Metabolism 29, 1243-1257.e1-e10, June 4, 2019

The shRNA targeting mouse *gapdh* was obtained using the oligonucleotide sequences forward 5'-GATCCCCGTATGATGACATC AAGAAGTTCAAGAAGACTTCTTGATGTCATCATACTTTTA-3' and reverse 5'-AGCTTAAAAAGTATGATGACATCAAGAAGTCCTTG AACTTCTTGATGTCATCATACGGG-3' and inserted into the pSUPER-retro.neo+ gfp vector (VEC-PRT-0006, Oligoengine) following the manufacturer's instruction. Self-inactivating retroviruses were generated by transient transfection of 293T cells (ATCC, #CRL-1573) and tittered as described previously (Frecha et al., 2011). Briefly 8.6 µg of plasmid of interest was co-transfected with 3 µg of envelope plasmid phCMV-VSV-G, 8.6 µg of MLV-Gag-Pol (gift from Dr. Verhoeyen) using the classical calcium phosphate method. 72 hours after transduction, GFP positive cells were sorted (FACS Aria, BD Biosciences).

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from cells (RNeasy mini kit, #74104, Qiagen) according to the manufacturer's instructions. 2µg of RNA were added to 20 µl reverse transcription-PCR using the Omniscript RT kit (#205113, Qiagen). The relative mRNA levels of *gapdh* expression (mouse or human) were obtained by real-time quantitative PCR (qPCR), using the TaqMan Fast Universal PCR Master Mix (#4366072, Fisher Scientific) and TaqMan assay primer sets (mouse *gapdh*: Mm99999915_g1; human *gapdh*: Hs02758991_g1, Thermo Fisher Scientific) on the Step One (Applied Biosystems) according to the manufacturer's instructions. For mouse primary malignant B cells, samples were normalized to mouse *rn18s* (Mm03928990_g1, Thermo Fisher Scientific). For human DLBCL cell lines, samples were normalized to human *rplp0* (Hs99999902_m1, Thermo Fisher Scientific).

To measure gapdh mRNA stability, cells were treated with 5 µg/ml of Actinomycin D (A9415, Sigma-Aldrich) for indicated period of time.

Western Blot Analysis

Cells were washed in PBS and lysed in laemmli buffer. Tissue samples were collected and lysed in Precellys 24 (Bertin Instruments) homogenizer (3x30 s, 6500g) in laemmli buffer. After quantification (Pirece BCA Protein Assay kit, #23225, Thermo Fisher Scientific), 40 µg of proteins were separated on 8% to 13% SDS polyacrilamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blotted with antibodies against indicated proteins. Immunoreactive bands were detected with an anti-mouse (#7076, Cell Signaling Technology) or an anti-rabbit (7074S, Cell Signaling Technology) IgG horseradish peroxidase (HRP)-linked antibodies. Immunoblots were visualized (FUJIFILM LAS4000) by chemoluminescence using Pierce ECL Western Blotting substrates (#32106, Pierce ECL, Thermo Fisher Scientific). When indicated, Western blot quantification was performed using ImageJ software.

Anti-V5 (R96025) was purchased from Invitrogen. Anti-Erk2 (sc-1647) was purchased from Santa Cruz. Anti-GAPDH (ab9485), anti-LDH (ab52488), anti-LDH-B (ab85319), anti-GLS (ab93434) were purchased from Abcam. Anti-HKII (#2106), Anti-ENO1 (#3810), anti-PKM2 (#3198), anti-p53 (#2524), anti-phospho-ACC (Ser 79) (#11818), anti-ACC (3676), anti-phospho-p70-S6K (T389) (#9234), anti-p70S6K (#9202), anti-phospho-Syk (Tyr 525/526) (#2710), anti-Syk (#2712) were purchased from Cell Signaling Technology.

Flow Cytometry Analysis

Intracellular staining for GAPDH was performed using the Cytofix/Cytoperm kit (#554714, BD Biosciences), anti-GAPDH (ab9485, Abcam) and Alexa-Fluor 647 goat anti-rabbit IgG secondary antibody (ab150087, Abcam).

IgG or IgM expressions on the surface of DLBCL cell lines was analyzed using FITC-anti-IgG (#130-093-192, Miltenyi Biotec) and APC-anti-IgM (#130-093-076, Miltenyi Biotec).

Staining were analyzed by flow cytometry (Miltenyi Biotec). Analysis of the FACS data was performed using Macsquantify Version 2.11 (Miltenyi).

Targeted LC-MS Metabolites Analyses

Metabolites were extracted as described (Mackay et al., 2015). Briefly, 1.10^{6} Eµ-Myc cells (4 replicates per clones) were used for extraction. Extraction solution was composed of 50% methanol, 30% ACN, and 20% water. The volume of extraction solution added was adjusted to the number of cells (0.5 ml/1x10⁶ cells). After addition of extraction solution, samples were vortexed for 5 min at 4°C and then centrifuged at 16,000 *g* for 15 min at 4°C. The supernatants were collected and stored at -80° C until analyses. Liquid chromatography/Mass Spectrometry (LC/MS) analyses were conducted on a QExactive Plus Orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe and coupled to a Dionex UltiMate 3000 UPLC system (Thermo, USA). External mass calibration was performed using the standard calibration mixture every 7 days as recommended by the manufacturer. 5 µl of each sample was injected onto Zic-pHilic (150 mm × 2.1 mm i.d. 5 µm) with the guard column (20 mm × 2.1 mm i.d. 5 µm) (Millipore) for the liquid chromatography separation. Buffer A was 20 mM ammonium carbonate, 0.1% ammonium hydroxide (pH 9.2); buffer B was acetonitrile. The chromatographic gradient was run at a flow rate of 0.200 µl/min as follows: 0–20 min; linear gradient from 80% to 20% B; 20–20.5 min; linear gradient from 20% to 80% B; 20.5–28 min: hold at 80% B (Mackay et al., 2015). The mass spectrometer was operated in full scan, polarity switching mode with the spray voltage set to 2.5 kV, the heated capillary held at 320°C. The sheath gas flow was set to 20 units, the auxiliary gas flow was set to 5 units, and the sweep gas flow was set to 0 unit. The metabolites were detected across a mass range of 75–1,000 m/z at a resolution of 35,000 (at 200 m/z) with the AGC target at 106, and the

maximum injection time at 250 ms. Lock masses were used to insure mass accuracy below 5 ppm. Data were acquired with Thermo Xcalibur software (Thermo Fisher Scientific). The peak area of metabolites was determined using Thermo TraceFinder software (Thermo Fisher Scientific), identified by the exact mass of each singly charged ion and by known retention time on the HPLC column. Statistical analyses were performed using Metaboanalyst 4.0 software.

¹H HRMAS NMR Spectroscopy

Cell pellets (10 million cells) were frozen at -80°C. After thawing cell pellets, 10 microliters of D_2O were added. Solvent and cells were then placed into a 30 µl disposable insert introduced into a 4mm ZrO₂ HRMAS rotor before NMR analysis. All the NMR experiments were recorded on a Bruker Avance III spectrometer operating at 400 MHZ and 100MHZ for ¹H and ¹³C, respectively, and equipped with a ¹H/¹³C/³¹P HRMAS probe. All the spectra were recorded at 4000 Hz spinning rate and a temperature of 277 K. For each sample, ¹H HRMAS NMR spectrum was acquired using the Carr-Purcell-Meiboom-Gill NMR sequence, preceded by a water presaturation pulse during relaxation time of 2 s. This sequence enables us to reduce the macromolecule and lipid signal intensities using a T2 filter of 50 ms synchronised with the rotor rotation speed. For each spectrum, 256 free induction decays of 20 800 data points, were collected using 8000 Hz of spectral window. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz and zero-filled prior to Fourier transformation. The calibration on the alanine doublet (δ =1.47 ppm) and the phase and baseline corrections were performed manually for all the spectra. The identification of the NMR signals was carried out using ¹H-¹H TOCSY, ¹H-¹³C HSQC NMR experiments, online databases (HMDB, BRMB) and literature (Nicholson et al., 1995; Wishart et al., 2013). Each spectrum was divided into 0.005 ppm-width integrated buckets and normalized to total spectrum intensity after having removed the presaturated water signal region (4.85 -5.10 ppm) using AMIX software (Bruker Biospin GmbH, Karlsruhe, Germany). The water region must be removed due to inconsistent signal suppression.

A matrix of 42 samples and 1700 buckets was then created and exported to the software Simca-P v13 (Umetrics, Umea, Sweden) for statistical analysis. Principal component analysis was first performed in order to detect intrinsic clusters and outliers within the dataset. Since expected discrimination was not achieved using PCA, the data were analyzed with orthogonalized projections to latent structures discriminant analysis (OPLS-DA). The OPLS algorithm derives from basic partial least-squares (PLS) regression and allows a more effective use of the relevant discriminating variables by removing information orthogonal to the Y matrix (matrix containing the sample classes), i.e., not relevant for this particular discrimination. The resulting scores and loading plots were used to visualize respectively the samples and the NMR frequency signals (variables) in the predictive and orthogonal reduced component frame. Resampling the model 999 times under the null hypothesis performed OPLS-DA model validation, i.e. by generating 999 models for each between 50 to 100 % of the Y matrix were permutated. The quality of the model was assessed by monitoring changes in goodness-of-fit and predictive statistics, R2Y and Q2, respectively, between the models calculated from the permuted Y-matrix and the original one.

The normalized signals of each discriminant metabolite were then integrated for each spectrum in order to calculate their respective fold change between the two groups. The significance of the fold change was tested using a two-tailed t-test with a significance level of 0.05.

Plasma samples (200 µl) were frozen at -80 C. After thawing, 400 µl of 0.9% saline solution in D2O was added. Samples were then placed into a 5mm NMR tube and NMR experiments were recorded on a Bruker Avance III spectrometer operating at 600 MHz and 150MHz for ¹H and ¹³C, respectively, and equipped with a ¹H/¹³C TXI probe. Spectra were recorded at 300 K. A Carr-Purcell-Meiboom-Gill (CPMG) NMR spin echo sequence $[90^{\circ} - (\tau - 180^{\circ} - \tau)n]$ with an effective spin echo time of 75.6 ms, preceded by a water presaturation pulse during the relaxation time was employed to reduce the signal intensities of lipids and macromolecules. A relaxation time of 33 s was necessary to achieve full relaxation for all the metabolites in order to enable absolute quantification. For each sample, 128 free induction decays (FID) of 32768 complex data points were collected using a spectral width of 12 000 Hz. A sample of 1.67 mM of maleic acid was used as an external reference to calibrate the ERETIC signal (electronic signal) used for the quantification of glutamine.

QUANTIFICATION AND STATISTICAL ANALYSIS

All survival analyses were performed on an intention-to-treat basis. Categorical variables were compared using the chi-squared or Fisher exact tests. Progression-free survival (PFS) was defined as the time from study entry until disease progression or death. Overall survival (OS) was defined as the time from the start of treatment until death. Survival functions were estimated using the Kaplan-Meier method and compared with the log-rank test. Comparative tests were considered significant if a 2-sided p < 0.05. Because the LNH2003-6 trial was not stratified by biological data, we controlled for the effects of prognostic factors on outcome due to sampling fluctuations in the treatment groups with a multivariate analysis of survival in a Cox model. Statistical analyses of clinical data were performed using R project software or SAS 9.2 (SAS Institute) and S-Plus 6.2 (MathSoft) software. Data distributions are presented using boxplots when needed, to provide a graphical representation of the variance. Statistical significance was assessed using the Wilcoxon rank sum test. For all other clinical data, statistical analyses used for each of the figures is specified in

the legends. For *in vitro* experiments, results are expressed as mean (-/+ s.d.) and statistical analyses were performed using two-tailed Student's t-test. A p value of less than 0.05 was considered to indicate statistical significance (*p <0.05, **p <0.01 and ***p <0.001). n for the different groups are provided in the figure legends.

DATA AND SOFTWARE AVAILABILITY

Code Availability

R code for microarray analysis has been deposited to Mendeley Data and is available at https://doi.org/10.17632/f4f3wbszmd.1.

Article 8

www.moleculartherapy.org

Commentary

CONFLICT OF INTEREST

The authors declare that the consensus agreement was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ACKNOWLEDGMENTS

G.A.-P. and C.D.P. are supported by NHLBI R01 HL135853 and HL130856. T.M. is supported by NIH NIAID R01 AI116880-01, CIRM RN3-06532, and the UCSF Center for Maternal-Fetal Precision Medicine and has received research support from Ultragenyx Pharmaceuticals. S.N.W. is supported by MRC grants MR/P026494/1 and MR/ R015325/1 and SPARKS grant 17UCL01.

REFERENCES

1. U.S. National Institutes of Health. Recombinant DNA Advisory Committee (2000). Prenatal gene tranfer: scientific, medical, and ethical issues: a report of the Recombinant DNA Advisory Committee. Hum. Gene Ther. 11, 1211–1229.

- Porada, C.D., Tran, N., Eglitis, M., Moen, R.C., Troutman, L., Flake, A.W., Zhao, Y., Anderson, W.F., and Zanjani, E.D. (1998). In utero gene therapy: transfer and long-term expression of the bacterial neo(r) gene in sheep after direct injection of retroviral vectors into preimmune fetuses. Hum. Gene Ther. 9, 1571–1585.
- Frederickson, R.M., and Ylä-Herttuala, S. (2019). Two Decades of Molecular Therapy: The Journey Continues. Mol. Ther. 27, 1–2.
- Collins, F.S., and Gottlieb, S. (2018). The Next Phase of Human Gene-Therapy Oversight. N. Engl. J. Med. 379, 1393–1395.
- Almeida-Porada, G., Atala, A., and Porada, C.D. (2016). In utero stem cell transplantation and gene therapy: rationale, history, and recent advances toward clinical application. Mol. Ther. Methods Clin. Dev. 5, 16020.
- 6. Witt, R., MacKenzie, T.C., and Peranteau, W.H. (2017). Fetal stem cell and gene therapy. Semin. Fetal Neonatal Med. 22, 410–414.



- Massaro, G., Mattar, C.N.Z., Wong, A.M.S., Sirka, E., Buckley, S.M.K., Herbert, B.R., Karlsson, S., Perocheau, D.P., Burke, D., Heales, S., et al. (2018). Fetal gene therapy for neurodegenerative disease of infants. Nat. Med. 24, 1317–1323.
- Schneider, H., Faschingbauer, F., Schuepbach-Mallepell, S., Körber, I., Wohlfart, S., Dick, A., Wahlbuhl, M., Kowalczyk-Quintas, C., Vigolo, M., Kirby, N., et al. (2018). Prenatal Correction of X-Linked Hypohidrotic Ectodermal Dysplasia. N. Engl. J. Med. 378, 1604–1610.
- Chan, J.K.Y., Gil-Farina, I., Johana, N., Rosales, C., Tan, Y.W., Ceiler, J., Mcintosh, J., Ogden, B., Waddington, S.N., Schmidt, M., et al. (2018). Therapeutic expression of human clotting factors IX and X following adeno-associated viral vectormediated intrauterine gene transfer in early-gestation fetal macaques. FASEB J., Published online December 5, 2018. https://doi.org/10.1096/fj.201801391R.
- 10. Verma, J., Thomas, D.C., Sharma, S., Jhingan, G., Saxena, R., Kohli, S., Puri, R.D., Bijarnia, S., and Verma, I.C. (2015). Inherited metabolic disorders: prenatal diagnosis of lysosomal storage disorders. Prenat. Diagn. 35, 1137–1147.

The Future: *In Vivo* CAR T Cell Gene Therapy

Rana Mhaidly¹ and Els Verhoeyen^{1,2} https://doi.org/10.1016/j.ymthe.2019.03.012

Chimeric antigen receptor (CAR) T cells represent a revolutionary therapy for combatting cancers.¹ This novel immunotherapy was only approved in 2017 for treatment of advanced blood cancers in the US and Europe. The most frequently used vehicles for CAR gene delivery in autologous T cells are lentiviral vectors (LVs) pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G-LV). *Ex vivo* gene delivery employing these broad spectrum LVs might, however, lead to off-target transduction of contaminating cancer or antigen-presenting cells with these CARs leading to severe health risks and adverse events.² In a new study published in *Molecular Therapy* – *Methods and Clinical Development*, Jamali et al.³ limit these risks by using CD4⁺ or CD8⁺ T cell targeted LVs for CAR gene transfer. The authors engineered human CD4⁺ and CD8⁺ T cell-targeted LVs capable of selective gene delivery into human CD4⁺ or CD8⁺ T cells, respectively.^{4,5}

The targeting of LVs is based on the principle that fusion-activation of a chimeric envelope

should be triggered by the interaction of the ligand displayed on the vector surface with its specific receptor on the target cell. Buchholz and co-workers³ designed a novel strategy based on the fact that paramyxoviruses separated the two functions, cell binding and virus-cell fusion, into separate glycoproteins. In this case, redirection of the glycoprotein that confers binding to the target cell leaves the fusion glycoprotein (gp) "untouched" and functional. This strategy was initially successful for LVs pseudo-typed with measles virus (MV-LVs) retargeted glycoproteins and more recently LVs



¹Université Côte d'Azur, INSERM, C3M, Nice, France; ²CIRI - Centre International de Recherche en Infectiologie, Univ Lyon, Université Claude Bernard Lyon 1, INSERM, U1111, CNRS, UMR5308, ENS Lyon, 69007 Lyon, France

Correspondence: Els Verhoeyen, International Center for Infectiology Research, EVIR, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon, France and Université Côte d'Azur, INSERM, C3M, 151 Route St Antoine de Ginestière, Nice, France. E-mail: els.verhoeyen@ens-lyon.fr

www.moleculartherapy.org Commentary





Figure 1. Toward In Vivo CAR T Cell Therapy

Exclusive transduction of CD4⁺ or CD8⁺ T cells in total PBMCs with an anti-CD19 CAR encoding CD4^{darpin}MV- and CD8^{scFv}NiV-retargeted lentiviral vectors, respectively. CAR T cells were able to specifically deplete healthy and cancer CD19⁺ cells *ex vivo. In vivo* injection of CD8^{scFv}NiV-retargeted lentiviral vectors into non-obese diabetic/ severe combined immunodeficient (NOD/SCID) γ C^{-/-} mice that were engrafted with hCD34⁺ stem and progenitor cells resulted in expression of the CD19 directed CAR solely on CD8⁺ cells *in vivo* which, upon encounter with CD19⁺ B cells, were strongly expanded and resulted in B cell depletion *in vivo*. This represents a first step toward *in vivo* CAR therapy but evaluation in non-human primates will be required before these tools can enter the clinic for patient treatment.

pseudotyped with receptor-targeted Nipah virus glycoproteins (NiV-LVs). These effectively enter into cells when using cell surface proteins as receptors that bring them sufficiently close to the cell membrane.⁶

Jamali et al.³ demonstrated exclusive transduction of CD4⁺ or CD8⁺ T cells in total peripheral blood mononuclear cells (PBMCs) with an anti-CD19 CAR encoding CD4MV- or CD8NiV-retargeted LVs, respectively. Moreover, in the presence of an LV facilitating agent, Vectofusin, transduction with CD19-CAR-targeted LVs was comparable to T cell transduction levels achieved with VSVG-LVs currently used in

the clinic. This was achieved without compromising the specificity for the target T cells (Figure 1). Although such a targeted approach will make ex vivo CAR T cell therapy safer, it remains a highly personalized treatment that likely requires a costly clinical manufacturing grade and labor-intensive ex vivo T cell manufacturing process. These considerations highlight the obstacles associated with manufacturing of this kind of patient-specific therapy. Therefore, implementation of CAR T cell therapy for routine use in the clinic remains a real challenge and this new therapeutic product may be out of reach for many cancer patients in need of this novel therapy. A future approach avoiding the above concerns may be *in vivo* CAR T cell generation.

The Buchholz team⁷ has very recently performed a first step toward *in vivo* reprogramming of CAR T cells using CD8NiV-targeted LVs. A single systemic injection of CD19-CAR-encoding CD8-LVs into immune-deficient mice engrafted with a human blood system generated *in vivo* CAR T cells, which effectively wiped out the human B cells (Figure 1).⁷ This outcome supports the feasibility of *in vivo* CAR T cell therapy. Remarkably, NiV-LVs could be produced at up to two orders of magnitude higher titers compared to their MV-based counterparts and were at www.moleculartherapy.org

Commentary

least 10,000-fold less effectively neutralized than MV glycoprotein pseudotyped LVs by human serum, underlining their potential as a medicine that is applicable for *in vivo* delivery.⁴

However, some of the CD8NiV-LV injected humanized mice developed a cytokine storm equivalent to some CAR T cell treated patients, which warrants further preclinical testing of the *in vivo* approach. A next logical step will be to test the performance of *in vivo* CAR T cells therapy in mice carrying patient derived tumor xenografts (PDX mice models). However, humanized mice do not recapitulate a fully functional immune system and evaluation in large animal models such as non-human primates may be needed before a first clinical trial on *in vivo* CAR T cell gene therapy can commence (Figure 1). Nonetheless, once these steps have been successfully completed future CAR T cell therapy might consist of a single injection of a vectorized medicine into the blood stream, circumventing the current cost-intensive *ex vivo* production of CAR T cells, and thus making this therapy more broadly available to patients.

REFERENCES

- Guedan, S., Calderon, H., Posey, A.D., Jr., and Maus, M.V. (2018). Engineering and Design of Chimeric Antigen Receptors. Mol. Ther. Methods Clin. Dev. 12, 145–156.
- Ruella, M., Xu, J., Barrett, D.M., Fraietta, J.A., Reich, T.J., Ambrose, D.E., Klichinsky, M., Shestova, O., Patel, P.R., Kulikovskaya, I., et al. (2018). Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. Nat. Med. 24, 1499–1503.
- Jamali, A., Kapitza, L., Schaser, T., Johnston, I.C.D., Buchholz, C.J., and Hartmann, J. (2019). Highly efficient and selective CAR-gene transfer using CD4and CD8-targeted lentiviral vectors. Mol. Ther.

Methods Clin. Dev., Published online March 16, 2019. https://doi.org/10.1016/j.omtm.2019.03.003.

- 4. Bender, R.R., Muth, A., Schneider, I.C., Friedel, T., Hartmann, J., Plückthun, A., Maisner, A., and Buchholz, C.J. (2016). Receptor-Targeted Nipah Virus Glycoproteins Improve Cell-Type Selective Gene Delivery and Reveal a Preference for Membrane-Proximal Cell Attachment. PLoS Pathog. 12, e1005641.
- Zhou, Q., Uhlig, K.M., Muth, A., Kimpel, J., Lévy, C., Münch, R.C., Seifried, J., Pfeiffer, A., Trkola, A., Coulibaly, C., et al. (2015). Exclusive Transduction of Human CD4+ T Cells upon Systemic Delivery of CD4-Targeted Lentiviral Vectors. J. Immunol. 195, 2493–2501.
- Frank, A.M., and Buchholz, C.J. (2018). Surface-Engineered Lentiviral Vectors for Selective Gene Transfer into Subtypes of Lymphocytes. Mol. Ther. Methods Clin. Dev. 12, 19–31.
- Pfeiffer, A., Thalheimer, F.B., Hartmann, S., Frank, A.M., Bender, R.R., Danisch, S., Costa, C., Wels, W.S., Modlich, U., Stripecke, R., et al. (2018). *In vivo* generation of human CD19-CAR T cells results in B-cell depletion and signs of cytokine release syndrome. EMBO Mol. Med. *10*, 10.



Article 9

CLEC12B regulates the color of the skin through SHP-1 and SHP-2/CREB pathway

Laura SORMANI LE BOURHIS¹, Hélène TAQUIN MARTIN², Rana MHAIDLY¹, Els VERHOEYEN¹, Claire REGAZZETTI¹, Marjorie HEIM¹, Nicolas NOTTET¹, Meri TULIC¹, Yann CHELI¹, Gian Marco DE DONATIS¹, Anne Sophie DABERT GAY⁴, Delphine DEBAYLE⁴, Nathalie CARDOT LECCIA³, Franck GESBERT⁵, Henri MONTAUDIE^{1,2}, Stéphane ROCCHI¹ and Thierry PASSERON^{1,2}

¹ Université Côte d'Azur. Centre Méditerranéen de Médecine Moléculaire (C3M). INSERM U1065 – Nice – France

² Université Côte d'Azur. Centre hospitalier universitaire Nice. Department of Dermatology. Nice, France

³ Université Côte d'Azur. Centre hospitalier universitaire Nice. Department of Pathology. Nice, France

⁴ Université Côte d'Azur. Institut de pharmacologie moléculaire et cellulaire (IPMC) CNRS, CAPABIO Platform, Sophia Antipolis, France.

⁵University Paris-Sud, University Paris-Saclay. Institut Curie, PSL Research University, INSERM U1021, Normal and Pathological Development of Melanocytes, Orsay, France. CNRS, UMR 3347, Orsay, France. Equipe Labellisée Ligue Contre le Cancer, Orsay, France

Correspondences:

Pr. Thierry Passeron, MD, PhD Department of Dermatology and C3M INSERM U1065 151 route Saint-Antoine de Ginestière 06200 Nice, France Email: <u>passeron@unice.fr</u>

Manuscript word count: 3 993

Figures: 4 Tables: 0

Keywords: Skin pigmentation; CLEC12B; melanogenesis; SHP-1; SHP-2.

ABSTRACT

Human skin pigmentation is a complex and tightly regulated process. Using a transcriptome analysis performed in pigmented skin compared to skin devoid of melanocytes, we identified CLEC12B as a new melanocytic gene. We demonstrated that CLEC12B is selectively expressed in melanocytes, and that its expression is decreased in highly pigmented skin compared to white skin. CLEC12B directly recruits and activates SHP-1 and SHP-2 through its ITIM domain and promotes CREB degradation, leading to a downregulation of the downstream MITF pathway and ultimately controls melanin production and pigmentation *in vitro* and in reconstructed human epidermis model. The identification of CLEC12B fosters a new therapeutic target for the development of melanogenic agents in the clinical and cosmetic fields, but it also provides novel insights in the understanding of the melanocyte biology and regulation of melanogenesis.

Cet article est soumis à Journal of Clinical Investigation

Curriculum Vitae

INFORMATIONS PERSONNELLES :

Rana MHAIDLY

N° étudiant: 21408350 Date de naissance: 22/11/1993 Tel: 00 33 6 59 65 23 43 Email : Rana.mhaidly27@gmail.com INSERM U1065, C3M Equipe 3 : « Metabolism, Cancer & Immune Response » Bâtiment Universitaire Archimed 151 Route de Sainte Antoine BP 2 3194 06204 NICE Cedex 3, France

DIPLOMES:

- ⇒ <u>2011-2014 :</u> License en Sciences de Laboratoire, Université Libanaise, Faculté de Santé Publique, Université Libanaise, mention Très Bien
- ⇒ <u>2014-2016</u>: Master en Sciences de la vie et de la santé, parcours Génétique Immunité Développement, Université Nice Sophia Antipolis Nice
- ⇒ <u>2016- à présent:</u> Doctorante Sciences de la vie et de la santé, Spécialité Immunologie et Microbiologie, Université Cote d'Azur Nice
- ⇒ <u>Octobre 2015</u>: Formation A spécialisée en expérimentale animale « Approche législative, éthique et pratique du rongeur de laboratoire ».

EXPERIENCES PROFESSIONNELLES :

- ⇒ Octobre 2016-à présent : Doctorante dans l'équipe de Dr Jean-Ehrland Ricci, Centre Méditerranéen de Médecine Moléculaire, Equipe 3 "Métabolisme, Cancer et Réponses Immunes"
 Encadré par Dr Els VERHOEYEN
 Financé par le « Ministère de Recherche »
- ⇒ Janvier 2016: Stage de Master 2, Institut de Biologie, Valrose Inserm U1091 Encadré par Dr. Elvire GOUZE
- ⇒ <u>Février 2015</u>: Stage de Master 1, Centre Méditerranée de Médecine Moléculaire Inserm U1065 Encadré par Dr Els VERHOEYEN

COMPETENCES ACQUISES :

Techniques de biologie moléculaire et cellulaire :

- Production de vecteurs rétroviraux et lentiviraux.
- Clonage plasmidique
- Amplification et quantification des ADN
- Culture des lignées cellulaire (HEK, ATDC5, NIH3T3, Cellules souches mésenchymateuses)
- Isolation des cellules souches hématopoïétiques à partir du sang du cordon
- Isolation des cellules primaires CD4⁺ et CD19⁺

- Transduction des cellules
- Western Blot
- Extraction d'ARN à partir des organes
- PCR quantitative
- Technique d'immuno-histologie
- Technique de Culture 3D
- Analyse de cytométrie de flux
- Différenciation des cellules immunitaires in vitro
- Analyse métabolique (seahorse, Mesure d'ATP, Mesure de lactate)

Approches d'expérimentation animale :

- Dissection des souris et isolation des organes
- Isolation des cellules souches mésenchymateuses à partir des fémurs
- Injection Intraveineuse et intrapéritonéale des souris

Compétences Informatiques :

- GraphPad Prism, Excel, Flow-Jo, Power Point, ImageJ, Pannoramic Viewer, Wave, Chromas

Language:

- French, English

Encadrement des étudiants:

- 2018-2019: Encadrement d'un étudiant en Master pendant 5 mois

COMMUNICATIONS ORALES :

- ⇒ <u>03-2018</u>: Séminaires jeunes Chercheurs au C3M, Nice, France (Présentation Orale)
- ⇒ <u>04-2018</u>: C3M & MetaCan Marie Curie ITN "inflammation & Disease Symposium", Nice, France (Présentation Orale)
- \Rightarrow <u>05-2018</u>: JEDNs (Journées de l'école doctorale 2018, Présentation orale de Poster)
- ⇒ <u>06-2018</u>: Canceropôle annual meeting, Saint Raphael, France (Présentation Orale de Poster)
- \Rightarrow <u>04-2019</u>: 3rd edition Metabolism & Cancer, Marseille, France, (Présentation Orale)
- \Rightarrow <u>05-2019</u>: JEDNs (Journées de l'école doctorale 2019, Présentation Orale)
- \Rightarrow <u>10-2019</u>: 4th International Symposium of the Cancer Research Centr of Lyon (Présentation Orale)

FORAMTIONS SCIENTIFIQUES (WORKSHOP):

⇒ <u>Octobre 2018:</u> EMBO Workshop "From epigenome towards epitranscriptome in cell fate choice", Capri, Italy (Poster presentation)

Liste de Publications

Liste de Publications

GAPDH overexpression in the T cell lineage promotes angioimmunoblastic T cell lymphoma through an NFKB dependent mechanism

Laura Mondragón^{1,*}, **<u>Rana Mhaidly^{1,*}</u>**, Gian Marco De Donatis¹, Marie Tosolini², Pascal Dao³, Anthony R. Martin³, Caroline Pons¹, Johanna Chiche¹, Marie Jacquin¹, Véronique Imbert¹, Emma Proïcs¹, Laurent Boyer¹, Anne Doye¹, Frédéric Luciano¹, Jaap G. Neels^{1,} Frédéric Coutant^{4,5}, Nicole Fabien⁴, Laura Sormani¹, Camila Rubio-Patiño¹, Jozef P. Bossowski¹, Florian Muller¹, Sandrine Marchetti¹, Elodie Villa¹, Jean-François Peyron¹, Philippe Gaulard⁶, François Lemonnier⁶, Vahid Asnafi⁸, Laurent Genestier⁹, Rachid Benhida³, Jean-Jacques Fournié¹⁰, Thierry Passeron^{1,11}, Jean-Ehrland Ricci^{1,12} and Els Verhoeyen^{1, 9,12,13} (* : Equal contribuation as co-first authors) (Paper In press, Cancer cell, June 2019)

The Future : In Vivo Car T Cell Gene Therapy

Mhaidly R, Verhoeyen E (Molecular therapy, 2019, 30914238)

BaEV-LVs transduce efficiently progenitor T cells and restored SCID-X1 T-cell deficiency

Bernadin O, Amirache F, Girard-Gagnepain A, Moirangthem RD, Lévy C, Ma K, Costa C, Nègre D, Reimann C, Fenard D, Cieslak A, Asnafi V, Sadek H, <u>Mhaidly R</u>, Cavazzana M, Lagresle-Peyrou C, Cosset FL, André I, Verhoeyen E. (Blood Advances, 2019, 30755435)

GAPDH expression predicts the metabolic state and the response of DLBCL patients to metabolic inhibitors

Chiche J, Reverso-Meinietti J, Mouchotte A, Rubio-Patiño C, <u>Mhaidly R</u>, Villa E, Bossowski JP, Proics E, Grima-Reyes M, Paquet A, Fragaki K, Marchetti S, Briere J, Ambrosetti D, Michiels JF, Molina TJ, Copie-Bergman C, Lehmann-Che J, Peyrottes I, Peyrade F, de Kerviler E, Taillan B, Garnier G, Verhoeyen E, Paquis-Flucklinger V, Shintu L, Delwail V, Delpech-Debiais C, Delarue R, Bosly A, Petrella T, Brisou G, Nadel B, Barbry P, Mounier N, Thieblemont C, Ricci JE. (Cell Metabolism, 2019, 30827861)

Low protein diet induces IRE1 dependent anticancer immunosurveillance

Rubio-Patiño C, Bossowski JP, De Donatis GM, Mondragón L, Villa E, Aira LE, Chiche J, <u>Mhaidly R,</u> Lebeaupin C, Marchetti S, Voutetakis K, Chatziioannou A, Castelli FA, Lamourette P, Chu-Van E, Fenaille F, Avril T, Passeron T, Patterson JB, Verhoeyen E, Bailly-Maitre B, Chevet E, Ricci JE (Cell Metabolism, 2018, 29551590)

HSP110 sustains chronic NF-kB signaling in activated B-cell diffuse large B-cell lymphoma through MyD88 stabilization

Boudesco C, Verhoeyen E, Martin L, Chassagne-Clement C, Salmi L, <u>Mhaidly R</u>, Pangault C, Fest T, Ramla S, Jardin F, Wolz OO, Weber ANR, Garrido C, Jego G. (Blood, 2018, 29871863)

IL-34 and CSF-1 display an equivalent macrophage differentiation ability but a different polarization potential

Boulakirba S, Pfeifer A, <u>Mhaidly R,</u> Obba S, Goulard M, Schmitt T, Chaintreuil P, Calleja A, Furstoss N, Orange F, Lacas-Gervais S, Boyer L, Marchetti S, Verhoeyen E, Luciano F, Robert G, Auberger P, Jacquel A. (Scientific Reports, 2018, 29321503)

Résumé

<u>Résumé</u>

L'activation des lymphocytes T (LT) et l'acquisition des fonctions effectrices sont des phénomènes récemment décrits pour être régulés par le métabolisme cellulaire, introduisant ainsi la notion d'« immunométabolisme » dans de nombreuses études biologiques.

Dix enzymes métaboliques participent à la conversion du glucose en énergie cellulaire (glycolyse). Parmi celles-ci, des études récentes ont fait émerger la glycéraldéhyde 3-phosphate déshydrogénase (GAPDH) comme un acteur principal de la survie et de la différenciation des LT. Dans ce rôle, la balance entre ses fonctions glycolytiques (associées à la catalyse du glycéraldéhyde-3-phosphate en le 1,3-bisphosphoglycérate) et non-glycolytiques (stabilité des ARNm des cytokines et activation des voies de signalisation) est un phénomène majeur contrôlant la sélection et l'activation des LT.

Afin d'étudier les fonctions glycolytiques et non-glycolytiques de la GAPDH dans le développement lymphocytaire, nous avons généré un modèle de souris transgéniques surexprimant la GAPDH exclusivement dans les LT (plck-GAPDH).

Une première caractérisation de ces souris montre que la surexpression de la GAPDH n'a pas d'effet sur les stades précoces de développement des LT. De façon intéressante, nous avons observé dans les souris âgées (>18 mois) des changements phénotypiques évidents, notamment : une splénomégalie, des ganglions lymphatiques hypertrophiés et une infiltration lymphocytaire majeure au niveau du foie et de la moelle osseuse. Un immunophénotypage détaillé de ces tissus a montré une augmentation de la proportion des LT folliculaires helpers (Tfh) CD4⁺ Bcl-6⁺CXCR5⁺ PD-1^{high}. De plus, ces cellules, fortement prolifératives et clonales, interagissent avec les lymphocytes B CD95⁺ GL7⁺ du centre germinatif, eux-mêmes associés à des cellules dendritiques folliculaires, formant ainsi un microenvironnement tumoral dans notre modèle plck-GAPDH.

À la recherche d'un équivalent humain à notre modèle, nous avons découvert que les caractéristiques pathologiques, l'immunophénotypage et le profil génétique des souris plck-GAPDH correspondaient étroitement au lymphome T angio-immunoblastique humain (AITL). Il s'agit d'une hémopathie maligne rare pour laquelle aucune stratégie thérapeutique n'a été clairement décrite. Nous avons également mis en évidence dans les souris plck-GAPDH une activation de la voie NF-kB non canonique, également activée chez les patients AITL. Le ciblage de cette voie par un nouvel inhibiteur associé à une immunothérapie anti-PD1 a augmenté la survie des souris. Ce traitement permet une induction efficace de la réponse immunitaire anticancéreuse et représente une nouvelle perspective thérapeutique pour les patients atteints d'AITL.

En conclusion, ce nouveau modèle de souris AITL capable de mimer étroitement les caractéristiques du lymphome humain, permettra d'élucider l'origine possible de la maladie, d'identifier d'autres voies de signalisation impliquées et de tester de nouvelles lignes thérapeutiques.