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Andrea Ávila Ávila. Characterization of the anti-leukemic properties of anti-CD3 antibodies in T-cell acute lymphoblastic leukemia (T-ALL). Cancer. Université Paris Cité, 2023. English. NNT: 2023UNIP5188. tel-04866403

HAL Id: tel-04866403

<https://theses.hal.science/tel-04866403v1>

Submitted on 6 Jan 2025

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Université Paris Cité

École doctorale Hématologie, Oncogénèse et Biothérapies (ED 561)

Intégrité du génome, ARN et cancer (UMR 3348)

Characterization of the anti-leukemic properties of anti-CD3 antibodies in T-cell acute lymphoblastic leukemia (T-ALL)

Par Andrea ÁVILA ÁVILA

Thèse de doctorat d'**Oncogénèse**

Dirigée par Christine TRAN QUANG

Présentée et soutenue publiquement le 07 décembre 2023

Devant un jury composé de :

Olivier AYRAULT	DR - Université Paris-Saclay	Président du jury
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Philippe KASTNER	MCU-PH HDR - Université de Strasbourg	Rapporteur
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Diana PASSARO	CR HDR - Université Paris Cité	Membre invité
Christine TRAN QUANG	CR HDR - Institut Curie / Université Paris Cité	Directrice de thèse

*In loving memory of María Martha Méndez Murillo,
whose loving eyes are kept in my heart.*

*In loving memory of Alicia Báez Vacio,
who taught me the profound significance of family.*

*To my beloved family,
whom I missed every day while being so far from home.*

Acknowledgements

I would like to express my sincere acknowledgments to the members of the jury who kindly accepted the invitation to review the present work; it is an honor for me to have your expertise in the field. Thanks to the reporters who evaluated this manuscript in depth. I owe a special debt of gratitude to Dr. Philippe Kaster, who accompanied me throughout my PhD journey as a member of my yearly thesis committees. The questions, comments, and discussions we had over these years were unquestionably fruitful for me. Thanks to Dr. Dominique Payet Bornet for providing opportune and helpful revisions and supportive commentaries on my work. Thanks to Dr. Olivier Ayrault for kindly accept the invitation to be an examiner of this thesis.

I would like to extend my sincere gratitude to the Marie Skłodowska-Curie Actions-Innovative Training Network and La Fondation pour la Recherche sur le Cancer for the financial support during these years; as well as the Mexican Council CONACYT, which made it possible for me to pursue my studies abroad.

I would like to thank Dr. Diana Passaro and Dr. Julien Calvo; while they couldn't officially join the jury due to external circumstances, their enthusiastic support during this challenging final period meant a lot to me. Additionally, I would like to thank Dr. Giorgio Seano and Dr. Frédérique Coquelle, who very kindly accepted to be part of my thesis committees every year. All the questions and discussions in these committees were an essential part of my scientific training.

Thanks to my thesis supervisors, Dr. Christine Tran Quang and Dr. Jacques Ghysdael, for accepting me into your laboratory and trusting me to work on this project. Thank you, Christine, for sharing your enthusiasm with me, for consistently fostering a team spirit in the laboratory, and for caring about my professional growth by always providing the necessary tools for me to have a successful PhD. I will always be grateful. Thank you, Jacques, for your guidance, for showing me your passion for science, and for teaching me the scientific rigor with which you conduct your research. I will always hold it as a true treasure.

Thanks to former and present members of the team, in chronological order: Benedetta Zaniboni, Romain Humeau, Gregoire Huré, Kanokporn Nuantang, and Mariana Lobato de Oliveira. All of you are part of a very precious and important period of my life. Benne, thank you for all your guidance as a senior PhD student, all your advice in the lab, but more importantly, for helping me navigate the life of a PhD student over these four years. Thank you for asking how I was doing from time to time; you always asked at key moments when the overview was more down than up, and talking with you always cheered me up. Thanks!!. Romain, thank you for being an excellent teacher for mouse manipulation, for your patience and care during my learning process, and more importantly, thank you for your friendship and the joyful moments we shared. Gregoire, thank you for looking out for me over these four years. I truly feel lucky to have your friendship. Thank you for all the happy moments, and for your company and support during the tough times. Kanok, what can I say? We spent a lot of time together in the lab, just the two of us! Thanks for being the perfect lab mate, French teacher, biscuit provider, and friend. Despite my bad mood (sometimes frequent) in the lab, you always provided me with constant support and understanding. Mariana, your arrival to the lab was the cherry on top of my PhD journey! You are a true gift. You came full of enthusiasm and a great sense of humor at a time when a PhD student specially needs it. Thanks for all the scientific discussions; they provided me with more confidence and passion for what we do. And of course, thank you for your friendship and the special moments in and out of the lab.

I want to thank the laboratory that hosted me at the Institut Curie: the Laboratoire du Biologie de l'ARN, Signalization et Cancer (U1278) led by Dr. Stéphan Vagner. Thank you, Stéphan, and all the team members: Patricia Uguen, Martin Dutertre, Lucilla Fabbri, Dorothee Baille, Amélie Heneman, Sylvain Martineau, Eva Guerin, Céline Labbe, Ana Dian, Clara Bonnet, Alexandre Devaux, Alexia Le Barch, Lucie Lagadec, Siddhant Kalia, and Quentin Fouilleul. I have learned a lot from every lab meeting and journal club that we had. Thank you for all your questions, discussions (and cakes!!) during these years.

Thanks to my ITN-network ARCH, with whom I shared exciting moments throughout my PhD journey and made me feel part of a scientific community. A special thanks to the 14 talented PhD students and now many of them already doctors: Eirini Sofia Fasouli, Chiara Taroni, Natalia Giner Laguarda, Sandra Alonso Rubido, Christina Pitsillidou, Athanasios Oikonomou, Clara Téllez Quijorna, Maria Eleni Psychogyiou, Mari Carmen Romero Mulero, Guillermo Fernández Rodríguez, Hillary Maniriho, Natalia Skinder, Agata Labeledz, Ludovica Proietti. Thank you, guys! I consider myself really lucky to have gone through very challenging moments, such as the COVID-19 lockdown, with your company. And, of course, to have experienced thrilling moments in each of our ARCH meetings where we shared our passion for science.

I also want to thank all the members of the unit my host laboratory belonged to, the Unité Intégrité du Génome, ARN and Cancer (UMR3348). Every unit meeting broadened my scientific perspective, I have learned a lot from you. From the unit, I want to thank specially to Kamila Schirmeisen and Ana Minello, with whom I went through the exciting process of defending our thesis. Thank you, girls! Sharing with you our feelings and concerns helped make this process smoother.

I would like to thank the staff of the animal facility and flow cytometry, which were the two facilities that I used on a daily basis during these years. From the animal facility, I would like to especially thank Elodie Belloir, Christophe Alberti, Colin Jouhanneau, and Pauline Dubreuil, who were very helpful and kind during this time. From the flow cytometry platform, I would like to thank Léa Guyonnet, who very kindly helped me design the FACS panel I used in this work. And of course, Charlène Lasgi, who was an important support during my PhD journey. Not only in terms of FACS (which is already a lot), but also personally. Thank you, Charlène, for always listening to me and giving words of encouragement when I needed.

I would also like to thank the people from FlowMetric Europe in Milan, who welcomed me very warmly. Specially to Christina Pitsillidou and Alessandra Roberto, who taught me priceless knowledge in managing multicolor flow cytometry. I would

also like to thank Sabine Druillennec who collaborated on this work, conducting the bioinformatic analysis and being readily available for discussions.

Thanks to the people from the administration desk: Laurence Jagot, Pascale Leon, and Morgane Jaunay, who very warmly helped me a lot during these four years to navigate the very complicated French bureaucracy. Very specially, I would like to thank Christophe Patry, who also assisted me in understanding French procedures inside and outside work. I really appreciate all your kindness and help during this time Christophe.

Thanks to colleagues and friends from the institute who always greeted me a good morning, a warm smile, or a nice discussion during my days at the institute: Jean-Jacques Lopes, Cédric Duprilot, Julie Talbot, Jeremy Raymond, Floriane Pereira, Celio Pouponnot, Liliana Mirabal, Laura Medina Cuadra, Laurence Mery, Sara Chabi, Magalie Larcher, Dave Yong Xiang, Morgane Lannoy, Moustapha Ka, Hua Yu, Irene Basili, Jacob Torrejón Díaz, Guillaume Bourmeau, Jean Chatain, Annaig Bertho, Joel Montalvao Pinto and Pasquale Rotondo, all of you undoubtedly made of my time at Curie a very pleasant place to work.

Thanks to my family, who, despite the distance, stood by my side during this part of my life and made possible for me to arrive at this moment. To my mom, Cecilia Ávila Méndez, gracias por apoyarme incondicionalmente, la fuerza, valentía y disciplina que me inculcaste desde chiquita han sido clave para conseguir terminar esta etapa. To my dad, Efrén Ávila Báez, gracias por hacerme creer en mí, por apoyarme en cada decisión en mi vida, gracias por sembrar en mí la semilla de la curiosidad. Los amo siempre. Thanks to my older sister, Altaira Ávila Ávila, for taking care of me, supporting me, advising me, and sharing her peace with me every time I needed it. Love you! Thanks to my entire family, very specially to my beloved MamáMartha, mamita gracias por todo el amor que me dió durante todos estos años, gracias por todas sus oraciones yo sé que ahora usted cuida de mi desde el Cielo. Thank you to my aunts Claudia, Martha, and Diana, and my uncle Hugo, as well as my cousins Jenny, Charis, and Peggy, gracias por estar tan cerca de mí sin importar la distancia, por echarme porras y hacerme sentir querida y apoyada. I

would also like to thanks to people that I already consider my family to Don Luis, Meztli, Salvador, Vane, Adri y Tía Rochi, gracias por hacerme parte de su amorosa familia, gracias por su cariño y apoyo. Thanks to my unconditional friends Iván, Mateo, Liliana and Mickey thank you for your full support, for being there in every break down episode thank you for being there regardless of the time and distance. Thanks to my precious friend Cecy. Your friendship throughout my life has strongly influenced the woman I have become. Thank you for being there in very important moments of my life. Thanks to Ale for being a friend and family here far away from Mexico. Thanks for your support and for teaching me your strength and share with me your sense of curiosity. Finally, thanks to my life partner, Luis Gómez Morales, for being by my side throughout this challenging but exciting part of my life. Thank you for your loving kindness that makes me stronger and capable of doing things I could never have imagined. Thanks for your unique advices that goes beyond the ordinary, thanks for your beautiful mind that helps me see things in the right perspective. Without your love, support and companionship, I would have never reached the place where I am now. I love you so much. Thanks to life for placing me in a very fortunate position surrounded by amazing people that I will always carry in my heart.

Titre: Identification des voies de signalisation essentielles aux propriétés anti-leucémiques des anticorps anti-CD3

Résumé:

La leucémie lymphoblastique aiguë à cellules T (LAL-T) est une pathologie hématologique agressive. Les traitements actuels, basés sur des schémas de chimiothérapie intensive, offrent des taux de survie globale ~ 85% chez les enfants et < 50% chez les adultes, ce qui appelle à la recherche de nouvelles options thérapeutiques. Notre équipe a montré que l'activation du récepteur des cellules T (TCR) est fortement anti-leucémique dans des modèles murins de LAL-T. Par ailleurs, les anticorps anti-hCD3 (aCD3) OKT3 ou Teplizumab et Foralumab, cliniquement pertinents, induisent la mort des cellules leucémiques, la régression de la leucémie et une amélioration de la survie des souris leucémiques dans les modèles de PDXs (Patients Derived Xenografts) de LAL-T. Bien que l'utilisation des anticorps aCD3 ouvre une nouvelle option immunothérapeutique pour traiter les LAL-T, en particulier lorsqu'elle est associée à la chimiothérapie, la monothérapie aCD3 conduit à une rechute chez les souris greffées avec les PDXs. Afin de rechercher les mécanismes moléculaires qui président aux propriétés anti-leucémiques des aCD3, nous avons identifié la signature transcriptomique induite par ces anticorps. L'analyse GSEA et KEGG/GO de cette signature a permis d'identifier la voie de signalisation "TNFa via NF-kB" comme la plus dérégulée et qui comprend les gènes codant pour les ligands (TNFa, LTa), les récepteurs (TNFR2), et les adaptateurs (IAP2, TRAFs) de cette voie. L'induction de ces gènes a été confirmée et généralisée à des PDX de LAL-T indépendantes. Fonctionnellement, grâce à l'utilisation d'Etanercept qui neutralise TNFa/LTa, nous avons montré que la voie TNFa favorise la survie des cellules leucémiques. Cependant, l'utilisation de Birinapant qui induit la dégradation de cIAP1/2 permet de rediriger la signalisation TNF d'un programme de survie pour induire un programme de mort des cellules leucémiques et par conséquent prolonger la survie des souris leucémiques. Nous

avons montré que cette synergie dépend de l'activité de RIPK1. En effet, nous avons constaté que la phosphorylation de RIPK1 est spécifiquement induite lors d'un traitement combiné aCD3/Birinapant. De plus, l'inhibition de RIPK1 in vitro en utilisant un shRNA, ou in vivo en utilisant un inhibiteur pharmacologique, réverte la coopération entre aCD3 et Birinapant. Ainsi, nos résultats plaident en faveur de l'utilisation du Birinapant ou d'autres SMAC mimétiques pour améliorer les propriétés thérapeutiques des aCD3. Nous avons également étudié le rôle d'autres candidats qui ont été trouvés dérégulés en réponse à l'aCD3, notamment: i) *PCD1* codant l'inhibiteur physiologique de la signalisation du TCR, PD-1 et ii) *TNFRSF4*, *TNFRSF9* codant les corécepteurs du TCR, 4-1BB et OX40. Nous avons validé et généralisé à d'autres PDX, l'induction de ces gènes en réponse à OKT3. Fonctionnellement, nous avons démontré que l'inactivation de PD1, soit par shRNA, soit grâce à l'utilisation d'un anticorps antagonistique, le Pembrolizumab, renforce les propriétés thérapeutiques des anti-CD3. L'analyse par RNA-seq du transcriptome des cellules co-traitées par OKT3 et Pembrolizumab montre une augmentation générale de l'ensemble des gènes induits par OKT3, plutôt que la détection d'une nouvelle signature spécifique de cette combinaison. Ces résultats étant en accord avec le rôle inhibiteur aPD1 sur le TCR. Nous avons aussi montré que l'anticorps agoniste de 4-1BB, Urelumab, induit également une amélioration des effets anti-leucémiques d'OKT3. L'ensemble de ces résultats suggère que l'utilisation d'outils renforçant l'activation du TCR améliorera les propriétés anti-leucémiques des aCD3. Alors que nos études précédentes ont identifié les anticorps aCD3 comme une nouvelle immunothérapie potentielle pour la LAL-T, ce travail montre que l'analyse de la signature induite par ces anticorps dans la LAL-T a révélé des voies moléculaires qui peuvent être ciblées pour améliorer l'efficacité thérapeutique des aCD3.

Mot clés: T-ALL, PDX, Thérapies ciblées, Immunothérapie, Mimétiques de SMAC, PD-1.

Title: Characterization of the anti-leukemic properties of anti-CD3 antibodies in T-cell acute lymphoblastic leukemia (T-ALL)

Abstract:

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological malignancy. Current treatments, based on intensive chemotherapy regimens provide overall survival rates of ~85% in children and <50% in adults, calling for the search of new therapeutic options. Our team previously showed that T cell receptor (TCR) triggering/activation is strongly anti-leukemic in mouse T-ALL models and that targeting the TCR of diagnostic T-ALL-derived PDX with anti-hCD3 (aCD3) monoclonal antibody (mAb) OKT3 or the clinically relevant Teplizumab and Foralumab induced leukemic cell death, leukemia regression and improved host survival. Although use of aCD3 mAbs opens a new immune-therapeutic option in T-ALL, in particular when associated with chemotherapy, aCD3 monotherapy results in relapse in T-ALL PDX. Thus, identification of the molecular mechanisms underlying the anti-leukemic properties of the aCD3 mAbs may lead to discovery of new T-ALL vulnerabilities to improve the aCD3 therapeutic effects. We identified the transcriptomic signature induced by either OKT3 and Teplizumab and show that 932 genes are commonly deregulated. GSEA and KEGG/GO analyses found the “TNF α signaling via NF- κ B” pathway as the top deregulated pathway which comprises genes encoding ligands (TNF α , LT α), receptors (TNFR2), adaptors (IAP2, TRAFs) of this signaling pathway. Induction of these genes was confirmed and generalized at the RNA and protein level to independent T-ALL PDXs. We further demonstrate that while TNF signaling promotes leukemic cell survival, it can be re-routed toward death upon cIAP1/2 degradation, thus enhancing anti-CD3 anti-leukemic properties. Indeed, the use of Etanercept, a sink for TNF α /LT α , was found to enhance aCD3-antileukemic effects, indicating that TNF/TNFR survival pathways counteract TCR-induced leukemic cell death. In contrast, the use of Birinapant, a SMAC mimetic that

ultimately results in cIAP1/2 degradation, acts synergistically with aCD3 to induce leukemic cell apoptosis, impair leukemia expansion and improve mice survival. We also demonstrate aCD3/Birinapant synergism to rely on RIPK1 activity. Indeed, we found that RIPK1 phosphorylation is specifically induced upon combinatorial treatment while inhibition of RIPK1(i) in vitro using shRNA or (ii) in vivo using a pharmacological inhibitor revert aCD3/Birinapant cooperation. Thus, our results advocate the use of Birinapant or other SMAC mimetics to improve aCD3 immunotherapy in T-ALL. In parallel, we also investigated the role of other candidates that were found deregulated in response to aCD3, including: i) *PCD1* encoding for the physiological inhibitor of TCR-signaling PD-1 and ii) TNFRSF4, TNFRSF9 encoding for the TCR co-receptors 4-1BB and OX40. Similarly, validation of their induction was confirmed and generalized at both RNA and protein levels in independent T-ALL PDXs. We further demonstrated that PD-1 shRNA-mediated downregulation in T-ALL cell lines enhanced aCD3-mediated leukemic cell death. Moreover, the use of the therapeutic aPD1 mAb Pembrolizumab, in combination with aCD3, demonstrated a synergistic anti-leukemic effect as measured by leukemia load in blood overtime leading to further prolonged mice survival compared to T-ALL PDX treated with aCD3 alone. RNA-seq analysis show a general enhancement of aCD3 transcriptomic signature upon Pembrolizumab treatment, in agreement with the known role of PD1 as a negative regulator of TCR. As using the agonistic mAb Urelumab that targets 4-1BB, a TCR co-activator, also results in improved aCD3 anti-leukemic effects, we suggest that tools re-enforcing TCR activation will improve the aCD3 anti-leukemic properties. While our previous studies identified aCD3 mAbs as a potential new immunotherapy for T-ALL, this work reveals that the signature evoked by these mAbs in T-ALL cells can unravel molecular pathways that can be targeted to further improve the efficacy of aCD3-based immunotherapy.

Keywords: T-ALL, PDX, Targeted therapies, Immunotherapy, SMAC Mimetics, PD-1.

Résumé substantiel

Les leucémies lymphoblastiques aiguës du lignage T (LAL-T) sont des pathologies hétérogènes qui résultent de multiples altérations oncogéniques amenant à l'expansion de lymphoblastes bloqués dans leur différenciation. Elles représentent 10 % des cas pédiatriques de leucémies lymphoblastiques aiguës (LAL-B/T) et 25 % des cas chez les adultes. Les analyses cytogénétiques et transcriptomiques globales classent les LAL-T en groupes moléculaires caractérisés par l'expression anormale de facteurs de transcription spécifiques (e.g. TAL ; LMO1/2 ; TLX1/3 ; HOXA) et un bloc de différenciation à des stades spécifiques. Les protocoles de chimiothérapie actuels utilisés dans le traitement des LAL-T sont longs, associés à une toxicité immédiate ou retardée et induisent un taux de survie de l'ordre de 85-90% pour les LAL-T pédiatriques et inférieurs à 50% chez l'adulte, les rechutes étant alors de très mauvais pronostic. Ceci appelle à la recherche de nouvelles options thérapeutiques. Si les approches récentes d'immunothérapie ont considérablement amélioré l'arsenal thérapeutique dans le traitement des LAL-B, elles sont sous-développées dans les LAL-T en raison d'un manque de marqueurs spécifiques dans ces cellules leucémiques. Grâce à une approche génétique, mon laboratoire d'accueil a montré que le développement d'une LAL-T est inhibée lorsque le récepteur de cellules T est activé. Cette activation peut être induite grâce à l'utilisation d'anticorps anti-CD3e dont ils ont montré qu'ils induisaient la mort des cellules leucémiques et par conséquent prolongeait considérablement la survie des animaux leucémique. Puisqu'environ 33% des LAL-T pédiatriques (et 23% des adultes) présentent l'expression d'un TCR (TCRab ou TCRgd) à leur surface, l'immunothérapie anti-CD3 représente une nouvelle option thérapeutique spécifique de ces patients. Plus particulièrement, Teplizumab qui est un anticorps approuvé cliniquement pour le traitement de patients atteints de diabète, a été montré comme induisant la mort des cellules leucémiques de LAL-T humaines xéno greffées dans des souris NSG. Cependant, bien que l'utilisation des anticorps α CD3 ouvre une nouvelle option immunothérapeutique dans la LAL-T, en particulier lorsqu'elle est

associée à la chimiothérapie, nous avons observé la récurrence de la maladie chez les souris traitées. Il est donc essentiel d'identifier les mécanismes moléculaires qui sous-tendent les propriétés antileucémiques des anticorps α CD3 et de cibler ces mécanismes pour accroître la vulnérabilité des LAL-T à l'immunothérapie α CD3. Le projet actuel a pour but -sur la base d'observations préliminaires solides- d'identifier certains des mécanismes moléculaires impliqués dans le programme de mort cellulaire induit par les α CD3 dans les LAL-T et d'exploiter cette connaissance du point de vue pharmacologique pour augmenter encore la vulnérabilité des LAL-T à l'immunothérapie basée sur les anticorps monoclonaux α CD3. Afin d'identifier les voies instrumentales de l'activité anti-leucémique liée à l'activation du TCR par les anticorps α CD3 dans la LAL-T, nous avons analysé le transcriptome global induit in vivo par les anticorps OKT3 et Teplizumab dans les PDX de LAL-T. Conformément aux résultats précédents de notre laboratoire, l'analyse d'enrichissement de l'ensemble des gènes (GSEA) a mis en évidence un enrichissement très fort et significatif des gènes induits par le TCR associés à la sélection négative des progéniteurs thymiques normaux, dans les cellules leucémiques traitées par l'OKT3 et le Teplizumab. Il convient de noter que NR4A1 et BCL2L11, les seuls gènes dont il a été démontré jusqu'à présent qu'ils étaient essentiels à la sélection négative des thymocytes, sont fortement induits en réponse aux α CD3.

En plus de cette signature "sélection négative", la voie "TNF α Signaling via NF-kB" s'est systématiquement classée au premier rang quand des analyses comparatives parmi les 50 GSEA Hallmarks ont été réalisées.

Cet enrichissement s'est avéré profond et significatif dans les cellules leucémiques prélevées sur des PDX LAL-T traités par OKT3 ou Teplizumab. En outre, les gènes codant pour les ligands (TNF α , LT α) et les adaptateurs/effecteurs (TRAF1, TRAF3, BIRC3/c-IAP2, NFKBIA, NFKBID, NFKBIE, NFKB1, NFkB2, c-REL, REL-B de la voie TNF/TNFR ont été induits dans les cellules LAL-T provenant de PDX LAL-T traitées à l' α CD3. En utilisant la technologie d'expression génique multiplex (ncounter Nanostring), nous avons confirmé l'augmentation de ces gènes induite par les α CD3 dans un panel de 7 PDX de LAL-T indépendantes, montrant

que les acteurs critiques de la signalisation $TNF\alpha/LT\alpha$ sont régulés à la hausse dans les cellules LAL-T en réponse aux AcM $\alpha CD3$. Ceci suggère un impact possible de la signalisation $TNF/TNFR$ sur le potentiel anti-leucémique de ces anticorps. Pour étudier le rôle du $TNF\alpha/LT\alpha$ dans les propriétés anti-leucémiques des $\alpha CD3$, nous avons interféré avec cette voie en utilisant l'éta nercept, une protéine de fusion recombinante qui se lie au TNF et à la lymphotoxine- α , agissant ainsi comme un récepteur soluble qui neutralise les ligands $TNF\alpha/LT\alpha$. L'administration d'éta nercept dans des PDX LAL-T n'a pas eu d'effet significatif sur la charge tumorale alors que, comme prévu, le traitement par OKT3 a entraîné une inhibition de la charge leucémique au cours du temps. En revanche, nous avons montré que l'administration d'éta nercept renforce les propriétés antileucémiques d'OKT3 notamment en améliorant la survie des souris leucémiques par rapport aux souris traitées avec OKT3 en monothérapie. Ceci indique que l'induction de $TNF\alpha/LT\alpha$ en réponse à OKT3 interfère avec la mort cellulaire induite par les $\alpha CD3$ dans les cellules LAL-T.

L'engagement de TNFR1 par $TNF\alpha/LT\alpha$ déclenche la formation de différents complexes de signalisation, chacun entraînant des réponses cellulaires distinctes. Le complexe I est assemblé à la membrane plasmique par le recrutement de protéines sur les queues cytoplasmiques du TNFR1. Ces complexes multiprotéiques comprennent des adaptateurs tels que TRADD et TRAF, des ubiquitines ligases IAP1 et IAP2 et des protéines kinases telles que RIPK1. De multiples événements d'ubiquitination dans le complexe TNFR1 conduisent à l'activation de l'I κ B kinase (IKK) et du NF κ B pour finalement favoriser la survie cellulaire. La perturbation du complexe I, par exemple par l'inactivation de l'ubiquitine E3 ligase cIAP1/2 par le SMAC, entraîne la formation de plusieurs complexes cytoplasmiques qui médient la mort cellulaire dépendante et indépendante de RIPK1. Etant donné que TNFR1 et c-IAP1 sont exprimés et que c-IAP2 augmente fortement en réponse au traitement $\alpha CD3$, nous avons ensuite cherché à savoir si l'interférence avec c-IAP1/c-IAP2 en utilisant un mimétique de smac tel que le Birinapant, pouvait rediriger la signalisation TNFR1 de la survie cellulaire vers la mort cellulaire et ainsi renforcer les propriétés

anti-leucémiques des AcM α CD3 pour induire l'apoptose des cellules de la LAL-T in vivo. Les résultats obtenus démontrent que le Birinapant agit en synergie avec les α CD3 pour induire l'apoptose des cellules leucémiques, qui s'accompagne d'une augmentation de l'activité de la caspase 8, ce qui retarde la progression de la leucémie et améliore la survie des souris leucémiques dans tous les cas de LAL-T testés. Ceci indique que la mort cellulaire induite par les α CD3 et ses propriétés anti-leucémiques peuvent être renforcées en altérant l'expression de c-IAP1/2 à l'aide de médicaments mimétiques de SMAC. La mort cellulaire médiée par le TNFR1 peut reposer sur l'activité kinase de RIPK1. Nous avons donc ensuite cherché à savoir si l'augmentation de l'apoptose induite en réponse à l' α CD3/Birinapant était associée à une augmentation de l'activité de RIPK1. Pour ce faire, nous avons analysé l'état de phosphorylation de RIPK1 dans les cellules de la lignée de LAL-T, CEM traitées soit avec l' α CD3, soit avec le Birinapant, soit avec la combinaison des deux composés. Les résultats obtenus montrent que si le traitement α CD3 n'induit pas l'activation de RIPK1, le co-traitement anti-CD3/Birinapant augmente fortement la phosphorylation de RIPK1. En accord avec nos données in vivo, le traitement au Birinapant renforce la mort cellulaire des cellules CEM induite par l' α CD3 in vitro. En outre, l'inhibition de RIPK1 par voie pharmacologique (Nécrostatine 2) ou génétique (shRNA) inhibe la mort cellulaire induite par la combinaison α CD3/Birinapant. De façon similaire, l'inhibition pharmacologique de l'activité de RIPK1 in vivo entraîne l'inhibition de la synergie de la combinaison α CD3 + Birinapant dans les PDX LAL-T. Ces résultats nous permettent de conclure que l'induction de la mort des cellules LAL-T par le traitement α CD3/Birinapant repose sur l'activité de la kinase RIPK1.

En plus des acteurs de la voie "TNF α Signaling via NF- κ B", nous avons identifié la forte augmentation des gènes codant pour des candidats potentiels qui pourraient servir de cibles attrayantes pour améliorer les propriétés antileucémiques de l' α CD3. Ces gènes comprennent : i) le gène de la protéine de mort cellulaire programmée 1 (*PDCD1*), qui code pour PD-1, un inhibiteur physiologique de la signalisation du TCR, et ii) les récepteurs de la superfamille TNFR connus sous le

nom de récepteurs de co-signalisation du TCR, notamment *TNFRSF9*, qui code pour 4-1BB, et *TNFRSF4*, qui code pour OX-40.

L'expression de l'ARN et des protéines de ces candidats a été évaluée dans 7 PDX de LAL-T indépendants lors de l'administration de l'anticorps α CD3. Les résultats obtenus montrent que dans tous les cas de LAL-T testés, ces récepteurs ainsi que leurs ligands respectifs sont fortement induits par le traitement α CD3. Ceci suggère qu'ils pourraient jouer un rôle dans les propriétés anti-leucémiques des α CD3. Etant donné que la liaison de PD-1 à ses ligands entrave la signalisation du TCR, nous avons cherché à savoir si l'interférence avec la signalisation de PD1 pouvait renforcer les effets antileucémiques induits par les α CD3. Pour ce faire, nous avons d'abord réduit l'expression de PD1 par shRNA dans les lignées cellulaires LAL-T Jurkat et CEM et évalué la mort cellulaire induite par l' α CD3. Alors que les cellules contrôles (scrambled-shRNA) meurent après un traitement à l' α CD3, les cellules PD-1 knock-down montrent une augmentation significative de la mort cellulaire induite par les α CD3. Ceci suggère que l'inhibition de PD-1 lors d'un traitement α CD3 pourrait être une stratégie utile pour potentialiser l'effet anti-leucémique de l' α CD3 dans la LALT. Nous avons donc ensuite cherché à savoir si l'inhibition pharmacologique de PD-1 avec l'anticorps antagoniste cliniquement pertinent Pembrolizumab pouvait améliorer les effets anti-leucémiques induits par l' α CD3 in vivo. Alors que l'administration d'OKT3 inhibe l'expansion de la leucémie comme prévu, l'administration individuelle de Pembrolizumab n'a montré aucun effet antileucémique. En revanche, l'administration conjointe des deux anticorps renforce l'effet antileucémique d'OKT3, comme le montre l'inhibition de l'expansion de la leucémie dans le sang et l'amélioration de la survie de l'hôte par rapport aux souris traitées avec l'OKT3 seul. Ces résultats nous ont permis de conclure que la régulation à la hausse de PD-1 induite par l' α CD3 dans la LAL-T est un récepteur fonctionnel et cliniquement ciblable pour améliorer les propriétés anti-leucémiques induites par les anticorps monoclonaux α CD3. PD1 étant un inhibiteur physiologique du TCR, nous avons ensuite voulu déterminer si la coopération OKT3/Pembrolizumab reposait sur une augmentation "générale" du programme de

mort induit par OKT3, ou si cela permettait la mise en place d'une nouvelle signature moléculaire. Pour répondre à cette question, nous avons identifié les profils transcriptomiques (RNA-seq) des cellules leucémiques des souris traitées par l'OKT3 ou la combinaison OKT3-Pembrolizumab. Ces analyses montrent une similarité importante des gènes différentiellement exprimés (DEG). En revanche, en comparant l'intensité de l'induction des gènes induits par OKT3 et ceux induits par la combinaison OKT3-Pembrolizumab, nous avons observé:

- En ce qui concerne les gènes très fortement induits par OKT3, la combinaison OKT3-Pembrolizumab induit une augmentation supplémentaire mais non significative
- En revanche, pour les gènes trouvés plus faiblement induits par OKT3, la combinaison permet une meilleure induction de ces gènes et de façon très significative. Parallèlement à ces résultats, l'analyse de l'expression du marqueur d'activation du TCR, CD69, a montré une augmentation supplémentaire dans les cellules LAL-T des souris traitées avec l'association OKT3-Pembrolizumab par rapport aux cellules LAL-T des souris traitées avec l'OKT3 seul.

Sur la base de ces résultats, nous concluons que l'inhibition de PD-1 pendant le traitement anti-CD3 dans les PDX LAL-T renforce les propriétés anti-leucémiques en amplifiant la force de la signalisation du TCR.

L'ensemble de ce travail a permis de caractériser le profil transcriptomique induit par les AcM α CD3 thérapeutiques, que nous avons exploité afin d'identifier certains des acteurs essentiels aux propriétés anti-leucémiques des anti-CD3. Le ciblage spécifique de ces acteurs notamment par des agents pharmacologiques (Birinapant pour IAP1/2, Pembrolizumab pour PD1) démontre qu'il est possible de renforcer les propriétés anti-leucémiques des anti-CD3 et ouvre donc de nouvelles perspectives quant au traitement des patients atteints de LAL-T.

Abbreviations

aBFM	Augmented Berlin-Frankfurt-Münster
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
ALL	Acute Lymphoblastic Leukemia
AICD	Activated Induced Cell Death
AML	Acute Myeloblastic Leukemia
APAF-1	Apoptosis Protease-Activating Factor-1
B-ALL	B-cell Acute Lymphoblastic Leukemia
BCL-2	B-cell lymphoma-2
BCR	B-cell Receptors
BiTE	Bispecific T-cell engagers
BM	Bone Marrow
CAR-T	Chimeric Antigen Receptor T-cells
CDC	Complement-Dependent Cytotoxicity
CLL	Chronic Lymphocytic Leukemia
CNS	Central nervous system
Con-A	Concanavalin A
CR	Complete Remission
CSK	C-terminal Src kinase
cTEC	cortical thymic epithelial cells
Cyt C	Cytochrome C
DD	Death Domain
DED	Death Effector Domain
DFS	Disease-Free Survival
DISC	Death-Inducing Signaling Complex
DN	Dominant Negative
DN	Double Negative

EMA	European Medicines Agency
ETP	Early T-cell Progenitor
FADD	Fas-Associated protein with Death Domain
FasL	Fas Ligand
FDA	US Food and Drug Administration
FISH	Fluorescence in situ hybridization
GEF	Guanine nucleotide Exchange Factors
GMP	Granulocyte Myeloid Progenitors
GRB2	Growth-factor Receptor-Bound protein 2
GVHD	Graft-versus-host disease
HPK1	Hematopoietic progenitor kinase 1
HR	High risk
HS	Hematopoietic System
HSC	Hematopoietic Stem Cells
HSCT	Hematopoietic Stem Cell Transplantation
IAP	Inhibitor of Apoptosis Proteins
ICD	Intracellular domain
IFN	Interferon
IL2	Interleukin 2
I κ B	Inhibitory of nuclear factor kappa B
IKK	I κ B kinase
ITAM	Immunoreceptor Tyrosine-based Activation Motifs
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
ITSM	Immunoreceptor Tyrosine-based Switch Motif
JAK	Janus kinase
LAT	Linker of Activation of T-cells
LCK	Lymphocyte-specific protein tyrosine kinase
LIC	Leukemia Initiating Cells
LUBAC	Linear Ubiquitin Chain Assembly Complex

LT α	Lymphotoxin alpha
mAb	Monoclonal antibody
MALT1	Mucosa-associated lymphoid tissue translocation protein-1
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEP	Megakaryocyte-Erythroid Progenitors
MHC	Major Histopathology Complex
MLKL	Mixed Lineage Kinase domain-like protein
MM	Multiple myeloma
MOM	Mitochondrial Outer Membrane
MOMP	Mitochondrial Outer Membrane Permeabilization
MPPs	Multipotent Progenitor cells
MR	Medium risk
MRD	Minimal Residual Disease
mTEC	Medullar thymic epithelial cells
mTOR	Mammalian target of rapamycin
mTNF α	Membrane-bound tumor necrosis factor α
MTX	Methotrexate
NEMO	NF- κ B Essential Modulator
NFAT	Nuclear Factor of Activated T-cells
NF- κ B	Nuclear Factor- κ B
NIK	NF- κ B inducing kinase
NK	Natural Killer cells
NLS	Nuclear localization signal
OVA	Ovalbumin
OS	Overall survival
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death protein-1
PDX	Patient-Derived Xenografts

PDK1	3-phosphoinositide-dependent kinase
PI3K	Phosphatidylinositol-3-kinases
PIP ₂	Phosphatidylinositol-4,4, biphosphate
PIP ₃	Phosphatidylinositol-3,4,5-triphosphate
PKCθ	Protein kinase C theta
pMHC	Peptide-MHC complex
PRC2	Polycomb Repressor Complex 2
pTα	pre-TCRα chain
PTEN	Phosphatase and tensin homologue
PTK	Protein Tyrosine Kinases
PTP	Protein Tyrosine Phosphatases
PTPN2	Protein tyrosine phosphatase non-receptor type 2
RIPK1	Receptor Interacting Protein Kinase 1
RIPK3	Receptor Interacting Protein Kinase 3
ROS	Reactive oxygen species
RTK	Receptor Tyrosin Kinase
SCZ	Subcapsular Zone
SEB	Staphylococcus enterotoxin B
SH-2	Src Homology 2 domain
SHP1	SH2 domain-containing phosphatase 1
SHP2	SH2 domain-containing phosphatase 2
SLE	Systemic lupus erythematosus
SLP76	SH2 domain containing leukocyte protein of 76kDa
SM	Smac-Mimetic
SMAC	Second Mitochondria-derived Activator of Caspases
SP	Single Positive
SR	Standard Risk
STAT	Signal Transducer and Activator of Transcription
STK	SRC family kinase

sTNF α	soluble tumor necrosis factor α
T-ALL	T-cell Acute Lymphoblastic Leukemia
TCR	T-cell Receptor
TNF α	Tumor necrosis factor α
TNFR	TNF Receptor
TNFSF	Tumor Necrosis Factor Superfamily
TKI	Tyrosine kinase inhibitors
TRAF	Tumor necrosis factor receptor (TNF-R)-associated factor

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Chapter I.

Introduction

1. T-lymphocytes

1.1. Introduction

The hematopoietic system (HS) comprises blood cells of different lineages, including erythrocytes (red blood cells), lymphocytes, monocytes (white blood cells), and thrombocytes (platelets). The diverse biology of these cells is indicative of the wide range of functions in which the HS is involved. Red blood cells supply oxygen to our body, while platelets prevent or stop bleeding in wounded tissues. Similarly, white blood cells encompass a variety of cell types that are highly specialized in detecting and eliminating pathogens that can threaten our health, in this way constituting the immune system.

1.2. Hematopoiesis

Hematopoiesis is the process by which the hematopoietic system is generated from multipotent Hematopoietic Stem Cells (HSCs). During human embryogenesis, the sites of hematopoiesis vary. Initially, HSCs arise in the aorta-gonad-mesonephros (AGM) region and then migrate to the placenta, followed by the embryonic liver and spleen. Eventually, prior to birth, hematopoiesis becomes established in the bone marrow (BM), which then serves as the site where HSCs maintain hematopoietic homeostasis (Jagannathan-Bogdan and Zon, 2013). One of the highlights of HSCs is their self-renewal capacity, which gradually diminishes in their progeny, while their lineage commitment becomes defined towards specific cell types. As observed in **Figure 1**, HSCs remain quiescent at the top of the lineage hierarchy. When hematopoietic homeostasis is disrupted, such as during an infection or disease, the quiescence of HSCs is disrupted. Activation of HSCs leads to the generation of progeny that maintains stemness potential, thereby replenishing the system with more HSCs. Other cells derived from HSCs lose self-renewal capacity but retain multipotent potential; these cells are known as Multipotent

Progenitor cells (MPPs) (Morrison and Weissman, 1994). The identification of each differentiation stages has been determined by enrichment of specific cell surface markers. Human hematopoietic cells exhibiting the phenotype CD34+ CD38- CD90+ have been identified as HSCs, in turn MPPs share some markers but with a decreased CD90 expression (CD34+ CD38- CD90) (Wang and Wagers, 2011).

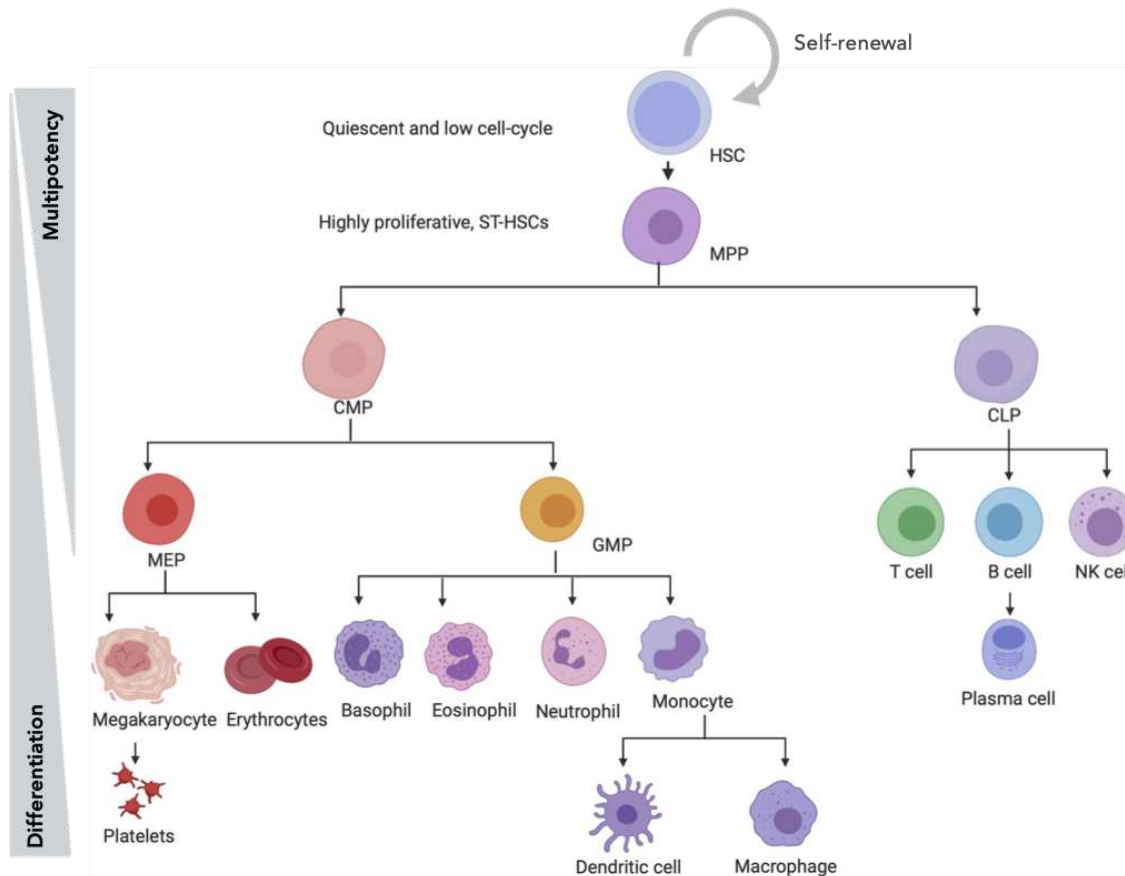


Figure 1. Scheme of hematopoietic process. HSCs sit at the top of the hierarchy, possessing self-renewal and multipotent capacity. The hierarchical tree bifurcates into two main lineages, headed by the oligoprogenitors CLP (right) and CMP (left). CMP gives rise to the bipotent progenitors MEP and GMP. The gray bars on the left depict the loss of multipotent capacity and the acquisition of maturation at each differentiation stage. Scheme modified from (Monga, Kaur and Dhanda, 2022)

Further in the differentiation process, although some studies that have proposed intermediate cell states in between (Naik *et al.*, 2013), in general terms MPPs will give rise to two oligopotent progenitors, the Common Myeloid Progenitors

(CMP) and Common Lymphoid Progenitors (CLP)(Monga, Kaur and Dhanda, 2022),(Akashi *et al.*, 2000). Likewise, the differential expression of IL-7R α is widely accepted to discriminate between CMPs and CLPs, thus identifying CMPs as cells IL-7R α negative, c-Kit $^{+}$, Sca-1 $^{-}$, and CLPs as IL-7R α positive, ckit lo , Sca-1 lo (Akashi *et al.*, 2003; Wang and Wagers, 2011). Downstream, CMPs give rise to Granulocyte Myeloid Progenitors (GMPs), which, as their name suggests, generate both granulocytes and myeloid lineages. These lineages further subdivide into neutrophils, eosinophils, basophils, monocytes, macrophages, and dendritic cells, respectively. These matured cells are part of the historically denominated white cells or leukocytes, with a majority of them actively involved in innate immunity. They possess specialized tasks, including highly efficient phagocytosis, production of enzymes, growth factors, and inflammatory cytokines, aimed at eradicating potential pathogens or dysfunctional cells from our body (De Kleer *et al.*, 2014). Furthermore, CMPs can also generate Megakaryocyte-Erythroid Progenitors (MEPs) that differentiate into cells with completely different functionalities. While megakaryocytes are polyploid cells primarily responsible for platelet production, erythrocytes are responsible for delivering oxygen to the body's tissues (Xavier-Ferruccio and Krause, 2018). In contrast, Common Lymphoid Progenitors (CLPs) exclusively give rise to lymphoid lineages, maturing into T lymphocytes, B lymphocytes, and NK cells, (Kondo, Weissman and Akashi, 1997). NK cells, as part of the innate immunity, exert cytolytic activities against various targets such as tumor and virally-infected cells, without prior immune sensitization (Vivier *et al.*, 2011)(Papamichail *et al.*, 2004). Conversely, T and B lymphocytes play a central role in carrying out the adaptive immune response, which is highly specific to pathogens. A key aspect of this response is the distinction between foreign and self entities. To achieve this, T and B lymphocytes express a diverse repertoire of antigen receptors. While T-cell Receptors (TCRs) are exclusively expressed in T cells, B-cell Receptors (BCRs) are expressed in B cells ('Adaptive immunity', 2010). The expression of these hallmark receptors is ultimately achieved in the final stages of B and T cell development,

respectively. In the following section, T-cell commitment and the appearance of T-cell receptors (TCRs) will be described.

1.3. T-cell development

1.3.1. Early stages

T-cells are an integral part of the lymphoid compartment in the hematopoietic system (HS). The distinguishing feature of T-cells is their ability to recognize foreign molecules or antigens through the T-cell antigen receptor (TCR). Consequently, during their development, lymphoid progenitors (CLPs) undergo rigorous maturation steps that ensure the generation of an exhaustive variety of TCR's and guarantee its proper functioning, from which only a minor percentage of cells succeed.

T-lymphocytes derive its name from “**thymus**”, which is the organ where these cells commit to the T lineage and complete their maturation. The thymus is an encapsulated organ formed by lobules that are histologically divided into two well-defined regions: the cortex and medulla. Each of these regions has stable microenvironments established by stromal cells. Therefore, the interaction of thymocytes (CLPs in the thymus) with each microenvironment is crucial for their further development (Petrie and Zúñiga-Pflücker, 2007). It's important to note that most of the knowledge on T-cell development is based on data generated from mouse models. Unless indicated otherwise, the information described here is based on such models.

Early T-cell development begins with the migration of CLPs from the bone marrow to the thymus through blood vessels located in the cortico-medullary junctions (CMJ). At this stage, the precursor cells are referred to as Double Negative cells (DN) because they do not express the TCR signaling coreceptors CD4 and CD8. DN cells have the potential to give rise to both T and non-T lineages, and they undergo sequential changes that lead to T-cell commitment (Rothenberg, Moore and Yui, 2008). Based on the phenotypic expression of CD117 (c-kit), CD44 and CD25 they have been classified into four subsets (from DN1 to DN4) (Carpenter and

Bosselut, 2010),(Robey and Fowlkes, 1994). In brief, these changes goes from the earliest thymocytes expressing c-kit⁺ CD44⁺ CD25⁻ (**DN1** or Early T-cell Progenitor, **ETP**), and c-kit⁺ CD44⁺ CD25⁺ (**DN2**) located in the inner cortex, to c-kit^{lo} CD44⁻ CD25⁺ (**DN3**) and c-kit^{lo} CD44⁻ CD25⁻ (**DN4**) located in the outer cortex or subcapsular zone (SCZ) (Pearse *et al.*, 1989),(Godfrey and Zlotnik, 1993) **Figure 2**.

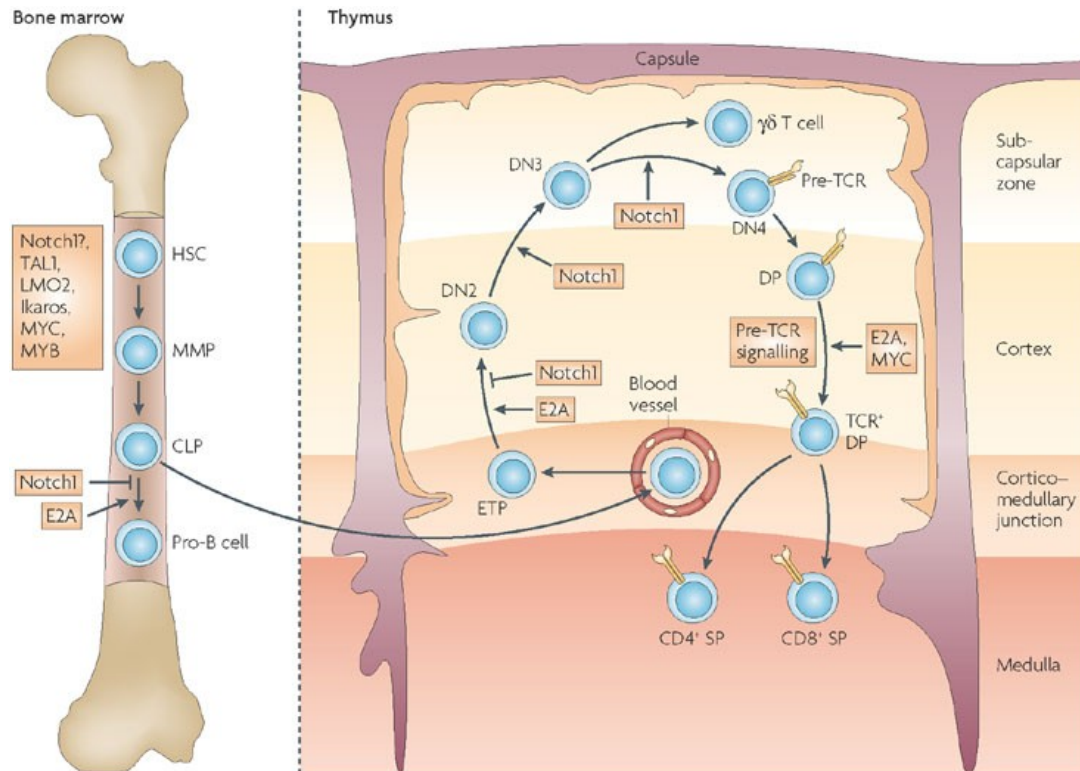


Figure 2. Overview of T-cell development HSCs self-renew and differentiate into CLPs in the bone marrow (left). CLPs then migrate through the bloodstream to reach the thymus. CLPs extravasate from the blood vessels at the cortico-medullary junction (CMJ). Once inside the thymus, CLPs undergo T-cell commitment and maturation. Schematic modified from (Aifantis, Raetz and Buonamici, 2008)

1.3.2. T-cell commitment

Through this journey, T-cell lineage commitment begins only in the DN2 cells, which undergo the initial gene rearrangements encoding for TCR β , TCR γ , and TCR δ chains. This process results in the generation of a diverse TCR repertoire

through V-D-J recombination, mediated by RAG1 and RAG2 proteins (Rothenberg, Moore and Yui, 2008),(Godfrey *et al.*, 1994). Notably, the stochastic nature of this process leads to both functional and non-functional rearrangements (Ciofani and Zúñiga-Pflücker, 2007). The majority of DN2 cells are destined to generate TCR $\alpha\beta$ -bearing cells, while only a small proportion of cells [$<10\%$] end up expressing TCR $\gamma\delta$ (Esin *et al.*, 1996). This outcome leads to a divergent transition to the DN3 stage. Cells that successfully rearrange TCR $\gamma\delta$ can directly transmit TCR signals, while TCR β -bearing cells, before they are able to signal, need to first associate with an invariant pre-TCR α chain (pT α) and CD3 signaling chains, forming the denominated pre-TCR complex. Further differentiation of thymocytes relies not only on the TCR expression on the cell surface but also on its capacity to actively transduce signals and induce cell cycle activation. This process represents a critical checkpoint in T cell differentiation, known respectively as β -selection or $\gamma\delta$ -selection. TCR $\gamma\delta$ -cells displaying active TCR signaling commit to the $\gamma\delta$ -lineage directly from the DN3 population, completing their maturation earlier without undergoing further phenotypic changes as compared with TCR $\alpha\beta$ -bearing cells. Meanwhile, cells expressing an active pre-TCR complex ($\alpha\beta$ lineage) downregulate CD25 expression and instead induce the expression of both CD8 and CD4 receptors on the cell surface, thus becoming Double Positive thymocytes (**DP**) (Dutta, Zhao and Love, 2021). DP cells lose expression of pT α and initiate VJ recombination at the TCR α locus, which leads to the formation of a mature TCR composed of functional recombined α and β chains. Ultimately, thymocytes from either lineages with defective TCR rearrangement and/or failing to transduced TCR-mediated signals will die by neglect (Groettrup and von Boehmer, 1993),(Dutta, Zhao and Love, 2021). The critical role of TCR signaling in overcoming this developmental checkpoint has been demonstrated in various studies, which showed that mice deficient in TCR components, including RAG recombinase proteins, or key signaling molecules downstream of TCR, such as the kinase LCK, experience an arrest in the DN3 thymocyte population (Germain, 2002; Ciofani and Zúñiga-Pflücker, 2007).

1.3.3. Shaping the TCR repertoire: Positive and Negative selection

Mature T-cells perform complex activities that contribute to the adaptive immune response, and these tasks rely on their refined capacity to distinguish self from non-self-entities through the TCR. During their development, T-cells undergo a series of challenges involving the presentation of self-antigens. Only the thymocytes that are unresponsive to self are privileged to complete their maturation. TCR can recognize peptides only when presented by the Major Histopathology Complex or MHC (Hennecke and Wiley, 2001; Germain, 2002). The MHC is encoded by a complex of genes divided into three regions on the chromosome 6p21.3. Polypeptides encoded by region I and II are glycoproteins that assemble the MHC class I (MHC-I) and the MHC class II (MHC-II), respectively. Whereas MHC-I is expressed in all nucleated cells, MHC-II expression is restricted to antigen presenting cells (APC), including B lymphocytes, dendritic cells and cells of the monocyte-macrophage lineage. The interaction between the TCR and MHC leads to lineage commitment in DP thymocytes. MHC-II-restricted thymocytes differentiate into CD4+ CD8- single positive (SP) cells, while MHC-I-restricted thymocytes differentiate into CD4- CD8+ SP cells (Klein *et al.*, 2014). MHC-bearing cells process proteins into peptides and load them onto the MHC within the endoplasmic reticulum, forming the peptide-MHC complex (pMHC), which is then displayed on the cell membrane (Lankat-Buttgereit and Tampé, 1999). In this context, presentation of the pMHC in the thymus challenge and shape the TCR repertoire of the TCR $\alpha\beta$ -bearing thymocytes in selection checkpoints denominated positive and negative selection. These events are not subsequent but rather independent processes, in general terms, thymocytes audition to the positive selection in the intrathymic cortex and to the negative selection in the medulla, the reason of this occurring is due to the specific features of each region that include the enrichment of specific MHC-carrier cells. While the intrathymic cortex is enriched in great extent by cortical thymic epithelial cells (cTECs), the medulla have enhanced populations of medullar thymic epithelial cells (mTECs) and hematopoietic antigen-presenting cells (APC) including

macrophages, dendritic cells and B-cells (Robey and Fowlkes, 1994), (Klein *et al.*, 2014),(Pieters, 1997).

Positive selection represents the most stringent checkpoint in T-cell development, where more than 90% of DP thymocytes are eliminated due to insufficient intracellular signaling resulting from weak TCR-pMHC recognition (Merkenschlager *et al.*, 1997). In contrast, DP cells that successfully engage in TCR signaling upon recognition of self-peptide-MHC complexes are rescued from programmed cell death. This survival mechanism is mediated by the induction of the early response transcription factor c-Fos, which promotes the upregulation of anti-apoptotic proteins of the BCL-2 family (described in section “5.Cancer and cell death resistance: a close relationship”, page 68) (Sentman *et al.*, 1991).

Several factors have been identified to influence a cell's ability to succeed positive selection. These factors include the nature of the presented peptides either “private” that are generated by cTECs or “public” produced by mTECs , the lineage of the carrier cell presenting the pMHC to the TCR (hematopoietic or epithelial), the location of the interaction (cortex or medulla), and the strength of TCR signaling upon self-peptide-MHC recognition (Klein *et al.*, 2014). Deficiencies in signaling molecules downstream of the TCR, such as CD3 δ signaling chain, LCK, ZAP70, and ERK2, result in impaired positive selection (Molina *et al.*, 1992; Dave *et al.*, 1997). Additionally, the level of TCR expression at the cell surface has been found to be critical, as mice deficient in *Cbl-b* an E3 ubiquitin ligase, exhibit increased levels of TCR at the cell surface, leading to enhanced positive selection (Starr, Jameson and Hogquist, 2003; Shamim *et al.*, 2007)

Negative selection is the developmental checkpoint by which auto-reactive TCRs are eliminated from the repertoire to avoid autoimmune diseases. TCR $\alpha\beta$ -bearing thymocytes, whether DP or SP, exhibiting high-affinity recognition of self-peptide-MHC, are induced to undergo apoptosis. Approximately 5% of the thymocytes will overcome this selection and to become mature T-cells unable to react against self-tissues (van Meerwijk *et al.*, 1997).

As mentioned before, thymic microenvironments play an important role during negative selection as it has been observed that it occurs more efficiently when is mediated by the main cell residents of the medulla: hematopoietic APCs or mTECs (Klein *et al.*, 2014). Despite interesting, the microenvironment influence will not be described to rather focus in the thymocyte intracellular processes during negative selection.

TCR signaling consequent of TCR-self-peptide-MHC interaction intriguingly orchestrate the divergent fates of cell survival during positive selection, and cell death induction in negative selection. Serial studies have been performed over the years trying to elucidate the molecular mechanisms behind these divergent processes. One of the most studied models that explain the opposite thymocyte fates is the strength and duration of the TCR signaling consequent on the TCR-pMHC affinity. This model is based on diverse studies from independent groups (Robey and Fowlkes, 1994; Hernández-Hoyos *et al.*, 2000; Yasutomo *et al.*, 2000; Zeidan *et al.*, 2019; Karimi *et al.*, 2021) and stands out that low affinity ligands lead to weak TCR signaling that consequently promote thymocyte survival (positive selection) and in turn, high affinity ligands leading to strong TCR signaling induce thymocyte cell death (negative selection).

To dissect the specific mechanisms involved particularly in negative selection, a series of mice models have been established. These models include mice bearing clonal transgenic TCRs whose ligands can be from endogenous or exogenous origin. Among the most used they are: (i) the H-Y TCR that specifically recognize male-specific HY antigen, thus exerts negative selection only in male mice, and (ii) OT-I, OT-II and DOI I.IO TCR that specifically recognize Ovalbumin (OVA) peptides. Other models to induce and assess negative selection comprise the direct administration of superantigens like Staphylococcus enterotoxin B (SEB) or administration of anti-murine α CD3 or α TCR antibodies (Liu *et al.*, 1994; Starr, Jameson and Hogquist, 2003)

Despite great similarities between positive and negative selection, some intriguing distinctions have been spotted to be restricted to negative selection,

including, players of cell death pathways, TCR signaling and MAPK signaling pathways.

1.3.3.1. Cell death in negative selection

Studies on cell death during negative selection have explored both extrinsic and intrinsic cellular signals that contribute to this process. While intrinsic apoptosis plays a pivotal role in thymic negative selection (as detailed below), the involvement of extrinsic apoptosis and TNF family members will be shortly described as it is a point of interest in the present study.

The extrinsic apoptosis refers to cell death induced by pro-apoptotic ligands that bind to specialized death receptors. Both the ligands and receptors of this signaling pathway belong to the tumor necrosis factor superfamily (TNFSF), which participates in signaling pathways with pleiotropic effects, including cell death (MacEwan, 2002) (further aspects of this pathway are detailed in the 'Extrinsic apoptosis' section on page 77). Since TNF is constitutively expressed in the human and murine thymus, and transgenic mice constitutively expressing TNF in the T cell compartment result in reduced thymus size and decreased populations of DP cells (Probert, 1993), it was thought that TNF could play a role in thymic development. However, functional studies in genetically-modified double knockout *Tnfr1*^{-/-} *Tnfr2*^{-/-} mice (Page *et al.*, 1998) exhibited normal thymocyte deletion in two antigen-induced in vivo models of negative selection, suggesting no major implication of TNF receptors in thymic negative selection. Additionally, similar observations were found in *Fas*^{-/-} (Adachi *et al.*, 1995) and *FasL*^{-/-} (Castro *et al.*, 1996) mice.

In contrast, a transcriptional comparison of clonal deletion in vivo in a diversity of models including endogenous and exogenous antigens, identified the consistent induced high expression of the proapoptotic proteins NR4A1 and BIM (Baldwin and Hogquist, 2007). NR4A1, also known as NUR77, encodes a zinc-finger transcription factor. Mice induced to undergo negative selection treated with an anti-CD3 antibody or bearing a transgenic DOI I.IO TCR showed increased NUR77 at the mRNA and

protein levels in thymocytes specifically undergoing negative selection. In line with these studies, the expression of a dominant negative mutant of NUR77, which encodes non-functional gene product and acts as a competitive inhibitor, in mice carrying a transgenic F5 TCR that recognizes exogenous antigens, leads to the protection of thymocytes from induced apoptosis. Consistent with these observations, mice constitutively expressing full-length NUR77 showed an increased number of thymocytes undergoing apoptosis and a reduced population of mature T-cells. (Liu et al., 1994; Woronicz et al., 1994; Cainan et al., 1995). Effectors of intrinsic apoptosis include members of the BCL-2 family, master regulators of the pathway (description in section “Intrinsic apoptosis”, page 69). Early observations on the role on the negative selection of one member of the BCL-2 family, the pro-apoptotic BH3-only protein BIM, showed that *Bim*^{-/-} mice displayed lymphoid hyperplasia and progressive autoimmune disease (Bouillet et al., 1999). Moreover, *Bim*^{-/-} thymocytes in mice models for negative selection, such as mice bearing transgenic autoreactive TCRs or injected with a murine α CD3 antibody or the superantigen SEB, showed severely decreased negative selection as observed by reduced DP thymocytes apoptosis and an accumulation of these cells in the thymus (Bouillet et al., 2001, 2002; Hu and Baldwin, 2015). In line with this, recent studies have identified a T cell-specific genomic enhancer *E^{BAB}* that regulates *BIM* expression, the deletion of which leads to the accumulation of autoreactive thymocytes (Hojo et al., 2019). Furthermore studies conducted in transgenic TCR-HY mice and double knockout for *Bim*^{-/-} and *Nur77*^{-/-} demonstrated an impairment of negative selection and a significant increase in positive selection efficiency (Hu and Baldwin, 2015). In contrast, analysis of the overexpression of the anti-apoptotic protein BCL-2 in mice models for negative selection, expressing the transgenic TCR HY (Strasser et al., 1994) or the TCR V β (Sentman et al., 1991), show that BCL-2 expression do not protect thymocytes from negative selection. Considering that BCL-2 can counteract BIM pro-apoptotic activity during intrinsic apoptosis, these observations highlight the particular role of BIM on the negative selection. The consistent results across the different models mentioned here reveal a major role of

BIM in conducting apoptosis during negative selection. Conclusively, NUR77 and BIM have emerged as the few key players shown in several models to be critically involved in mediating cell death during thymic negative selection.

2. T-cell activation

2.1. TCR signaling

As described before, signaling through TCR in T-cells is critical for their commitment and fate during T-cell development. In mature T-cells, TCR activation is the primary trigger throughout their lifespan for carrying out physiological functions during the adaptive immune response. Activation of the TCR in mature T-cells through the recognition of foreign peptides associated with MHC-I and MHC-II complexes promotes intracellular signaling, resulting in cellular and molecular changes that lead to diverse outcomes. This includes cell survival, proliferation, clonal expansion, cytokine production, and eventually, the induction of its own death in a process denominated Activated Induced Cell Death (AICD).

2.2. TCR complex

The TCR complex is composed of a heterodimer of TCR chains, which result from the association of the four TCR variants expressed in humans (α , β , γ , δ), and couple either as α - β or γ - δ . The TCR chains consist of a cytoplasmic tail, a transmembrane region, and an extracellular region containing the constant and variable domains. While the variable domain is responsible for recognizing and binding antigens, the constant domain is the site where the TCR is associated with two CD3 signaling heterodimers (CD3 $\epsilon\delta$ and CD3 $\epsilon\gamma$) and one CD3 ζ homodimer intracellularly (**Fig. 3**). A critical feature of these signaling chains is their immunoreceptor tyrosine-based activation motifs (ITAM), whose tyrosine residues are phosphorylated upon TCR activation. As observed in the **Figure 3** the CD3 signaling chains contain per TCR complex a total of 10 ITAMs, which is a significantly higher number compared to its homologue in B-cells (BCR) that has only 2 ITAMs (Kurosaki, 2000). These motifs serve as cytoplasmic docking sites for

protein kinases, adaptors, and signaling mediators necessary to T-cell signaling propagation.

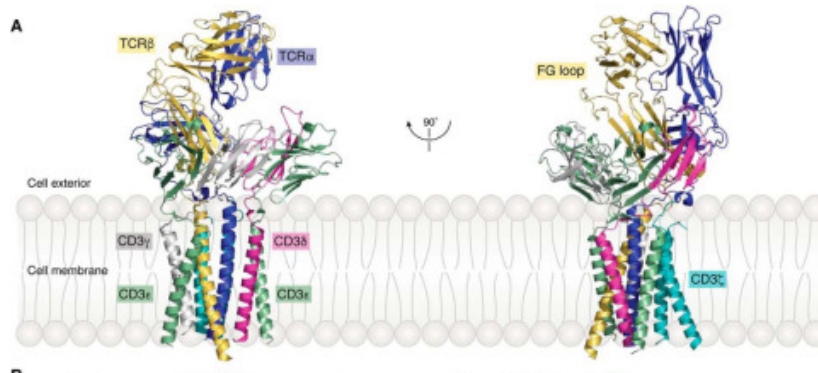


Figure 3. Structure of the TCR:CD3 complex. Schematic modified from (Mariuzza, Agnihotri and Orban, 2020)

2.3. TCR-associated cytoplasmic proteins and proximal signaling

The earliest phosphorylation event following TCR binding with pMHC is carried out by the lymphocyte-specific protein tyrosine kinase (LCK). LCK is a protein tyrosine kinase from the SRC family kinase (STK) that is expressed in T-cells in a closed conformational state (inactive). LCK is associated through its SH4 domain to the cytoplasmic tails of CD4 and CD8 co-receptors. Upon pMHC recognition LCK get activated through transphosphorylation by another LCK molecule. LCK phosphorylation at the Tyr³⁹⁴ residue promotes an open/active conformation that allows it to interact and phosphorylate CD3's ITAMs (Straus et al., 1996; Simeoni, 2017).

ITAM phosphorylation depends to a great extent, but not exclusively, on LCK. Indeed, while LCK-deficient mice showed only a partial impairment of TCR signaling, the Lck^{-/-} and Fyn^{-/-}, double knockout mice (Fyn is also a STK) showed entirely compromised TCR signaling, clearly exhibiting the redundant roles of LCK and FYN (Groves et al., 1996; Van Oers et al., 1996). The phosphorylated tyrosine residues of ITAMs create binding sites for Src Homology 2 (SH2)-domain holder proteins which include ZAP70, a Src family tyrosine kinase that can bind to the CD3 ζ

signaling chains, and the inducible T-cell kinase (ITK). Their recruitment makes them targets for LCK-mediated phosphorylation, ultimately leading to their activation. Targets of ZAP70 and ITK include the linker of activation of T-cells (LAT), which serves as the primary signaling scaffold downstream of the TCR (Balagopalan et al., 2010),(L. Berg et al., 2005).

LAT is a transmembrane protein that serve as main signaling scaffold downstream TCR. It has a short extracellular domain and an extensive cytoplasmic tail, harboring four tyrosine residues (Y132, Y171, Y191, and Y226), which are phosphorylated by ZAP70. Each of these residues recruits new molecules that can later be activated by proximal proteins in the hub, thereby creating a signalosome complex (Guy and Vignali, 2009). Tyrosine-to-phenylalanine substitution mutations in each residue uncovered that pY132 recruits PLC γ 1, while pY171, pY19, and pY226 promote the recruitment of the adaptor proteins growth-factor receptor-bound protein 2 (GRB2) and the GRB2-related adaptor downstream of Shc (GADS) to the LAT signalosome. Mutation of Tyr¹³² disrupts calcium flux and block of ERK, NFAT, PKC activation normally induced upon TCR activation (Zhang et al., 2000), showing that PLC γ 1 is an upstream modulator of these signaling effectors. Additional proteins are recruited to LAT signalosome through GRB2 and GADs, notably the adaptor protein SLP76 (SH2 domain containing leukocyte protein of 76kDa) that together with LAT built the core of the signalosome complex to propagate signaling (**Figure 4**). LAT deficient cell lines and LAT^{-/-} mice -but also conditional deletion of SLP-76 in mouse T-cells- result in TCR signaling disruption, both during thymic development and in mature peripheral T-cells, thus demonstrating the essential role of the signalosome formation to conduct TCR activation (Zhang et al., 1999; Wu et al., 2011).

Through GRB2, VAV1, a guanine nucleotide exchange factors (GEFs), is recruited to the signaling scaffold. VAV1 activates the Rho-family GTPases Rac1 and Cdc42 that are essential for conducting actin cytoskeletal rearrangements (Crespo et al., 1997; Labno et al., 2003). Consequently, VAV1-deficient- T cells

exhibit defects in calcium influx, inside-out activation of the integrin LFA-1, and cell polarization (Tybulewicz, 2005).

Depicted in general terms in the **Figure 4**, there is a diversity of signaling molecules that join the TCR complex, which signaling culminates in activation of further pathways, including Ca^{2+} -calcineurin-NFAT, PKC-IKK-NFkB, RasGRP1-Ras-ERK1/2, TSC1/2-mTOR. In this way, TCR signaling through these pathways induces global cellular changes, including the activation of transcriptional regulators and protein synthesis, cytoskeleton reorganization, and metabolism activation. All of these changes are crucial for naïve T-cells to undergo clonal expansion and differentiate into effector subsets, ultimately achieving T-cell physiological functions.

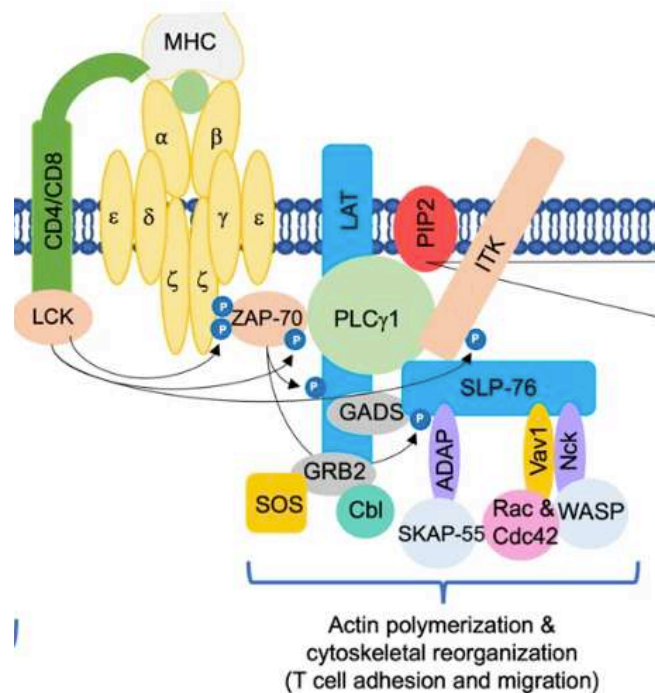


Figure 4. Proximal signaling events upon TCR-pMHC binding. TCR ligation with a peptide-MHC leads to the activation of LCK. LCK then phosphorylates the tyrosine residues of the ITAMs present in the CD3ζ chains. The two phosphorylated motifs specifically recruit a second tyrosine kinase, ZAP70. Once recruited to the membrane, ZAP70 is phosphorylated by LCK, resulting in its activation. ZAP70 then phosphorylates four tyrosine residues of LAT, which serve as scaffold of recruitment of more signaling proteins that can be phosphorylated and activated by ZAP70 and LCK. Activation of these multiple molecules ultimately to lead to diverse and distal

signaling pathways important for TCR-activation. Schematic modified from (Shah et al., 2021),(Love and Hayes, 2010).

2.4. Signaling pathways downstream TCR-activation

The antigen-mediated response in T-cells results in the induction of genes that contribute to their physiological functions. These genes can generally be classified based on the time it takes for their expression upon TCR:pMHC ligation. Immediate genes that are induced within minutes include *IKB α* , *TNF α* , *CD69*, *cJUN* (Chauhan et al., 1993),(Liu et al., 2016),(Gallagher et al., 2021). Early genes, which are induced hours after TCR activation, include cytokines such as *IL2*, *IL3*, *IL6*, and *IFNGR1*. The expression of these genes is regulated by distal signaling pathways that include predominantly Ca²⁺-calcineurin-NFAT, PKC-IKK-NF- κ B and RasGRP1-Ras-ERK1/2, illustrated in **Figure 5** and described in more details thereafter.

2.4.1. Ca²⁺-calcineurin-NFAT

PLC γ 1 is one of the principal signaling molecules downstream of the TCR that links proximal and distal TCR signaling. PLC γ 1 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to convert it into inositol-3-phosphate (IP₃) and diacylglycerol (DAG), which are important second messengers. Specifically, IP₃ promotes calcium-dependent activation of the calcineurin-NFAT pathway. In brief, IP₃ binds to calcium-permeable ion receptors on the endoplasmic reticulum, resulting in the release of ER Ca²⁺ stores into the cytoplasm. The increased Ca²⁺ concentration in the cytoplasm activates calcineurin, which is a phosphatase that dephosphorylates members of the nuclear factor of activated T-cells (NFAT). NFAT's dephosphorylation lead to its nuclear translocation and subsequent gene regulation of its target genes (Oh-hora and Rao, 2008). The NFAT family consists of five members, with NFAT1, NFAT2, and NFAT4 being co-expressed in T-cells and

activated upon TCR ligation. All NFAT members share significant similarities, including DNA-binding domains, which can result in redundancy in their gene regulatory functions (Lyakh, Ghosh and Rice, 1997). Target genes regulated by NFAT proteins are essential for T-cells to accomplish their functions. NFAT1 and NFAT2 have been shown to be critical for transcriptional induction of cytokines. Deficiency of NFAT1 and NFAT2 in mice leads to compromised production of several cytokines by T cells, including importantly interleukin (IL)-2 and the tumor necrosis factor α (TNF- α) but also interferon (IFN)- γ , IL-4, IL-5, IL-10 (Macian, 2005),(Shaw et al., 1988),(Macián, López-Rodríguez and Rao, 2001). NFAT can act either alone or together with other transcription factors complexes such as AP1 formed by c-JUN and c-FOS. Interestingly, individual gene regulation by NFAT without AP-1 promotes the expression of genes related to T-cell anergy or T-cell unresponsiveness, revealing NFAT members as regulators of the dual activation/anergy responses of T-cells (Heissmeyer et al., 2004).

2.4.2. RasGRP1-Ras-ERK1/2

Ras is a small GTPase located at the plasma membrane, in T cells, they are 2 guanine nucleotide exchange factors (GEFs) that can activate Ras: (i) RASGRP1 which is recruited and activated upon diacylglycerol (DAG) production in response to PLC γ 1 activation and SOS, a ubiquitously expressed GEF. Activated Ras initiates the RAS-MAPK signaling cascade by phosphorylating ser/threonine residues of the mitogen-activated protein kinase kinase kinase (MAPKKK) Raf1. In turn, Raf1 phosphorylates and activates the MAPK kinase (MAPKK) MEK1/2 that subsequently phosphorylate and activate the MAPK extracellular signal-regulated kinase-1 and 2 or ERK1/2(Shah et al., 2021),(Hwang et al., 2020). ERK activation results in the transcription of c-FOS and c-JUN, which can associate and form the AP-1 transcription factor. AP-1 regulates target inflammatory genes, including *IL2*, *TNFSF2* encoding for TNF- α , *IL1*, and *IFNG*. As mentioned before, many AP-1

target genes are being co-regulated with NFAT (Macián, López-Rodríguez and Rao, 2001),(Ye et al., 2014).

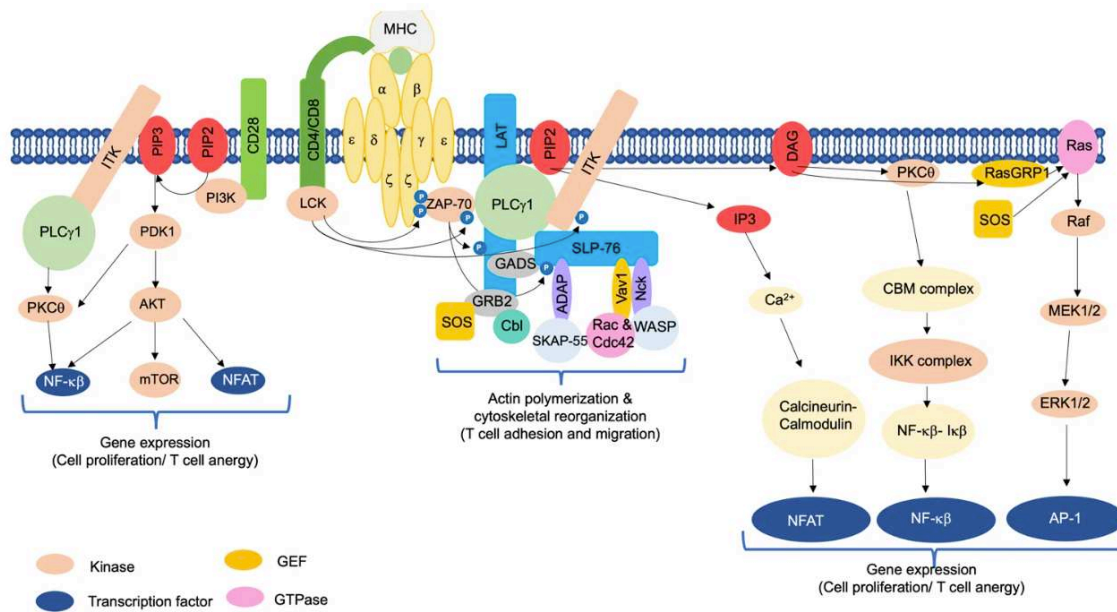


Figure 5. Propagation of TCR signaling. The proximal TCR signaling is amplified through the activation of various signaling pathways. The right side illustrates the Ca²⁺-calcineurin-NFAT, PKC-IKK-NF-κB, and RasGRP1-Ras-ERK1/2/PI3K pathways. On the left side, signaling pathways are depicted that are triggered upon CD28 co-stimulation. Schematic from (Shah et al., 2021)

2.4.3. PKC-IKK-NF-κB

There is a particular interest in this signaling pathway downstream TCR signaling that lead to the activation of nuclear factor-κB (NF-κB). NF-κB family is constituted by five members: c-REL, RELA/p65, RELB, NF-κB1/p50 and NF-κB2/p52. In absence of stimulatory signals these subunits are residing in the cytoplasm, sequestered by inhibitory proteins called inhibitory of nuclear factor kappa B (IκB). The activation of NF-κB is dependent on the degradation of IκB's and the translocation of NF-κB subunits into the nucleus (Hayden and Ghosh, 2014).

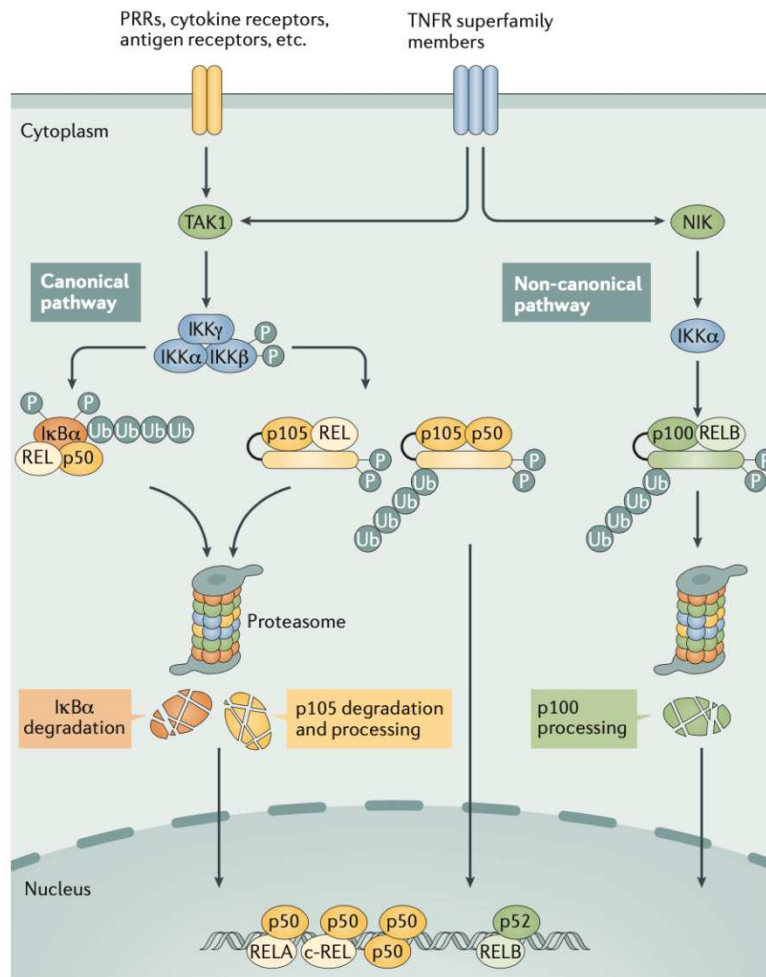
NF-κB activation occurs downstream of PLCγ1, which is activated immediately upon TCR:pMHC binding. This activation leads to an elevation in cytoplasmic concentrations of DAG, subsequently promoting the activation of

Protein kinase C theta (PKC θ). Upon activation, the caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1) is recruited to the plasma membrane. This recruitment allows its physical interaction with PKC θ and subsequent phosphorylation. Phosphorylated CARMA1 undergoes a conformational change, enabling its association with partner components such as B cell lymphoma/leukemia 10 (BCL10) and mucosa-associated lymphoid tissue translocation protein-1 (MALT1). The association of these three proteins forms the CBM complex, which serve as a molecular scaffold to connect IKK with its direct activators (Sun et al., 2004). NF- κ B can be activated by two main signaling pathways: the canonical and the non-canonical (illustrated in **Figure 6**). While the canonical pathway is directly induced by immune receptors like TCR, leading to rapid NF- κ B activation, the non-canonical pathway is typically induced by ligands of the tumor necrosis factor (TNF) family (Li et al., 2005; Sun, 2017). Given that TCR signaling and the canonical NF- κ B signaling itself (detailed later) induce the expression of a number of receptors and ligands of the TNF family (Pahl, 1999; Croft, 2009), the activation of the non-canonical NF- κ B signaling pathway is indirectly triggered. Of note, TNF receptors can also mediate NF- κ B activation via the canonical pathway.

The canonical pathway involves an IKK complex composed of three subunits: I κ B kinase (IKK) α , IKK β , and NEMO/IKK γ . IKK α and IKK β serve as catalytic kinase subunits, while NEMO/IKK γ functions as a structural and regulatory subunit. Recruitment of this IKK complex, via NEMO/IKK γ , to the CBM complex leads to TAK1-mediated phosphorylation of IKK β . Upon activation, IKK β phosphorylates I κ B α , resulting in its proteasomal degradation. Degradation of I κ B α releases NF- κ B heterodimer RELA/p65-NF- κ B1/p50, enabling its nuclear translocation and drive the expression of target genes (Zandi et al., 1997). It is important to note that the degradation of I κ B α , nuclear translocation of NF- κ B, and phosphorylation of RELA/p65 are indicative markers of NF- κ B activation (Trask, 2004).

The non-canonical pathway relies on NF- κ B inducing kinase (NIK), which activate the IKK α homodimer complex and bring it into close proximity to RelB-NF-

κ B2/p100. Upon activation, IKK α phosphorylates RELB-NF- κ B2/p100, leading to its partial degradation and subsequent release of the p52 fraction. The released p52 fraction is then translocated to the nucleus. Translocation of the NF- κ B subunits in



to the nucleus is critical for the transcriptional activation of specific NF- κ B-target genes.

Figure 6. NF- κ B signaling pathways: canonical and non-canonical. On the left the canonical pathway is initiated by numerous receptors, including TCR. The formation of CBM complex allows for the recruitment of TAK-1 that subsequently activates the IKK complex. IKK-mediated I κ B α phosphorylation lead to its degradation and thus nuclear translocation of NF- κ B heterodimer RELA/p65-NF- κ B1/p50. On the right non-canonical depends on NIK-mediated activation of IKK α . Activated IKK α promotes the partial degradation of RELB-NF- κ B/p52, resulting in its nuclear translocation. Schematic from (Sun, 2017).

There is a wide variety of NF- κ B target genes including cytokines and chemokines and their modulators, immunoreceptors, cell surface receptors, growth factors and their modulators, transcription factors and regulators of cell death detailed in **Table 1**.

Gene	Function
Cytokines/Chemokines	
CINC	Cytokine-induced neutrophil chemoattractant
*CXCL 11	Chemokine ligand for CXCR3
Eotaxin	β Chemokine, eosinophil-specific
Gro α - γ	Melanoma growth stimulating activity
IFN- γ	Interferon
IL-1 α	Interleukin-1 α
IL-1 β	Interleukin-1 β
IL-1-receptor antagonist	Inhibitor of IL-1 activity
IL-2	Interleukin-2
IL-6	Interleukin-6, inflammatory cytokine
IL-8	Interleukin-8, α -chemokine
*IL-9	Interleukin-9
IL-11	Interleukin-11
IL-12 (p40)	Interleukin-12
*IL-15	Interleukin-15
β -Interferon	Interferon
IP-10	α Chemokine
KC	α Chemokine
Lymphotoxin α	Anchors TNF to cell surface
Lymphotoxin β	Macrophage chemotactic protein, β Chemokine
MCP-1/JE	Macrophage inflammatory protein-1, β Chemokine
MIP-1 α , β	Macrophage inflammatory protein-1, β Chemokine
MIP-2	Macrophage inflammatory protein-1, β Chemokine
RANTES	Regulated upon Activation Normal T lymphocyte Expressed and Secreted, β Chemokine
TCA3, T-cell activation gene 3	T-cell activation gene 3, β Chemokine
TNF α	Tumor necrosis factor α
TNF β	Tumor necrosis factor β
Immunoreceptors	
B7.1 (CD80)	Co-stimulation of T cells via CD28 binding
BRL-1	B-cell homing receptor
CCR5	Chemokine receptor
CD48	Antigen of stimulated lymphocytes
F ϵ epsilon receptor II (CD23)	Receptor for IgE
IL-2 receptor α -chain	IL-2 receptor subunit
Immunoglobulin C γ 1	IgG heavy chain
Immunoglobulin ϵ heavy chain	IgE heavy chain
Immunoglobulin κ light chain	Antibody light chain
Invariant Chain I μ	Antigen presentation
MHC class I (H-2K ^b)	Mouse histocompatibility antigen
MHC Class I HLA-B7	Mouse histocompatibility antigen
β 2 Microglobulin	Binds MHC class I
T-cell receptor β chain	T-cell receptor subunit
*TNF-Receptor, p75/80	High-affinity TNF receptor
Regulators of apoptosis	
Bfl1/A1	Pro-survival Bcl-2 homologue
Bcl-xL	Pro-survival Bcl-2 homologue
Nr13	Pro-survival Bcl-2 homologue
cCD95 (Fas)	Pro-apoptotic receptor
Fas-Ligand	Inducer of apoptosis
IAPs	Inhibitors of Apoptosis
IEX-1L	Immediate early gene
Growth factors	
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
*IGFBP-1	Insulin-like growth factor binding protein-1
IGFBP-2	insulin-like growth factor binding protein-2
M-CSF (CSF-1)	Macrophage Colony Stimulating Factor
PDGF B chain	Platelet-Derived Growth Factor
Proenkephalin	Hormone
*Thrombospondin	Matrix glycoprotein t
VEGF C	Vascular Endothelial Growth Factor
Transcription factors	
A20	TNF-inducible zinc finger
c-myb	Proto-oncogene
c-myc	Proto-oncogene
c-rel	Proto-oncogene
IRF-1	Interferon regulatory factor-1
IRF-2	Interferon regulatory factor-2
I κ B α	Inhibitor of Rel/NF- κ B
junB	Proto-oncogene
nfk β 2	NF- κ B p100 precursor
nfk β 1	NF- κ B p105 precursor
p53	Tumor suppressor
Cell surface receptors	
A1 adenosine receptor	Pleiotropic physiological effects
Bradykinin B1-Receptor	Pleiotropic physiological effects
*CD23	Cell-surface molecule
CD69	Lectin mainly on activated T cells
Ga11 Receptor	Galanine receptor, neuroendocrine peptide
Lox-1	Receptor for Oxidized low density lipoprotein
Mdr1	Multiple drug resistance mediator (P-glycoprotein)
Neuropeptide Y Y1-receptor	Pleiotropic physiological effects
PAF receptor 1	Platelet activator receptor
RAGE- receptor for advanced glycation end products	Receptor for Advanced Glycation End products

Table 1. Target genes of NF- κ B. Modified from (Pahl, 1999)

2.5. TCR co-stimulation

Besides TCR:pMHC recognition, there are other elements that play a role in maintaining and propagating TCR signaling. One of these elements is the presence of coreceptors, which their assistance leads to a complete TCR activation. In this section it will be described some important TCR coreceptors, their recruitment, and their participation in maintaining, enhancing, or propagating TCR signaling.

A coreceptor is a receptor that participates in ligand-receptor binding or enhances downstream signaling of other receptors. Immunoglobulins such as CD4, CD8, CD28, and members of the TNF family like 4-1BB and OX40 are considered TCR coreceptors.

2.5.1. Immediate co-receptor: CD4 and CD8

CD4 and CD8 are immediate co-receptors that assist in the initial TCR:pMHC binding. They contain three main segments: an extracellular region with immunoglobulin-like domains, a transmembrane region, and a cytoplasmic tail that is the anchor site for LCK recruitment. The CD4 receptor is a monomer with four immunoglobulin (Ig)-like domains in its extracellular region, ranging from the outermost D1 to the innermost D4 domain attached to the plasma membrane. The D1 domain specifically binds to the conserved $\beta 2$ domain of pMHC-II. On the other hand, CD8 is a dimer primarily composed of an α -chain and a β -chain, with each chain containing one Ig-like domain that directly interacts with the conserved $\alpha 3$ domain of pMHC-I (**Figure 4**). The interaction between CD4:MHC-II and CD8:MHC-I leads to the recognition of MHC complexes and stabilizes TCR:pMHC binding, thus highlighting CD4 and CD8 co-receptors as facilitators of TCR signaling initiation (Rudd, 2021).

2.5.2. CD28

Even though there are multiple receptors that can act as co-stimulators along with the TCR, CD28 is one of the most robust and well-studied coreceptor, significantly influencing downstream TCR signaling. CD28 is a transmembrane immunoglobulin homodimer primarily expressed in T-cells. It has two ligands, CD80 (B7.1) and CD86 (B7.2), which are expressed on various cell types, including APC cells (Hathcock et al., 1994). When CD28 binds to its ligands upon TCR:pMHC engagement, it amplifies TCR signaling, leading to enhanced T-cell responses such as proliferation, survival, and cytokine production.

CD28 possesses a cytoplasmic tail that bears a pYMNM motif, which recruits PI3K. PI3K promotes the local generation of PIP₃, which in turn acts as a docking site for the kinase PDK1. PDK1 then recruits its target AKT, leading to the activation of mTOR, NF-κB, and NFAT signaling pathways (**Figure 5**). However, it is important to note that these signaling pathways can also be activated by TCR engagement alone, indicating that CD28 co-stimulation enhances the strength of TCR signaling rather than providing distinct functionalities to the signal (Acuto and Michel, 2003).

Despite *Cd28*^{-/-} mice exhibiting normal T-cell development, they demonstrate selective defects in T-cell activation. For instance, activation and proliferation of T-cells derived from *Cd28*^{-/-} mice are significantly decreased compared to wildtype cells when exposed to the T-cell mitogen that induces TCR activation (concanavalin A). However, when these cells are activated via PKC with phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore Ionomycin, their activation and proliferation remain unaffected despite the absence of CD28 (Smith-Garvin, Koretzky and Jordan, 2009). This indicates that CD28 co-activation impacts proximal TCR signaling.

2.5.3. Co-stimulatory receptors: members of the TNFR super family

There are several receptors belonging to the TNFR-Super Family (TNFRSF) that are expressed in T-cells and have been documented to function as TCR coreceptors. These include OX40, 4-1BB, HVEM, CD30, GITR, TNFR2, and DR3 (Ward-Kavanagh et al., 2016). (Figure 7). For the specific focus of this thesis, the attention will be directed towards the description of 4-1BB, OX-40 and TNFR2.

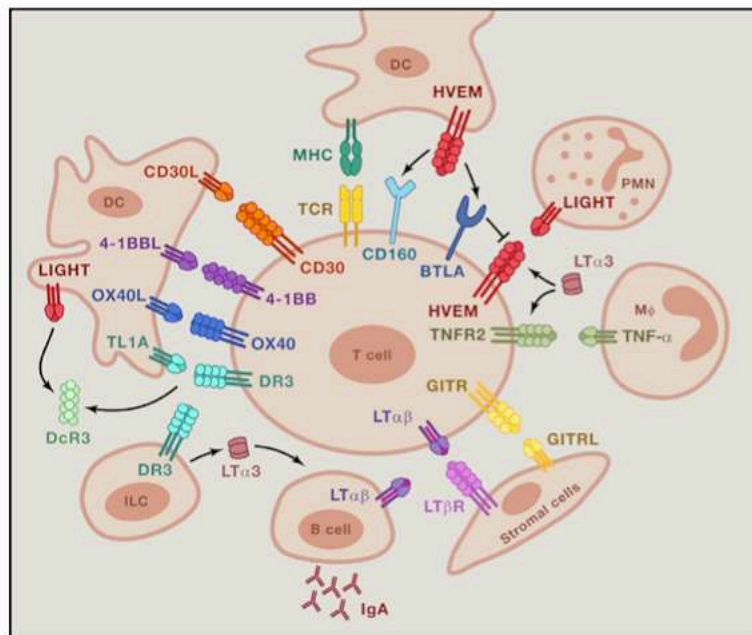


Figure 7. TNFRSF members expressed in activated T-cells. Activated T lymphocytes express a variety of co-signaling members of the TNFRSF. Their specific ligands from the TNFRSF are expressed in professional antigen-presenting cells (APCs), neutrophils, macrophages, or stromal cells. Schematic from (Ward-Kavanagh et al., 2016).

2.5.3.1. 4-1BB and OX40

4-1BB (CD137, TNFRSF9) and OX40 (CD134, TNFRSF4) are found induced at the plasma membrane upon TCR activation. Studies of these receptors in T-cells show that they can play a role in expansion, survival and enhanced effector functions through the induction of cytokines (Oh *et al.*, 2015). T-cells from 4-1BB^{-/-} (Kwon *et*

al., 2002) and *OX-40* *-/-* (Kopf *et al.*, 1999). mice show decreased T-cell functions, including cytokine production and reduced proliferation. Their ligands 4-1BBL and OX-40L respectively, are mostly expressed in immune cells that serve as APC including B-cells, macrophages and dendritic cells (DeBenedette *et al.*, 1997). The engagement with their ligands enables OX40 and 4-1BB activation that leads to induction of NF- κ B, AKT, p38 MAPK, and ERK signaling pathways (Mbanwi and Watts, 2014). Studies on the mechanisms behind the TNFR family members co-stimulation to the TCR signaling have proposed that the binding of TNFR with its ligands results in the formation of a signaling scaffold, facilitated by the adapter protein TNFR-associated factor (TRAF). This, in turn, leads to the recruitment and accumulation of PI3K and Akt in close proximity to the TCR signalosome (So and Croft, 2013). Ultimately, the activation of these signaling pathways upon 4-1BB and OX-40 co-activation promote the expression of survival genes encoding survivin, Bcl-2 and Bcl-XL, and decrease the expression of pro-apoptotic BIM (Croft *et al.*, 2009).

Through their co-stimulatory signaling 4-1BB and OX-40 prolong and sustain the immune response, which is essential during development of memory T-cell (Watts, 2005). Remarkably, much of what is known about these co-signaling receptors is attributed to research conducted in the field of oncology, where they are utilized as long-term enhancers of anti-tumor immunity of T-cells, in part due to their ability to rescue T-cells from anergy. As a consequence, agonistic antibodies targeting 4-1BB and OX40 have been developed in order to enhance/prolong the activity in endogenous T-cells (Bartkowiak and Curran, 2015), while engineered chimeric antigen receptor (CAR) T-cells (CAR-T) co-expressing 4-1BB or OX-40 receptors improve CAR-T efficiency (van der Stegen, Hamieh and Sadelain, 2015; Mascarelli *et al.*, 2021). Interestingly, various studies have observed that the administration of agonistic antibodies targeting 4-1BB (α -4-1BB ab) can lead to the deletion of over-stimulated pathogenic/autoreactive T-cells through activation-induced cell death (AICD). These studies comprise pathogenic models, such as chronic graft-versus-

host disease (cGVHD), as well as autoimmune diseases like systemic lupus erythematosus (SLE) (Sun et al., 2002; Foell et al., 2003; Kim et al., 2005).

2.5.3.2. TNFR2

Unlike TNFR1, which is expressed ubiquitously, TNFR2 (*TNFRSF1B*) is primarily expressed in immune cells, including T-cells. TNFR2 expression is induced upon TCR activation, however it can only exert co-stimulatory signals upon binding with its ligands TNF α and LT α , which are shared with TNFR1. Studies investigating TNFR2 co-stimulation in T-cells are complex in part due to the shared ligands with TNFR1. Additionally, TNF α can exist in a membrane-bound form (mTNF α) or a soluble form (sTNF α), and the biological effects upon binding to its receptors can vary depending on its physical state (Grell et al., 1995). Furthermore, numerous reports investigating the role of TNFR2 co-stimulation in T-cells have revealed that the outcomes are specific to T-cell subsets and cellular state. In this context, two contrasting outcomes of TNFR2 co-stimulation in T-cells will be described thereafter.

Upon the primary response in naïve T-cells (mature T-cells that have never encountered antigens), TNFR2 co-stimulation in both CD4 $^{+}$ and CD8 $^{+}$ T-cells is found to promote clonal expansion and survival, thereby allowing a larger accumulation of effector cells. In naïve CD8 $^{+}$ T-cells, TNFR2 depletion results in a loss of protection against apoptosis, which is correlated with a decreased expression of survivin and Bcl-2 (Kim *et al.*, 2006).

Mechanistically TNFR2 co-activation of the TCR signaling relies in the TNFR2-induced activation of the NF- κ B signaling pathway. TNFR2 does not possess Death Domains (DD) in its cytoplasmic region, as other members of the TNFR super family. Therefore, it relies on the recruitment of adaptor proteins like TRAF to form intracellular signaling complexes. The binding of TRAF proteins facilitates the recruitment of the ubiquitin ligases inhibitor of apoptosis 1 (IAP1) and inhibitor of apoptosis 2 (IAP2). IAP-dependent polyubiquitination of various proteins recruited to this complex, leads to canonical activation of NF- κ B. Moreover, several studies have

shown that TNFR2 activation can promote non-canonical NF- κ B activation. In unstimulated cells, NIK is kept inactive through K48-ubiquitination by IAP1 and IAP2, leading to its degradation. However, upon TNFR2 binding with its ligand, the recruitment of IAP proteins to TRAF adaptors prevents NIK's constitutive degradation, allowing its accumulation and subsequent activation (Varfolomeev et al., 2007; Hayden and Ghosh, 2008). This ultimately leads as described earlier to non-canonical NF- κ B activation (**Figure 8**). Ultimately, NF- κ B activation pathway lead to T-cell proliferation, survival, and lineage stability during T-cell primary response (Kim et al., 2006b; Chen et al., 2013)

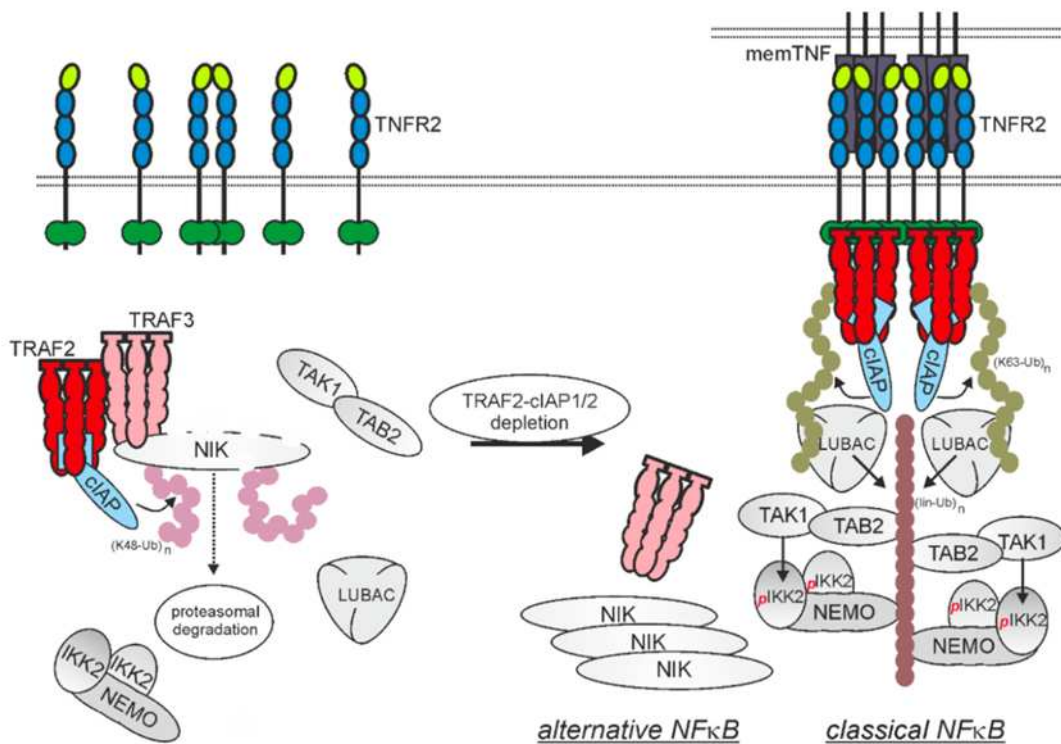


Figure 8. TNFR2 co-stimulation in naive T-cells. In unstimulated conditions (left), where the TNF ligand is absent, the TRAF3-NIK complex interacts with the TRAF2-cIAP1/2 complex in the cytoplasm. This interaction results in the cIAP1/2-mediated ubiquitination of NIK, maintaining NIK at basal levels through proteasomal degradation. In stimulated conditions (right) where TNF engages with TNFR2, the TRAF2-cIAP1/2 complex is recruited to the plasma membrane, forming the signaling complex that facilitates canonical NF- κ B activation. Concurrently, the disruption of the TRAF2-cIAP1/2-TRAF3-NIK complex hampers NIK degradation, leading to the

accumulation of NIK in the cytoplasm. NIK accumulation can ultimately promote the activation of non-canonical NF- κ B signaling. Schematic from (Medler, Kucka and Wajant, 2022).

Conversely, TNFR2's implication in activated T cells is primarily associated with cell death induction. The expression of TNFR2 in activated CD8⁺ T-cells has been linked to the contraction of this T-cell subset, resulting in decreased immune responses to viral and tumor challenges (Bertrand et al., 2015; DeBerge et al., 2015). Additionally, activated TNFR2^{-/-} T-cells exhibit resistance to TNF-induced cell death compared to TNFR2 wildtype cells (Yang, 2023). These observations categorized the function of TNFR2 as a co-activator of TCR signaling, particularly in naive T-cells.

2.6. TCR signaling regulation

TCR signaling begins with the rapid recruitment of protein tyrosine kinases (PTKs) to the plasma membrane, followed by their phosphorylation, which, in turn, propagates the signaling into downstream pathways. Given the diverse range of cellular responses that result from TCR signaling in T-cells, it is not surprising that the process is tightly regulated at different stages. This section will describe several regulatory mechanisms of TCR signaling initiation, including (i) inactivation of proximal signaling components, (ii) degradation of some components of proximal signaling through ubiquitination events, and (iii) PD1, a TCR co-inhibitory receptor.

2.6.1. Inactivation of proximal signaling proteins

LCK, FYN, and ZAP70 are crucial triggers for the initiation of TCR signaling. Consequently, inhibition of their activities leads to impairment of TCR signaling. The principal regulatory mechanisms involve phosphorylation and dephosphorylation at specific sites, which result in their inactivation. For instance, LCK and FYN get inactivated when phosphorylated at the conserved C-terminal regulatory tyrosine

residue. Specifically, phosphorylation of the Tyr505 residue in LCK and the Tyr528 residue in Fyn induces conformational changes that impair their physical interaction with CD4/CD8 and ZAP70 (Mustelin and Taskén, 2003). The C-terminal Src kinase (CSK) is the only protein found to have the specificity to phosphorylate LCK and FYN at these residues. Accordingly, studies conducted in T-cells overexpressing CSK have demonstrated a significant reduction in TCR-induced tyrosine phosphorylation and IL-2 production (Chow et al., 1993).

In contrast, TCR regulation can also be driven by dephosphorylation, which is carried out by protein tyrosine phosphatases (PTPs). PTPs are abundantly implicated in the regulation of TCR signaling. The human genome expresses 107 different PTPs, out of which 60 are expressed in T-cells, and many of them have been demonstrated to play a role downstream of TCR signaling (Alonso *et al.*, 2004). Selected PTPs extensively documented for their pivotal role in the regulation of TCR signaling are described below.

PTPN22 is a protein tyrosine phosphatase that in effector T-cells exerts robust inhibition of proximal TCR signaling (Yang *et al.*, 2020). PTPN22 upregulation in cell lines has shown to have a potent negative regulatory role in TCR signaling (Stanford, Rapini and Bottini, 2012). Through the use of PTPN22 "substrate-trapping" mutants to isolate their respective substrates and analyze them by mass spectrometry, PTPN22 has been found to dephosphorylate LCK at the Y394 residue, ZAP-70 at the Y493 residue, as well as the CD3 ζ and CD3 ϵ (Wu *et al.*, 2006; Shah *et al.*, 2021). Other phosphatases considered negative regulators of TCR signaling include SH2 domain-containing phosphatase 1 (SHP-1) and SH2 domain-containing phosphatase 2 (SHP-2). Studies on SHP-1 null mice (me/me) have shown that T-cell of these mice exhibit an increase in IL-2 production (Lorenz *et al.*, 1996). Consistent with these findings, SHP-1 in human T-cells has been found to indirectly mediate the dephosphorylation of LCK, FYN, and ZAP70 (Lorenz, 2009). Further evidence shows that SHP-1 and ZAP70 co-immunoprecipitate together, and the phosphatase activity of SHP-1 has been shown to decrease ZAP70 phosphorylation (Plas *et al.*, 1996). Moreover, mice conditionally deleted for SHP-2 in T-lymphocytes

exhibited widespread inflammation and autoimmunity. This was associated with increased serum levels of proinflammatory cytokines and infiltration of T cells into non-lymphoid tissues, thereby demonstrating the critical role of SHP-2 in negative regulation of T-cell activation. Furthermore, under inhibitory conditions SHP-2 has been found to dephosphorylate the inducible T cell kinase or ITK, resulting in the inhibition of cytokine secretion in T-cells. Interactome mapping of SHP-2 interactions has shown that other SHP-2 target phosphotyrosine sites are in CD3 ζ chain and ZAP70 (Strazza *et al.*, 2021). Although only most representative examples were described, to date, 15 PTPs have been documented to inhibit PTK involved in proximal TCR signaling, in this regard, PTPs are considered major inhibitory regulators of TCR signaling that impact activation thresholds and can fine-tune the intensity and duration of the TCR signal.

2.6.2. Ubiquitination

The ubiquitination of proteins by ubiquitin ligases can target them for degradation via the proteasome, providing an effective mechanism to inhibit a signaling pathway. The two E3 ubiquitin ligases, c-CBL and CBL-b, possess various protein-protein interaction domains, including the tyrosine kinase binding (TKB) domain and the really interesting new gene (RING) finger domain. These domains are responsible for their E3 ubiquitin ligase activity and have been shown to be implicated in downregulating TCR signaling. Studies on T-cells from mice with ablated expression of c-Cbl proteins (*c-Cbl*^{-/-}), Cbl-b (*Cbl-b*^{-/-}), or both (double knockouts) found that CBL proteins act as negative regulators of TCR activation by promoting TCR internalization upon engagement with a ligand (Naramura *et al.*, 2002). Consistently, *c-Cbl*^{-/-} and *Cbl-b*^{-/-} mice phenotype include hyperactive TCR signaling accompanied by elevated levels of LCK and FYN; and increased phosphorylation of ZAP70 (Thien and Langdon, 2005). Moreover, *in vitro* studies demonstrate that the CD3 ζ chain in T-cells are target of the ubiquitin ligase activity of the CBL proteins (Wang *et al.*, 2001).

2.7. PD1: a negative regulator of TCR

Programmed Cell Death protein-1 (PD1 or CD279) is a protein mainly expressed in activated T-cells, but it can also be expressed in several cell types of the innate and adaptive immune system (Francisco, Sage and Sharpe, 2010; Simon and Labarriere, 2018). Historically, PD1 was discovered in 1992 as a gene associated with cell death (Ishida *et al.*, 1992). However, it was later identified that PD1 expression was induced by TCR or BCR activation in lymphocytes (Agata *et al.*, 1996). In 2000, the discovery of PD1 ligands unveiled PD1's function as a brake for the adaptive immune response (Freeman *et al.*, 2000). Furthermore, in 2001, it was observed that cancer cells can express PD1 ligands and similarly promote inhibition of T-cell activation (Latchman *et al.*, 2001). This discovery revolutionized cancer therapy leading to subsequent decades of targeting the interplay between PD1 and its ligands using antagonistic antibodies to restore T-cell responses in cancer patients as an antitumor strategy. In this section will be described i) findings that demonstrates the major role of PD-1 as a negative regulator of TCR signaling and ii) the mechanisms by which PD1 can exert these negative regulatory effects in the proximal TCR signaling.

2.7.1. Inhibitory effect of PD1 in T-cells

Early observations indicating that PD-1 is involved in the inhibition of immune responses include the phenotype of *Pd-1* *-/-* mice, which, depending on the genetic background, develop different autoimmune diseases (Nishimura *et al.*, 1999; Wang *et al.*, 2005). Moreover, assessments of T-cell functionality have shown that PD1 strongly suppresses the production of cytokines, including IL-2 and IFN- γ (Mizuno *et al.*, 2019). Notably, the blockade of PD-1 by genetic deletions or by the use of antagonistic anti-PD1 antibodies shows that blocking the PD1 pathway can improve T-cell functions and even overcome T-cell exhaustion as shown by the

reestablishment of T-cell activation, proliferation, and cytokine production (Barber *et al.*, 2006). For instance in case of tumor infiltrating T-cells (TILs), that exert cytotoxic effects towards cancer cells, blockage of the PD1-PDL1/2 axis has been reported to induce re-activation of T-cells and thus tumor burden decreased in numerous cancer types (Pauken and Wherry, 2015). This includes melanoma which was the first cancer to be studied with such approach and the first cancer type to be approved to treat patients with an anti-PD1 antagonistic antibody (Robert *et al.*, 2015; Gellrich *et al.*, 2020).

2.7.2. Mechanisms of PD1 inhibitory effect in proximal TCR signaling

PD1 is part of the extended CD28 family of T cell regulators. The structure of PD1 is formed by an immunoglobulin extracellular domain, a transmembrane region, and an intracellular domain harboring two immunoreceptor tyrosine-based inhibitory motifs (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). PD1 has two ligands, PD-L1 (CD274) and PD-L2 (PDCD1L2), which are members of the B7 family (the same family as the CD28 ligands). While PD-L1 is widely expressed in both hematopoietic and non-hematopoietic cells, PD-L2 expression is predominantly restricted to DCs, macrophages, and B cell populations (Sharpe and Pauken, 2018). The most common activation of PD1 in T-cells occurs upon its binding in trans, where it interacts with PD-L1 expressed on another cell, often an antigen-presenting cell (APC). Upon ligation, the intracellular ITIM and ITSM motifs of PD1 undergo phosphorylation. While it has been previously demonstrated that phosphorylated ITIM and ITSM can bind to the phosphatases SHP-1 and SHP2 (Chemnitz *et al.*, 2004), recent studies have shown that the phosphorylation of ITSM at the Y248 residue is the only essential site for PD-1's inhibitory activity. The kinases LCK and FYN are responsible for carrying out this phosphorylation (Patsoukis *et al.*, 2020). Recruitment of SHP-2 at this Y248 via its SH2 domain, leads to the dephosphorylation and inactivation of the TCR proximal signaling proteins. More recent research suggests that PD1 also inhibits the formation of the trimeric protein

complex (pMHC/TCR/CD8), resulting in reduced LCK-mediated TCR signaling. This highlights a second mechanism by which PD1 is capable of inhibiting TCR signaling (Li *et al.*, 2021).

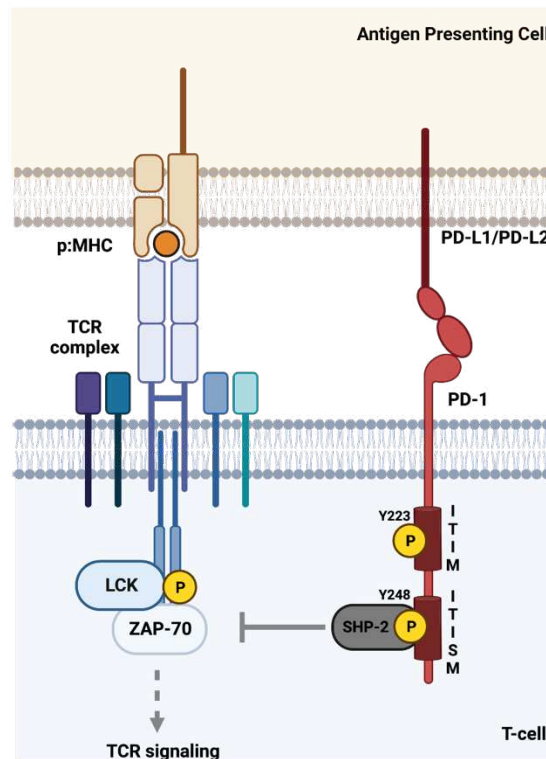


Figure 9. Mechanisms of PD1 regulation in proximal TCR signaling. The co-inhibitor PD-1 upon ligation with its ligands PDL-1/PDL-2 promote the recruitment of the protein tyrosine phosphatase SHP-2. SHP-2 negative regulation acts by dephosphorylating kinases and inhibiting proximal TCR signal transduction.

3. T-cell lymphoblastic leukemia

3.1. Generalities

Acute Lymphoblastic Leukemia (ALL) is an aggressive hematologic malignancy that results from a stepwise transformation process based on the acquisition and accumulation of genetic alterations. These alterations include changes in chromosome number (aneuploidy), chromosomal rearrangements leading to gene expression deregulation, deletions or gains of DNA sequences, and DNA mutations. The combination of a series of these alterations affects cell growth, proliferation, survival, and differentiation, leading to cellular transformation and arrest at distinctive stages of lymphoid progenitor differentiation. Ultimately, the accumulation of these alterations results in leukemia development. Leukemic cells gather and infiltrate different tissues including blood, bone marrow, thymus, spleen, liver, and lymph nodes. Consequently, ALL patients present high white blood counts in the blood and bone marrow and experience symptoms such as bleeding caused by thrombocytopenia, anemia, fatigue, and pallor, as well as infections caused by neutropenia (Hunger and Mullighan, 2015).

ALL is the most common cancer in children represents approximately 26% of cancer diagnoses among children younger than 15 years (Ward *et al.*, 2014). ALL comprises B-cell Acute Lymphoblastic Leukemia (B-ALL) that originates from B-cell lineage precursors and is the most common ALL subtype, followed by T-cell Acute Lymphoblastic Leukemia (T-ALL), which is characterized by proliferation of immature T-cell lymphoblast. T-ALL incidence varies with demographic characteristics. According to epidemiological studies in the United States, T-ALL is more common in young patients compared to adults and the elderly. Additionally, T-ALL is more prevalent in males, accounting for 67.3% of cases, compared to 32.7% in females. In terms of race, white individuals are more affected, comprising 74.6% of cases, while black individuals account for 14.9% (Guru Murthy *et al.*, 2019). T-ALL is a highly heterogeneous disease, with T-ALL blasts exhibiting phenotypic and

genetic diversity. Given this, T-ALL have been classified according to different criteria including i) phenotypic features, ii) ectopic expression of transcription factors and iii) gene expression profiles.

3.2. Classification according to phenotype

Immunophenotypes of T-ALL blast include the variable expression of CD1a, CD3, CD4, CD5, CD7 and CD8. Based on immunophenotyping and the physiological intrathymic differentiation, the WHO classification subdivides T-ALL into four categories from the most immature to the most mature: pro-T, pre-T, cortical T, and medullary T (Sin and Man, 2021) detailed in **Table 2**.

	cCD3	CD7	CD28	CD1a	CD34	CD4	CD8
Pro-T	+	+	–	–	+/–	–	–
Pre-T	+	+	+	–	+/–	–	–
Cortical T	+	+	+	+	–	+	+
Mature T* (medullary)	+	+	+	–	–	+/–	+/–

cCD3, cytoplasmic CD3.

*Also surface (membrane) CD3⁺.

Table 2. Immunologic classification in T-ALL. (Litzow and Ferrando, 2015)

3.3. Classification according to ectopic expression of transcription factors

A hallmark of T-ALL pathogenesis is the ectopic expression of specific transcription factors (TF), which often results from chromosomal translocation placing genes encoding TF under the control of strong enhancers. These include T-cell specific enhancers located in the TCR alpha and delta loci at 14q11, the TCR beta locus at 7q35, or the TCR gamma locus at 7p14-15 (Armstrong and Look,

2005). Depicted in **Table 3** are the major transcription factors, their partner genes, their locations, and the consequences of their rearrangement.

Chromosomal rearrangement or mutation	Partner gene 1 (oncogene)	Chromosome location	Partner gene 2	Chromosome location	Consequence of the rearrangement/mutation
Major transcription factors					
del(1)(p32p32)	<i>TAL1</i>	1p32	<i>SIL</i>	1p32	Ectopic <i>TAL1</i> expression driven by <i>SIL</i> promoter
t(1;14)(p32;q11)			<i>TCRα</i>	14q11	Ectopic <i>TAL1</i> expression driven by TCR enhancer
t(1;14)(p32;q11)			<i>TCRβ</i>	14q11	Ectopic <i>TAL1</i> expression driven by TCR enhancer
Small insertion			—	—	Ectopic <i>TAL1</i> expression driven by de novo enhancer
t(10;14)(q24;q11)	<i>TLX1</i>	10q24	<i>TCRα</i>	14q11	Ectopic <i>TLX1</i> expression driven by TCR enhancer
t(7;10)(q34;q24)			<i>TCRβ</i>	7q34	Ectopic <i>TLX1</i> expression driven by TCR enhancer
t(10;14)(q24;q11)			<i>TCRδ</i>	14q11	Ectopic <i>TLX1</i> expression driven by TCR enhancer
t(5;14)(q35;q11)	<i>TLX3</i>	5q35	<i>TCRδ</i>	14q11	Ectopic <i>TLX3</i> expression driven by TCR enhancer
t(5;14)(q35;q32)			<i>BCL11B</i>	14q32	Ectopic <i>TLX3</i> expression driven by <i>BCL11B</i>
inv(7)(p15;q34)	<i>HOXA9/HOXA10</i>	7p15	<i>TCRβ</i>	7q34	Ectopic expression of <i>HOXA</i> genes, predominantly <i>HOXA9</i> and <i>HOXA10</i> , driven by TCR enhancer
t(10;11)(p13;q14)	<i>PICALM (CALM)</i>	11q14	<i>MLLT10 (AF10)</i>	10p13	<i>PICALM-MLLT10</i> fusion transcript, resulting in upregulation of <i>HOXA</i> genes
del(9)(q34;q34)	<i>SET</i>	9q34	<i>NUP214</i>	9q34	<i>SET-NUP214</i> fusion transcript, resulting in upregulation of <i>HOXA</i> genes
inv(14)(q11;q13)	<i>NKX2-1</i>	14q13	<i>TCRα</i>	14q11	Ectopic <i>NKX2-1</i> expression driven by TCR enhancer
T(14;20)(q11;p11)	<i>NKX2-2</i>	20p11	<i>TCRδ</i>	14q11	Ectopic <i>NKX2-2</i> expression driven by TCR enhancer
t(11;14)(p15;q11)	<i>LMO1</i>	11p15	<i>TCRα</i>	14q11	Ectopic <i>LMO1</i> expression, mostly together with <i>LYL1</i> or <i>TAL1</i> expression
t(11;14)(p13;q11)	<i>LMO2</i>	11p13	<i>TCRα</i>	14q11	Ectopic <i>LMO2</i> expression, mostly together with <i>LYL1</i> or <i>TAL1</i> expression
del(5)(q14;q14)	<i>MEF2C</i>	5q14	—	—	Small deletions close to <i>MEF2C</i> leading to upregulation of <i>MEF2C</i> expression
t(7;19)(q34;p13)	<i>LYL1</i>	19p13	<i>TCRβ</i>	7q34	Ectopic expression of <i>LYL1</i> driven by TCR enhancer
t(11;14)(p11;q32)	<i>SPI1</i>	11p11	<i>BCL11B</i>	14q32	Ectopic expression of <i>SPI1</i> driven by <i>BCL11B</i>

Table 3. Chromosomal rearrangements resulting in ectopic expression of transcription factors. Modified from (Girardi *et al.*, 2017).

Major transcription factors ectopically expressed in T-ALL include *TAL1* and *LYL1*, which can be concurrently expressed with *LMO1/LMO2*. However, other transcription factors, such as *TLX1*, *TLX3*, *HOXA9/10*, *LMO2*, or *NKX2-1*, show mutually exclusive expression (Girardi *et al.*, 2017). The most frequent oncogenic TF in T-ALL is *TAL1* that is found in 30% of pediatric cases and 34% of adult cases. *TAL1* encodes a serine phosphoprotein containing a DNA binding domain called basic helix-loop-helix (bHLH), which binds specifically to the E-box DNA motif. Physiologically, *TAL1* is an important regulator of hematopoietic stem development and is normally expressed in hematopoietic progenitors (Aifantis, Raetz and Buonamici, 2008). However, *TAL1* expression is absent in differentiating T and B lymphocytes (Porcher, Chagraoui and Kristiansen, 2017). In T-ALL, *TAL1* is often

associated with a 90 to 100 kbp deletion that place *TAL-1* coding region under the control of *SIL* promoter resulting in the *SIL/TAL1* fusion product that lead to *TAL-1* overexpression (Brown *et al.*, 1990). In a smaller subset of cases (~3%), *TAL1* aberrant overexpression results from a chromosomal translocation to juxtaposes the promoter region of *TCRA/D* to the *TAL-1* (Bash *et al.*, 1995). Transgenic mouse models in which *TAL1* overexpression is induced specifically in lymphoid tissues result in clonal T-ALL development and reduced mice survival, thus demonstrating the *TAL1* oncogenic activity (Condorelli *et al.*, 1996; Kelliher, Seldin and Leder, 1996).

Other TFs often overexpressed in T-ALL include LMO1 and LMO2 which are members of the LIM-domain-only (LMO) family (Girardi *et al.*, 2017). LMO proteins are characterized by the presence of two cysteine-rich LIM domains, which are thought to be responsible for their DNA interaction due to their structural similarity to the GATA DNA binding zinc-finger-like domain (Omichinski *et al.*, 1993). However, no direct interaction of LMO proteins with DNA has been demonstrated. Instead, LMO proteins are found to be recruited to DNA by binding to hematopoietic basic helix-loop-helix factors like *TAL1* or *LYL1*, which are often overexpressed concomitantly with LMO2 (Vlierberghe and Ferrando, 2012). Studies conducted in double transgenic mice overexpressing *Tal1* and *Lmo2* in T-cells show that these TFs form dimer complexes, leading to rapid leukemia development (Larson *et al.*, 1996; Tremblay *et al.*, 2010). Likewise, LMO2-transgenic mice lacking *Lyf1* in the thymus show that *LYL1* is essential for LMO2-driven leukemogenesis (McCormack *et al.*, 2013).

Moreover, members of the HOX gene family, such as *TLX1* and *TLX3* are also considered major TF frequently found aberrantly in T-ALL. HOX genes encode transcription factors that contain a homeodomain (HD), which is the main domain responsible for their DNA-binding specificity. Physiological *TLX* expression is mainly restricted to embryonic development (Sun, Cui and Shi, 2017). Although no role of *TLX-1* and *TLX-3* has been reported specifically in the T cell lineage, *Tlx-1* (-/-) mice exhibit asplenia (Roberts, Shutter and Korsmeyer, 1994). Further investigations

revealed that *Tlx-1* (-/-) mutant embryos initially undergo spleen formation but is followed by rapid and complete resorption (Dear *et al.*, 1995), thus, highlighting the essential role of TLX-1 in the survival of splenic precursors. In T-ALL, the ectopic expression of *TLX1* is found in ~5-10% of the pediatric cases and 30% of the adult cases (Riz *et al.*, 2009). *TLX-1* was the first gene found to be translocated under the control of the *TCR* gene enhancers including t(10;14)(q24;q11) and t(7;10)(q34;q24) (**Table 3**). Similarly, *TLX3* can become aberrantly activated in T-ALL due chromosomal translocations. These include the t(5;14)(q33-34;q11) juxtaposing *TLX3* to the *TCR* delta locus and the t(5;14)(q35;q32) that result in *TLX3* location under regulatory sequences of *BCL11B* gene (Bernard *et al.*, 2001) (**Table 3**). *TLX1* and *TLX3* leukemias exhibit a shared mechanism of transformation as illustrated by an overlapping gene expression signatures (Vlierberghe and Ferrando, 2012). The oncogenic potential of aberrant TLX gene expression was observed in transgenic mice, in which *TLX1* expression was induced specifically in thymocytes using the *Lck* promoter. *TLX1* transgenic mice exhibited development of lymphoid tumors, with infiltration of immature lymphoblasts in several organs, including the thymus, bone marrow, spleen, and lymph nodes (De Keersmaecker *et al.*, 2010).

3.4. Classification according to gene expression profiles

Initial studies using oligonucleotide microarrays to identify the gene expression profiling of 27 T-ALL cases revealed gene expression signatures highly concordant with recognized stages of normal thymocyte development (Ferrando *et al.*, 2002). Among 39 T-ALL cases analyzed 27 exhibited ectopic expression of three major oncogenic TF: *LYL1*, *TLX1* and *TAL1*. Gene signatures marked by elevated *LYL1* expression were associated with a phenotype of lowly differentiated thymocytes, characterized by the upregulation of early hematopoietic markers such as *CD34*, the cell adhesion gene L-selectin (*SELL*) and the antiapoptotic gene *BCL2*. Moreover, cases with ectopic expression of *TLX1* (also known as *HOX11*) were linked to gene signatures resembling thymocytes at the early cortical stage, which

included the upregulation of CD1, LAR, and CD10. In contrast, gene signatures of T-ALL cases with *TAL-1* overexpression exhibited a phenotype akin to thymocytes at the late cortical stage of differentiation, marked by the upregulation of *CD3E*, *TCRA*, *TCRB*, and *LCK*.

More recent gene expression profiling performed with genome-wide sequencing analyses in 264 T-ALL cases have integrated the expression of oncogenic TF with recurrently mutated genes and signaling pathways as well as differentiation stage of T-ALL. Remarkably, hierarchical clustering analysis result in defined groups according to transcription factor deregulation. These groups, as observed in **Figure 10**, included deregulation of TAL1 in 36% of the cases, TAL2 in 8%, TLX1 in 11%, TLX3 in 19%, HOXA in 14%, LMO1/LMO2 in 4%, LMO2/LYL1 in 7%, and NKX2-1 in 6%.

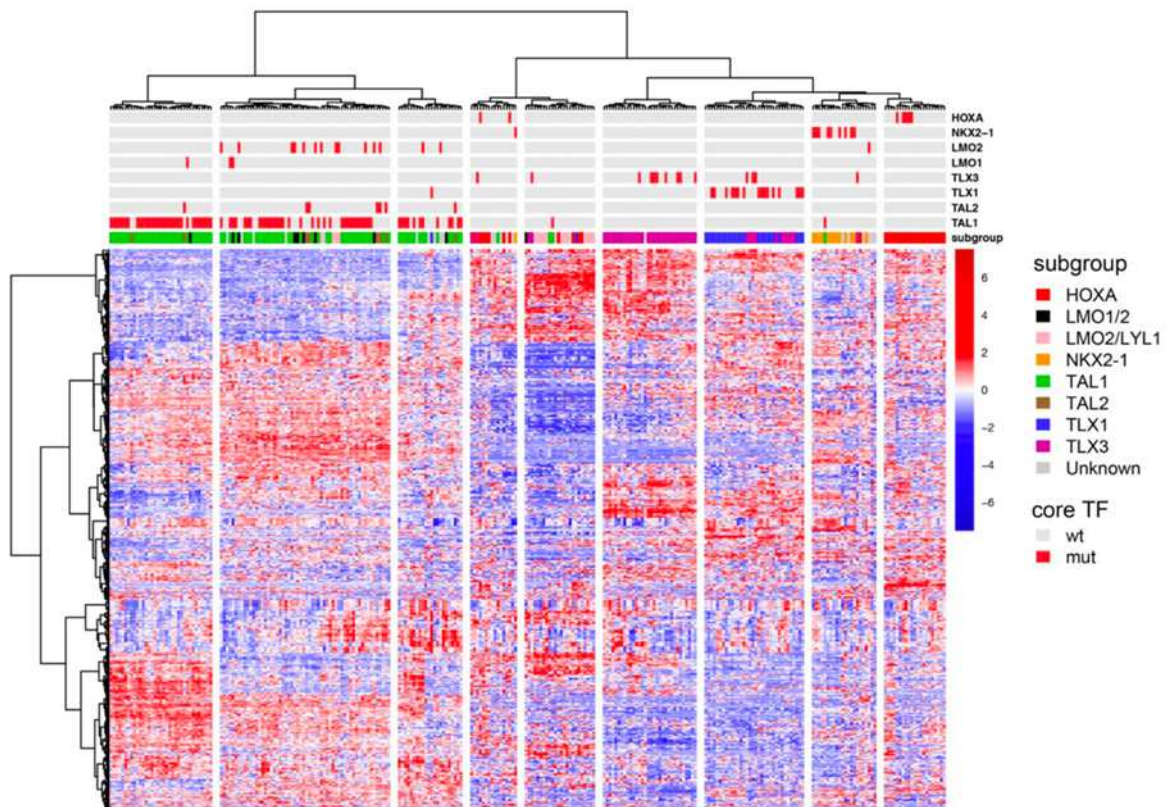


Figure 10. Hierarchical clustering of genome signatures of diagnosed samples of T-ALL patients. Hierarchical the top 500 most variably expressed genes from transcriptome sequencing data. Annotation of genetic alterations resulting in core

transcription factor deregulation are indicated at the top 7 rows. Transcription factor subgrouping indicated on the multicolored 8th bar. Figure from (Liu et al., 2017).

The genomic landscape of T-ALL proposed by (Liu et al., 2017) is in concordance with the seminal studies of (Ferrando *et al.*, 2002), in which the T-ALL molecular groups share genes critical to specific stages of T cell development. As illustrated in **Figure 11**, ETP T-ALL cases exhibiting an undifferentiated immunophenotype that includes the pro-T and pre-T classification, exhibit a higher occurrence of LMO2/LYL1 and HOXA deregulation (Liu et al., 2017). Early cortical T-ALL is primarily associated with aberrant expression of TLX1 and TLX3, and to a lesser extent, with NKX2-1. Finally, late cortical T-ALLs that experience arrest in differentiation at the DP or SP stage (expressing CD3, CD4, and CD8) are recurrently associated with the aberrant expression of TAL1 transcription factor (Vlierberghe and Ferrando, 2012).

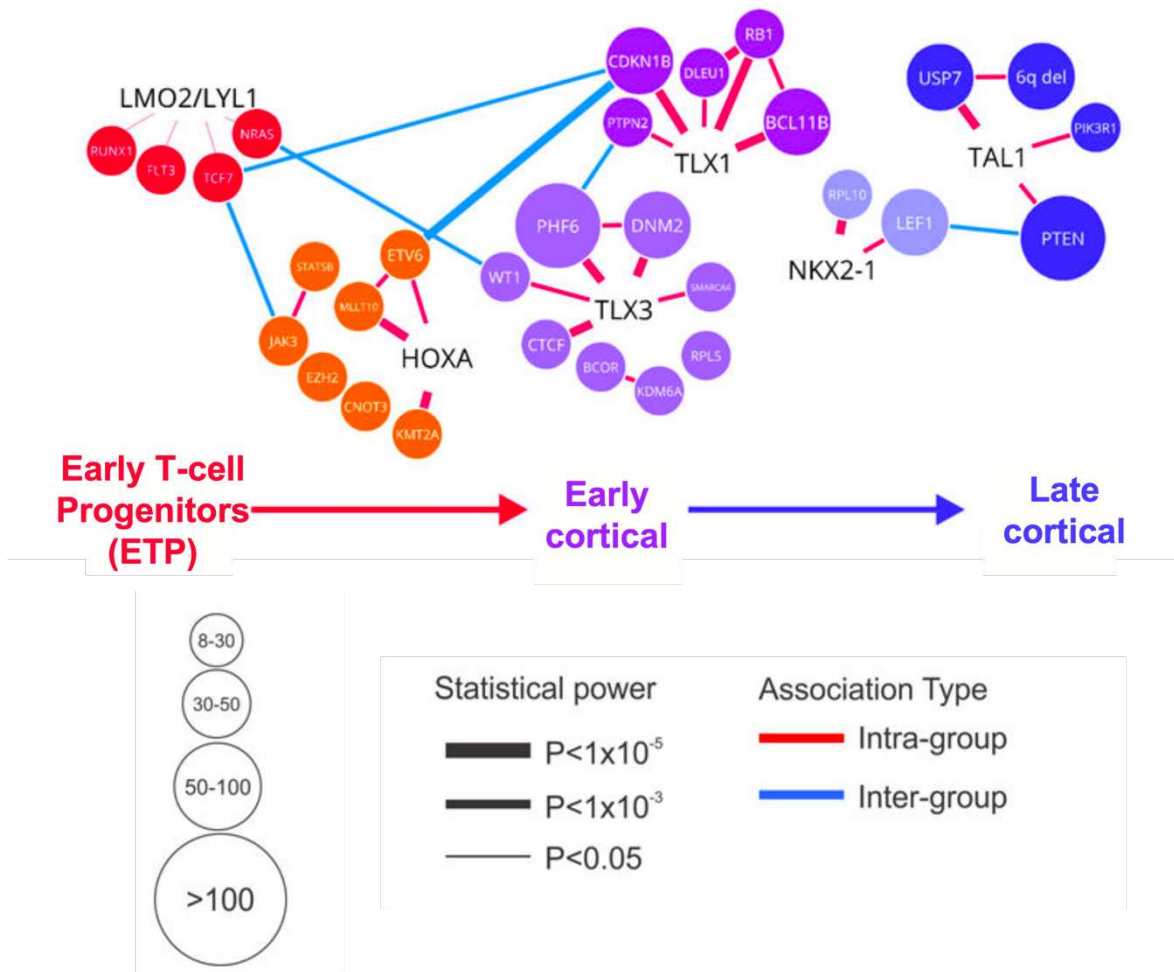


Figure 11. Network mapping the association between genetic alterations in T-ALL and stages of T-cell Development.

T-ALL subgroups are accompanied by nodes representative of genetic alterations. Color spectrum from red to blue is indicative of the T-ALL subgroup association with stages of T-cell development defined as Early T-cell Progenitors (ETP), Early Cortical, and Late Cortical. Frequencies of the genetic alteration across the entire cohort are represented by the size of each node. Lines connecting the nodes indicate statistically significant co-occurrence of a gene-gene pair or a gene-subtype pair. Intra- and inter- subtype cooccurrence is shown in red and blue, respectively with the thickness of each line represents the statistical significance. Schematic modified from (Liu et al., 2017).

3.5. Mutational landscape in T-ALL

T cell transformation involves a sequential accumulation of multiple genetic aberrations. In addition to chromosomal translocations, aberrant expression of oncogenic transcription factors can also be caused by mutations. Similarly, additional recurrent genetic alterations in T-ALL include mutations that result in constitutive activation of the NOTCH pathway and deletion of the tumor suppressor *CDKN2A*, which also represent major oncogenic pathways for T cell transformation (Vlierberghe and Ferrando, 2012). In line with this, sequence mutations, DNA rearrangements and alterations found by genome-wide sequencing profiling, resulted in the identification of 10 functional pathways recurrently mutated in T-ALL (Liu et al., 2017). Affected pathways include transcriptional regulation occurring in 91% of cases, that comprised mutations in the transcriptional regulators of *LMO2*, *TLX1*, *TLX3* and *TAL1*; cell cycle regulation and tumor suppression (84%) including mutations in the *CDKN2A* locus; mutation affecting NOTCH1 signaling (79%), epigenetic regulation (68%), PI3K-AKT signaling (29%), JAK-STAT signaling (25%), Ras signaling (14%), ribosomal function (13%), ubiquitination (9%) and RNA processing (9%) (**Figure 12**). The most representative genetic lesions found in T-ALL leading to cell cycle defects, constitutive activation of signaling pathways and aberrant chromatin remodeling will be described below.

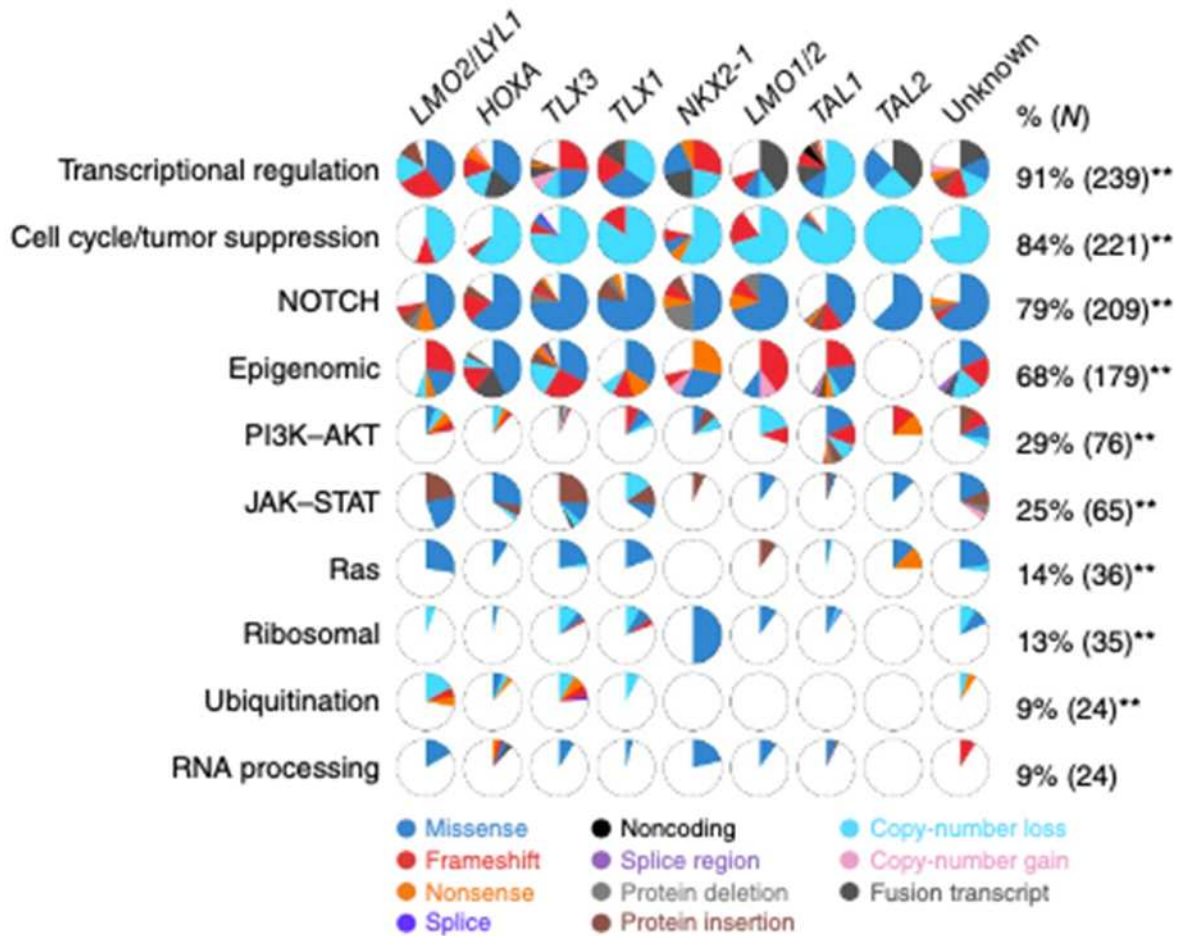


Figure 12. Correlation of transcription factor deregulation and recurrently mutated pathways in T-ALL. At the top are found eight molecular subgroups identified based on the malignant activation of specific transcription factors. On the left ten frequently mutated pathways in T-ALL are listed. On the right, the proportion of recurrence in T-ALL cases for each pathway is indicated. On the bottom, type of genetic alteration. Schematic from (Liu et al., 2017).

3.5.1. Cell cycle defects

Cell cycle defects in T-ALL are mostly related to *CDKN2A* deletions, which are the most frequent abnormality in T-ALL, existing in more than 70% of patients. The *CDKN2A* locus encodes the p16/INK4A and p14/ARF tumor suppressor genes, which function as cell cycle regulatory proteins. While P16INK4A blocks the cyclin D/cyclin-dependent kinase CDK4 proteins (INK4) complex that promotes cell cycle

progression, p14ARF inhibits MDM2, which is a negative regulator of the TP53 tumor suppressor gene. Consequently, genetic alterations leading to the inactivation of these genes not only promote uncontrolled cell cycle entry but also hamper the p53-controlled cell cycle checkpoint and apoptosis. Additional alterations leading to the inactivation of cell cycle regulators in T-ALL include the overexpression of cyclins (Van Vlierberghe *et al.*, 2008). An example of this is the identified chromosomal translocation t(7;12)(q34;p13) in T-ALL cases, which results in the placement of the *CCND2* gene, encoding for cyclin D2, proximate to TCRB regulatory sequences, leading to cyclin D2 dysregulation (Clappier *et al.*, 2006).

3.5.2. Dysregulation of Notch pathway

The NOTCH gene family encodes four receptors (Notch 1-4) that bind to different ligands encoded by the Delta and Jagged gene families, namely Delta-like 1 (DL1), Delta-like 3 (DL3), Delta-like 4 (DL4), Jagged1, and Jagged2. Notch receptors are highly conserved transmembrane receptors whose structure is mainly composed of an extracellular domain consisting of epidermal growth factor (EGF)-like repeats responsible for Notch-ligand interaction, followed by a negative regulatory region (NRR) that comprises a heterodimerization domain (HD) and a cysteine-rich region (CR), as well as a Notch intracellular domain (ICN) that mediates Notch signaling (Gordon *et al.*, 2007). The cognate interaction of the Notch receptor with its ligands promotes a proteolytic cleavage of the extracellular domain, which is mediated by the metalloprotease ADAM. This cleavage generates a truncated Notch that is then rapidly recognized and cleaved at the membrane by the γ -secretase complex, thereby releasing the active ICN from the plasma membrane. Upon release, ICN translocate to the nucleus to regulate the expression of Notch target genes (Demarest, Ratti and Capobianco, 2008). The negative regulation of Notch transcriptional activity is initiated by cyclinC:cdk8, which phosphorylates the ICN in its PEST domain. Phosphorylation of the PEST domain allows the binding of the

ubiquitin ligase FBXW7, which promotes the ubiquitination of Notch and its subsequent proteolytic degradation by the proteasome (Gupta-Rossi *et al.*, 2001).

Notch signaling plays a crucial role in T-cell development. Inducible loss of *Notch1* in newborn mice results in severe deficiency in thymocyte development without effecting the development of other hematopoietic lineages (Radtke *et al.*, 1999). In T-ALL, mutations affecting Notch signaling are present in approximately 50-79% of the cases (Weng *et al.*, 2004; Liu *et al.*, 2017). The implication of NOTCH in T-ALL was first discovered in a rare chromosomal translocation, specifically t(7;9)(q34;34.3), which occurs in less than 1% of T-ALL cases. The identified translocation in human T-ALL cases results in the juxtaposition of *NOTCH1* into the *TCRB* locus, which leads to the expression of a truncated NOTCH1 receptor (ICN, also named TAN-1) and the constitutive activation of Notch signaling (Ellisen *et al.*, 1991). Posterior studies performed sequence analysis in 96 T-ALL patients showed that more 50% cases exhibit gain-of-function mutations involving the HD and PEST domains (Weng *et al.*, 2004). While mutations in the HD region lead to ligand independent proteolytic cleavage of Notch, mutations in the PEST domain result in the shortening or loss of the PEST domain, which lead to impaired FBXW7-mediated targeting of ICN to proteasomal degradation (Thompson *et al.*, 2008). These mutations in *Notch1* have been demonstrated to result in augmented levels cellular ICN and increased NOTCH transcriptional activity (Demarest, Ratti and Capobianco, 2008). Additionally, 15% of T-ALL cases exhibit loss-of-function mutations or deletion in FBXW7, which result in the inhibition of NOTCH degradation and thus promoting activation of the pathway (Song *et al.*, 2008) .

3.5.3. Epigenetic alterations

T-ALL exhibits a very high frequency of mutations in genes that encode proteins involved in epigenetic regulation compared to other cancer types (Huether *et al.*, 2014). The most frequent genetic alterations leading to dysregulation of epigenetic regulators affect PHF6 and members of the Polycomb repressor complex

2 (PRC2): EZH2 and SUZ12. The plant homeodomain (PHD) finger 6 (PHF6) is a protein containing two zinc finger domains, enabling its chromatin-mediated regulation of gene expression. *PHF6* mutations are frequently observed in T-ALL, occurring in 5-16% of pediatric and 19-40% of adult patients (Van Vlierberghe *et al.*, 2010). These mutations are often nonsense and frameshift truncating alleles that result in the ablation of protein expression. Although the specific regulatory targets of PHF6 and its role in leukemogenesis are active areas of research, recent analysis of whole exome sequencing in T-ALL patients shows that *PHF6* represents an early mutational event in T-cell transformation (Wendorff *et al.*, 2019). Moreover, up to 25% of T-ALL cases exhibit nonsense and frameshift mutations leading to loss-of-function mutations targeting the *EZH2* and *SUZ12* genes, which are core members of the PRC2 complex. PRC2 is a central epigenetic regulator and transcriptional repressor that mediates the H3 lysine 27 trimethylation (H3K27me3) epigenetic mark. This results in the transcriptional repression of genes involved in development, stem cell maintenance, and differentiation (Margueron and Reinberg, 2011). Biallelic deletion of *Ezh2* in mice results in spontaneous $\gamma\delta$ T-ALL occurrence (Simon *et al.*, 2012), thus establishing the role of the PRC2 complex as a tumor suppressor gene in T-ALL pathogenesis.

3.5.4. Signal transduction dysregulation

3.5.4.1. PI3K-AKT signaling

Phosphatidylinositol-3-kinases (PI3Ks) are a family of lipid kinases that phosphorylate inositol phospholipids at the 3'-OH group of the inositol ring. This family comprises three classes of proteins, among which the class IA PI3K members are implicated in human cancer (Yuan and Cantley, 2008). Class IA PI3Ks are heterodimers consisting of a regulatory subunit (p85 α , p55 α , p50 α , p85 β , p55 γ) and a catalytic subunit (p110 α , p110 β , and p110 δ). Also, PI3Ks contain an SH2 domain that enables them to be recruited to phosphorylated tyrosine residues from RTK and other signaling receptors, thereby getting activated (Engelman *et al.*, 2005).

Activation of PI3K results in the production of the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP₃) from the substrate phosphatidylinositol-4,4, biphosphate (PIP₂) (Fruman, Meyers and Cantley, 1998). Increased levels of PIP₃ lead to the recruitment of AKT at the plasma membrane, where its phosphorylated and activated by 3-phosphoinositide-dependent kinase (PDK) 1 and PDK2. The AKT kinase has multiple substrates, through which it exerts regulatory effects on various cellular processes such as cell survival, cell cycle progression, and cell growth. In T-cells, some of the targets of AKT include the NFAT and NF-κB transcription factors, as well as the mammalian target of rapamycin (mTOR) (Shah *et al.*, 2021) **Figure 5**. Direct regulators of the PI3K pathway include the phosphatase and tensin homologue (PTEN), whose main physiological substrate is PIP₃, the primary product of PI3K activation. PTEN has a lipid phosphatase motif that dephosphorylates PIP₃ and turn it back to PIP₂ resulting in decreased cellular levels of PIP₃, thereby impairing PI3K signaling (Chen *et al.*, 2018).

Genetic lesions leading to the deregulation of the PI3K-AKT pathway in T-ALL are reported to occur in approximately 29% (Liu *et al.*, 2017) to 47% (Gutierrez *et al.*, 2009) of T-ALL cases. The most common alterations affecting the PI3K-AKT pathway are defects in *PTEN*. These defects include loss-of-function mutations frequently affecting the PTEN phosphatase domain in 27% of cases, as well as deletions in 9% of cases. Additionally, constitutive activation of the PI3K-AKT pathway can also occur as a consequence of mutations in *PI3K* and *AKT* genes. These alterations include gain-of-function mutations, such as mutations in the catalytic subunit of *PIK3CA* encoding p110α, which have been observed in 4.5% of cases. Another example is the AKT E13K point mutation, which promotes constitutive translocation of AKT to the plasma membrane and has been observed in 2.3% of T-ALL cases. Additionally, loss-of-function mutations or deletions in the regulatory subunit PIK3R1/p85α have been reported in 4.5% of T-ALL cases (Gutierrez *et al.*, 2009). The alterations described in *PTEN*, *PI3K*, and *AKT* were found to be mutually exclusive in almost half of the cases.

3.5.4.2. JAK-STAT signaling

JAK/STAT signaling pathway is mediated by the kinase and transcription regulation activities of these two protein families. Janus Kinase (JAK) family comprises four tyrosine kinases JAK1, JAK2, JAK3 and tyrosine kinase 2 [TYK2], while Signal transducer and activator of transcription (STAT) family is formed by seven members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6). The JAK/STAT pathway functions as the principal signaling pathway downstream of cytokine receptors with JAK3 particularly associated to the common γ chain (γ_c) of the interleukin (IL) receptors including IL-2, IL-4, IL-7, IL-9, and IL-15. JAK proteins associate with cytokine receptors through proline-rich regions in the receptors intracellular domain. In the absence of their specific ligands, cytokine receptors are expressed on the plasma membrane in an inactive preformed dimeric state. Upon ligand binding, conformational changes take place, causing a reorientation of the preformed dimeric receptor. This reorientation brings the JAK proteins into close proximity, facilitating their transphosphorylation and subsequent activation (Remy, Wilson and Michnick, 1999). Activated JAK then phosphorylates tyrosine residues in the cytoplasmic tail of the receptor, creating recruitment sites for effector molecules bearing SH2 domains including STAT which are then phosphorylated by JAKs (Ihle, 1995). This phosphorylation event promotes STAT homo- or heterodimerization and subsequent translocation into the nucleus, where act as transcription factors. Target genes positively regulated by STAT include genes that promote cell survival, proliferation, and cell cycle progression, among them *BCL2*, *IL2RA*, *MYC*, and *CDKN1A* (Lord *et al.*, 2000). Genetic studies in mice that selectively delete different JAKs provided insights into the crucial role of the JAK signaling pathway, specifically through JAK1 and JAK3, in the formation of lymphoid cells. On one hand, mice with homozygous ablation of the ubiquitously expressed *Jak1* were runted at birth with severe defects in lymphoid development, including a 260-fold reduction in thymocyte numbers (Rodig *et al.*, 1998). On the other hand, induced deficiency of *Jak3*, which

is exclusively expressed in lymphoid lineages, resulted in viable mice with a drastic population decline in thymocytes, B cells, and T cells (Nosaka *et al.*, 1995).

In T-ALL genetic alterations affecting JAK-STAT signaling pathway are observed in 25 – 30% of the cases (**Figure 12**) (Girardi *et al.*, 2017). The most common mutations affecting this pathway are somatic gain-of-function mutations in the IL-7 receptor (IL-7R), which are present in almost half of the cases (10% of the total T-ALL cases). These mutations all occur in exon 6, and result in the introduction of an unpaired cysteine in the juxtamembrane-transmembrane portion of the extracellular domain of IL-7R. This results in disulfide bond-dependent homodimerization of two IL-7R α chains, which consequently drive constitutive signaling via JAK1 and independently of IL-7, γc or JAK3 (Zenatti *et al.*, 2011). Additionally, gain-of-function mutations in JAK1(5%, 7%), JAK3 (8%, 12%) and STAT5 (6%, 6 %) have been observed in pediatric and adult T-ALL cases respectively (Girardi *et al.*, 2017). Additionally, a rare chromosomal translocation t(9;12)(p24;p13) in T-ALL results in the expression of the *TEL-JAK2* fusion gene, leading to the constitutive activation of JAK2 tyrosine kinase activity (Lacronique *et al.*, 1997). Notably, transgenic expression of the TEL-JAK2 fusion in mice results in the development of fatal leukemia, thus unveiling the role of aberrantly activated JAK2 signaling in leukemia pathogenesis (Carron *et al.*, 2000).

4. Treatment of T-ALL

4.1. Stratification of patients and treatment

T-ALL is an aggressive malignancy historically associated with a poor prognosis compared to other leukemias. Since Sydney Farber's discovery seven decades ago of aminopterin's ability to induce temporary remission in children with acute leukemia, antifolates have remained the front-line treatment for ALL patients. Nowadays, advancements in drug combinations and treatment protocol administration have led to better and more hopeful results compared to those in 1948 (Farber et al., 1948), achieving 5-year overall survival (OS) rates of approximately 85% for pediatric cases and over 50% for adult cases.

Diagnosis and stratification of patients are important strategies that have contributed to improved OS rates. Diagnosis involves analyzing cell morphology through microscopy, determining the immunophenotype using flow cytometry according to the WHO classification (**Table 2**), and performing cytogenetic analysis such as karyotyping and fluorescence in situ hybridization (FISH) to identify common translocations. The leukemia characteristics identified during diagnosis contribute to risk stratification of the patients. However, the most significant predictor of treatment response is the detection of minimal residual disease (MRD) at the end of the first treatment phase. Current treatment regimens are based on intensive chemotherapy that lasts from 2 to 3.5 years. Treatment approaches are refined and administered according to the patient's risk stratification. However, all patients commonly undergo three main phases of treatment: induction, consolidation, and maintenance, which will be described below (Raetz and Teachey, 2016) (**Figure 12**).

4.1.1. Induction phase

The induction phase is an intensive regimen administered to all ALL cases, including T-ALL patients. It lasts between 4-6 weeks and aims to achieve leukemia

remission. This initial regimen is based on a combination of glucocorticoids, anti-mitotic alkaloids, anthracyclines, and asparaginase.

Glucocorticoids (GCs) are small lipophilic molecules that can cross the plasma membrane and bind to intracellular GC receptors (GR). The binding of GCs with GR shapes the GC-GR complex, which can form homodimers that translocate to the nucleus and bind to glucocorticoid-responsive elements DNA sequences to regulate gene transcription. Synthetic analogues of GC including prednisone were commonly used as a treatment for ALL patients, however, in the last two decades, it has been replaced by dexamethasone, a synthetic GC with an extended half-life and higher penetrance in the central nervous system (CNS).

Vincristine is a plant alkaloid extracted from *Vinca rosea*. It was first isolated in 1961 and characterized for its oncolytic activity (Neuss, 1962). It exerts its effects by binding to the B-subunits of tubulin, leading to the disruption of microtubule dynamics, consequently affecting mitotic spindle formation and resulting in mitosis inhibition, an effect not restricted to cancer cells. Thus, vincristine administration in patients is associated with a variety of side effects, including gastrointestinal toxicity (Boussios *et al.*, 2012), myelosuppression (Maxwell and Maher, 1992), hair loss, skin reactions, and more severely, neurotoxicity. Anthracyclines were primarily discovered for their antibacterial properties in 1939 and later characterized as antitumor agents (Arcamone *et al.*, 1969). They exert their cytostatic and cytotoxic effects through various mechanisms. These mechanisms include DNA intercalation, which inhibits DNA biosynthesis; generation of free radicals that cause damage to cell membranes, proteins, and DNA; release of reactive oxygen species (ROS), resulting in oxidative stress and DNA damage; and inhibition of topoisomerase-II-mediated DNA repair, leading to DNA damage (Gewirtz, 1999). The conventional anthracyclines used in cancer therapy include doxorubicin, epirubicin, daunorubicin, and idarubicin. Similar to the other chemotherapy agents mentioned above, anthracyclines lack specificity for cancer cells, resulting in their impact on normal cells and subsequent side effects. Anthracycline-associated cardiotoxicity is frequently observed, which can lead to non-relapse mortality (Yu *et al.*, 2021).

Asparaginase is a chemotherapy drug that preferentially targets acute lymphoblastic leukemia cells. Asparaginase is an enzyme not expressed in humans that hydrolyzes L-asparagine (Asn), thereby depleting serum levels of this amino acid. Since leukemic cells are incapable of synthesizing Asn, asparaginase-containing regimens lead to Asn starvation in leukemic cells, resulting in leukemic cell death. There is a consistent observation that the addition of asparaginase to treatment regimens significantly improves patient outcomes (Kumar *et al.*, 2014). The current form of L-asparagine used is its pegylated version, which stays in circulation for five times longer, allowing for less frequent treatment administrations.

During the induction phase, analyzing the treatment response by monitoring leukemic expansion in the patient is an essential criterion and represents the strongest prognostic factor in ALL patients so far. This evaluation is known as Minimal Residual Disease (MRD) and can be determined by flow cytometry and polymerase chain reaction (PCR) using personalized primers that detect rearrangement in immunoglobulin (*IG*) or T-cell receptor (*TCR*) genes. The risk of relapse is generally proportional to the level of MRD (Campana, 2010), thus based on the level of MRD after the induction phase patients are stratified in standard, medium and high risk (**Table 4**). MRD positivity is defined by the presence of 0.01% or more of ALL cells in the bone marrow. ALL patients who test negative for MRD after the induction phase are stratified as standard risk (SR). Patients who show MRD positivity are classified as medium risk (MR). Patients that show MRD positivity and additionally present other poor prognostic factors or maintain MRD positivity after the different phases of treatment are classified in the high risk (HR) category. Based on this stratification the treatment regimen administered to patients would then vary (**Figure 13**).

Risk	COG T-ALL risk group definitions
Standard risk	M1 day 29 marrow; Day 29 MRD* <0.01%; CNS 1; no testicular disease; no steroid pretreatment
Intermediate risk	M1 or M2 day 29 marrow; day 29 MRD \geq 0.01%; end-of-consolidation MRD < 0.1%; any CNS and testicular disease status; any steroid pretreatment status
Very high risk	M3 day 29 marrow or end-of-consolidation MRD \geq 0.1%

Table 4. Risk groups stratification in patients with ALL.

M1: less than 5% blasts in the bone marrow; M2: 5-25% blasts in the bone marrow; M3: more than 25% blasts in the bone marrow. Central Neural System (CNS) 1: less than 5 White Blood Cells (WBC)/uL and no blast in the cerebro-spinal fluid (CSF). Table modified from (Raetz and Teachey, 2016).

4.1.2. Consolidation

Clinical trials conducted over the past two decades have demonstrated improved outcomes in T-ALL with protocols that incorporate intensive consolidation regimens. Among these, the augmented Berlin-Frankfurt-Münster (aBFM) regimen has shown promising results and is recommended for implementation (Teachey and O'Connor, 2020),(Chang et al., 2008). The aBFM regimen involves an 8-week administration of several drugs:

1) Cyclophosphamide, a DNA alkylating agent that compromises genetic integrity and cell survival (Ogino and Tadi, 2023). 2) Cytarabine and 3) Mercaptopurine, both anti-metabolites that antagonize pyrimidines and purines, respectively, leading to the inhibition of DNA, RNA, and protein synthesis, ultimately inducing cell death (Faruqi and Tadi, 2023), 4) Vincristine and 5) high doses of PEG-asparaginase, as previously described. Additionally, since T-ALL can infiltrate the central nervous system (CNS), during the first two weeks of consolidation, T-ALL patients also receive intrathecal administrations of 1) Methotrexate, another anti-metabolite drug that antagonizes folate, preventing de novo purine synthesis and 2) cranial and spinal radiotherapy (Nachman et al., 1998).

4.1.3. Maintenance

The maintenance or continuation treatment phase is based on the combination of weekly oral methotrexate (MTX) and daily oral mercaptopurine administration. The main purpose of this treatment is to maintain white cell counts per liter below 3×10^9 , a level that appears to be associated with a reduced risk of relapse. Therefore, doses of these drugs are adjusted to achieve this maintenance (Aricó *et al.*, 2005). A survey of ten cooperative ALL study groups shows that patient treatment typically lasts a total of 24 months, with a maintenance/continuation phase lasting from 12 to 18 months (Pui and Evans, 2006). For reasons not well understood, the length of this phase is important. This was demonstrated in a randomized, which showed that shortening the treatment duration in this phase by 6 months compromises the patient's disease-free survival by 10% (Schrappe *et al.*, 2000).

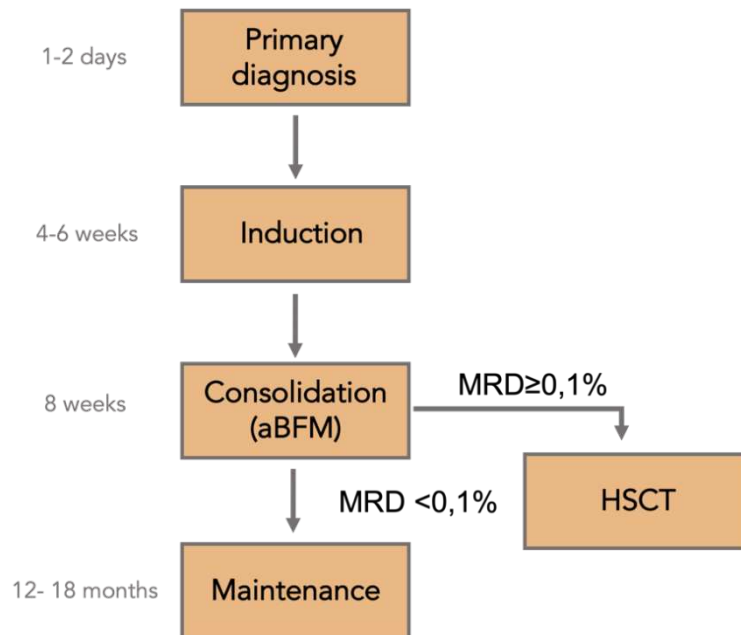


Figure 13. Schematic of established treatment timeline of ALL patients. Abbreviations. aBFM augmented Berlin-Frankfurt-Münster treatment regimen, HSCT hematopoietic stem cell transplantation. Schematic modified from (Teachey and O'Connor, 2020).

4.1.4. Updated recommendations for ALL treatment

Teachey and O'Connor, 2020 propose additional phases based on a series of trials that demonstrate better outcomes in T-ALL patients (**Figure 14**). This comprises the administration of a treatment regimen called provisional maintenance that consists of approximately 8 weeks of escalating doses of MTX (known as the Capizzi maintenance regimen) followed by a 4-week delayed intensification or re-induction phase, in which patients are treated with the same glucocorticoid-based 4-drugs induction regimen initially described. Clinical trials comparing patients who received traditional and augmented treatments showed that patients with augmented regimens have significant improvement in event-free overall survival (Teachey and O'Connor, 2020), (Nachman et al., 1998; Chang et al., 2008).

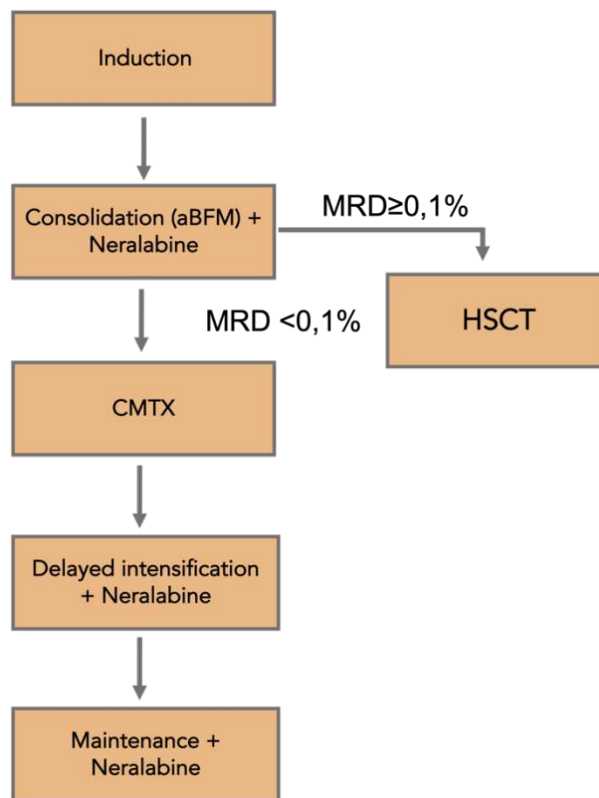


Figure 14. Schematic of updated recommendations of treatment for T-ALL patients. Abbreviations. aBFM augmented Berlin-Frankfurt-Münster treatment regimen, HSCT hematopoietic stem cell transplantation, CMTX Capizzi Methotrexate. Schematic modified from (Teachey and O'Connor, 2020).

Likewise, more recent clinical trials focused on refractory T-ALL patients have shown that the use of Nelarabine induces effective remission (S. L. Berg et al., 2005; Dunsmore et al., 2012; Zwaan et al., 2017). Nelarabine is a prodrug for 9- β -D-arabinofuranosylguanine (ara-G), a deoxyguanosine analogue. Similar to other purine analogues, once ara-G is incorporated into a replicating DNA strand, it interrupts DNA synthesis, which results in inhibition of DNA replication and ultimately leading to the induction of cell death (Buie, Epstein and Lindley, 2007). The rationale for treating T-ALL patients with Nelarabine is based on the observation that ara-G has a specific tendency to accumulate in immature T-lymphocytes, thus exhibiting selective cytotoxicity (Ullman and Martin, 1984; Shewach and Mitchell, 1989). Nelarabine was approved by the European Medicines Agency (EMA) and US Food and Drug Administration (FDA) in 2005. A clinical trial AALL0434 conducted by the Children's Oncology Group enrolled 1562 newly diagnosed cases. In the provisional maintenance phase, these cases were treated with MTX alone or in combination with Nelarabine. The results of the trial demonstrated that children and young adults with T-ALL who received nelarabine treatment had a significantly higher 5-year disease-free survival (DFS) rate of 88% compared to 82% in those without nelarabine (Dunsmore et al., 2020). Recent treatment approaches in north America and United Kingdom recommended the use of Nelarabine in a frontline setting (Abaza *et al.*, 2018; Teachey and O'Connor, 2020).

Another important explanation for the better outcomes observed in patients treated with Nelarabine is its ability to penetrate the blood-brain barrier, helping in the prevention and treatment of central nervous system (CNS) infiltration. Despite the proven selectivity and the significant improvements achieved with Nelarabine as a first-line treatment it has been associated with side effects. These side effects can notably affect the CNS and peripheral nervous system, comprising somnolence, seizures, dizziness, confusion, and ataxia, paresthesia (burning sensation) and pain in the extremities. In some cases, Nelarabine has even been linked to the

development of irreversible neurological toxicities, including paraplegia (Kuhlen *et al.*, 2017).

4.2. Treatment options for high-risk T-ALL patients

Allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT) is considered for high-risk patients with persistent MRD. Allo-HSCT is accompanied by a conditioning therapy regimen that comprise high-dose chemotherapy and radiotherapy, aiming to eradicate malignant cells and suppress the recipient's immune response to allow for durable allogeneic engraftment. Besides the anti-leukemic benefit of the acute conditioning regimen, allo-HSCT contains immunologically competent lymphocytes with potential immunologic reaction against malignant cells, an effect referred to as graft-versus-tumor (GvT) effect (Welniak, Blazar and Murphy, 2007). Despite the clear therapeutic advantage of allo-HSCT for T-ALL patients (Litzow and Ferrando, 2015), this approach is associated with a high rate of toxicity and late complications, including chronic graft-versus-host disease (Gyurkocza and Sandmaier, 2014; Luo *et al.*, 2023). Given this, modern treatment protocols recommend HSCT only for patients stratified as high-risk cases (**Figure 14**) (Teachey and O'Connor, 2020) as this group can benefit the most from this therapy despite its life-threatening side effects (Thiebaut *et al.*, 2000). Being a candidate for allogeneic transplantation involves a careful selection process, particularly in the search for HLA-identical sibling donors.

4.2.1. Targeted therapies for relapse cases

4.2.1.1. Inhibitors of the BCL2 family

The ETP-ALL group cases are associated with a poorer prognosis compared to patients with mature T-ALL cases. ETP's are often associated with chemotherapy resistance, high rates of remission failure, and relapse. A clinically relevant characteristic of ETP-ALL patients is their increased expression of the anti-apoptotic

BCL-2 protein (Maude et al., 2015), suggesting a potential therapeutic benefit of BCL-2 antagonists, such as the clinically approved ABT-199 (Venetoclax) (Chonghaile *et al.*, 2014). Indeed, recent case reports demonstrated that patients with refractory/relapsed ETP-ALL expressing BCL-2 protein achieved temporal remission when treated with the combination of standard chemotherapy and Venetoclax (McEwan, Pitiyarachchi and Viiala, 2020; Farhadfar *et al.*, 2021). Such results highlight the potential use of Venetoclax in newly diagnosed T-ALL cases which is an ongoing study in clinical trials [NCT05386576].

4.2.1.2. Src inhibitors

Unlike B-ALL cases, only a small subset of T-ALL cases harbors *ABL1* fusion (~8% T-ALL cases), *NUP214-ABL1* being the most frequent and *ETV6-ABL1* very rare (Hagemeijer and Graux, 2010). The *ABL1* fusion gene leads to the expression of tyrosine kinases that are constitutively activated, making them potential targets for tyrosine kinase inhibitors (TKIs). TKIs have been used to treat the *BCR-ABL1* lesion observed in chronic myeloid leukemia (CML) (Hantel and Larson, 2018) and in B-ALL Ph-like cases where *ABL1* lesions are frequently observed (Roberts et al., 2014). In a clinical trial involving refractory B-ALL Ph-like cases, Dasatinib, a second generation TKI, was administered in combination with chemotherapy, resulting in 78% of the patients achieving MRD negativity 2.5 months after TKI initiation (Tanasi et al., 2019). Following the same rationale, recent studies have reported case reports of refractory T-ALL patients with the *NUP214-ABL1* lesion who were treated with Dasatinib, showing an effective anti-leukemic response. This opens the perspective of targeted therapeutic options for this subset of T-ALL cases.

4.2.1.3. JAK inhibitors

Despite the high prevalence of mutations dysregulating the JAK-STAT signaling pathway in T-ALL and the availability of FDA-approved inhibitory targeted drugs, including Ruxolitinib and Tofacitinib inhibiting JAK1/2 and JAK3 respectively,

there are few studies of their potential application in T-ALL cases. Ongoing phase II clinical trials have documented the safety and tolerability of Ruxolitinib combination with intensive multi-agent chemotherapy in refractory B-ALL cases (Tasian et al., 2018) while preclinical studies showed the in vivo antileukemic effects of Ruxolitinib in ETP-ALL derived xenografts (Maude et al., 2015). Moreover, a case report study of a patient with refractory T-ALL harboring a *JAK3* mutation have reported to achieved complete remission (CR) after Ruxolitinib monotherapy regimen (Jaramillo et al., 2021). Notably, recent studies indicate that the use of Ruxolitinib can inhibit JAK-STAT signaling in T-ALL cases expressing IL7R, regardless of their mutational status (Courtois *et al.*, 2023). Considering that IL7R expression is more frequent (~70% of cases) in T-ALL compared to T-ALL cases with mutations in the IL7R-JAK-STAT pathway, these observations expand the potential candidates who can benefit from these specific inhibitors.

4.2.1.4. Immunotherapies

A healthy immune system plays a crucial role in detecting and eliminating abnormal cells, thereby preventing the growth and proliferation of malignant cells. More than a decade ago, it became increasingly clear that cancer cells, by employing diverse mechanisms, can establish local and systemic immunosuppression, leading to evasion of their detection and destruction by the immune system. In this context, immunotherapy is a type of cancer treatment that assists the host immune system in enhancing or reactivating its ability to effectively target and destroy cancer cells. Various immunotherapies, including antibody-based therapy and CAR-T cells have been successfully used in ALL patients.

4.2.1.4.1. Antibody-based therapy

Tumor-targeting mAbs are the most clinically used anti-cancer immunotherapy and, as a result, the most characterized. Some of the mechanisms by which tumor-targeting mAbs mediate tumor killing include cell signaling agonism

or antagonism, complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP) (Figure 15).

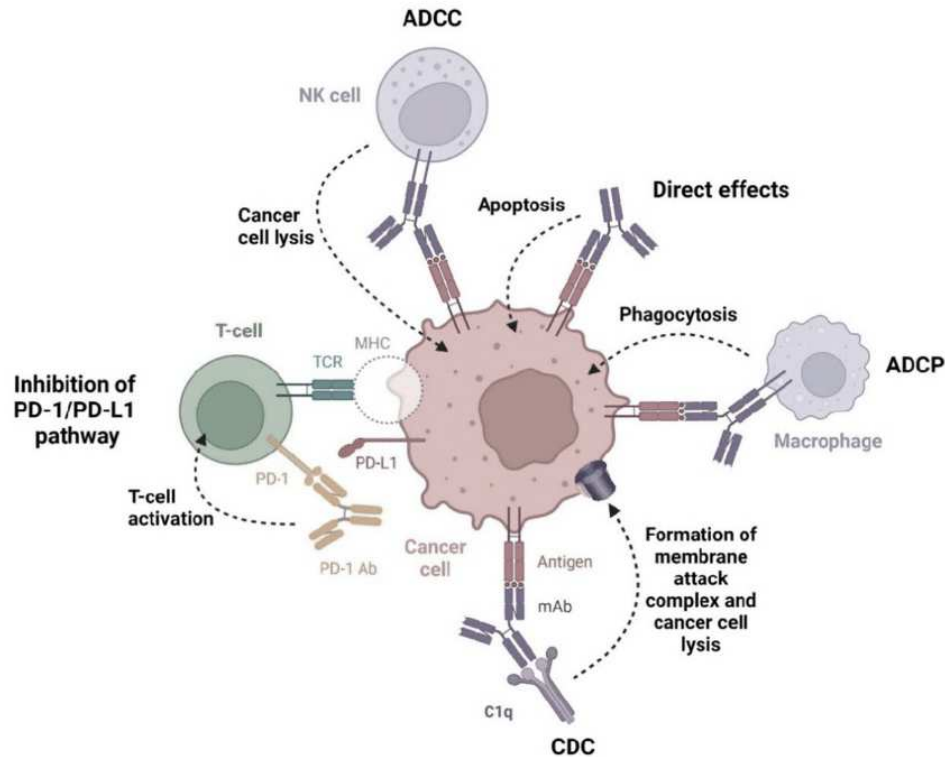


Figure 15. Mechanisms of action of anti-tumor monoclonal antibodies (mAbs). Abbreviations. TCR: T-cell receptor. MHC: major histocompatibility complex. NK: natural killer ADCC: antibody-dependent cellular cytotoxicity. ADCP: antibody-dependent cellular phagocytosis. CDC: complement-dependent cytotoxicity. C1q: complement component 1q. PD-1: programmed death-1 protein. PD-L1: programmed death ligand-1. PD-1 Ab: monoclonal antibody directed against PD-1. Scheme from (Sochacka-Ćwikła, 2022)

Among these mechanisms, ADCC is a predominant anti-tumor mechanism of the majority of the anti-tumor mAbs approved for clinical use in patients. Anti-tumor mAbs induce ADCC by recognizing the target antigen expressed on the cancer cell, resulting in a coating of cancer cells with these anti-tumor mAbs. This coating conducts the recruitment of Fc receptor-bearing cells, including macrophages, dendritic cells, and NK cells, which, in close proximity to a malignant cell, ultimately

lead to cytotoxic activities toward the cancer cell (Kohrt *et al.*, 2012) (**Figure 15**). In this context the identification of markers for cancerous cells is essential for anti-cancer immunotherapy. While treatment of B-ALL patients has advanced thanks to the development of several anti-tumor mAbs due to the identification of CD20 and CD19 markers (Levato and Molica, 2018), T-ALL patients have not benefited from any immunotherapy given the reduced number of specific markers identified.

Recent studies have found that both early T-cell precursor (ETP) and mature T-ALL patients can express high levels of CD38, a transmembrane glycoprotein that is physiologically expressed at low basal levels in lymphoid and myeloid cells (Tembhare *et al.*, 2020). Consequently, monoclonal antibodies specifically recognizing this target have been produced. Preclinical studies in T-ALL using an anti-CD38 monoclonal antibody, daratumumab, have shown that it has efficient antileukemic effects in independent T-ALL patient-derived xenografts (PDX). This preclinical study exhibit that the use of Daratumumab have immune-independent anti-tumor mechanisms, as it maintains its anti-leukemic properties even in NSG mice that are lacking of B, T, NK lymphocytes and complement factors. Still, additional pre-clinical studies with a PDX model, show that some T-ALL cases exhibit insensitivity to daratumumab despite the expression of CD38 on the cell surface (Bride *et al.*, 2018; Vogiatzi *et al.*, 2019).

4.2.1.4.2. Cellular therapy

4.2.1.4.2.1. CAR-T

Given the anti-tumor activity of T-cells against tumoral cells, enhancing T-cell responses has been an attractive anti-tumor strategy that has been investigated for decades. A technology that emerged based on this strategy is the genetic modification of T-cells to express recombinant receptors called Chimeric Antigen Receptors (CARs), designed to identify specific anti-tumoral targets/markers. Therefore, T-cells expressing CARs (CAR-T cells) redirect their immune reactivity

towards the cancerous cells bearing the chosen antigen. The strategy of CAR-T production is depicted in **Figure 16**.

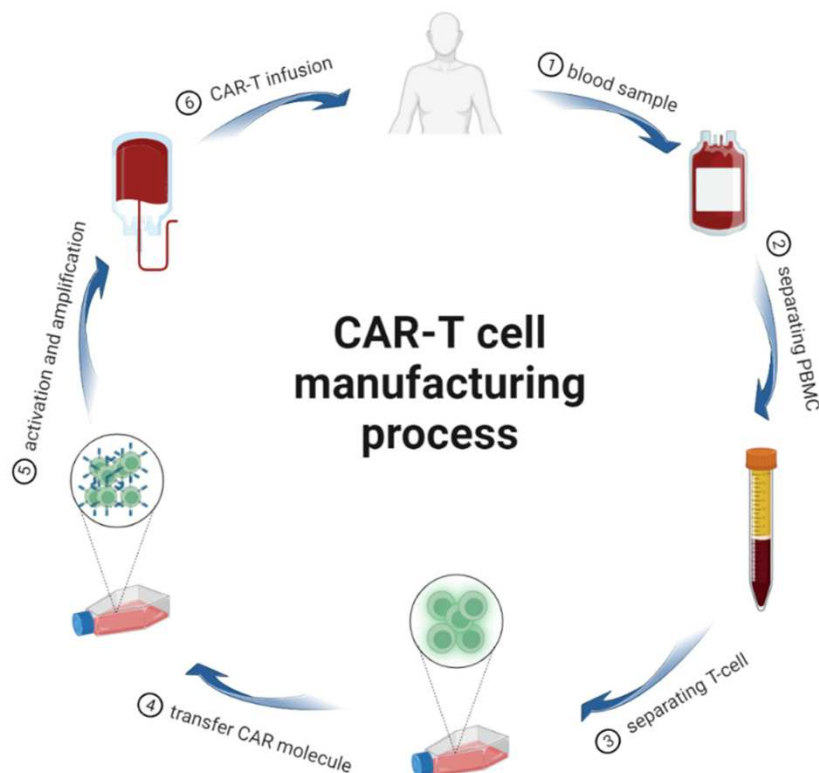


Figure 16. CAR-T structure and production Left: CAR components: (1) an extracellular target antigen-binding domain, (2) a hinge region, (3) a transmembrane domain, and (4) one or more intracellular signaling domains Right: The CAR-T production: T cells need to be cultured to allow for transduction of the designed CAR, transformation if followed by expansion of the successfully transduced cells. Scheme from (Wang *et al.*, 2023).

Despite de great outcomes of CAR-T administration in patients suffering different types of cancer including hematological malignancies and solid tumors (Sadelain, Brentjens and Rivière, 2013), the use of CAR-T in T-cell malignancies has been hampered due to several challenges. The first limitation is that both malignant T-cells and CAR-T cells share the same lineage, making the search for selective antigens a difficult task. As a result, CAR-T cells undergo fratricide, which refers to the self-killing of CAR-T cells. The second challenge is the collection of pure

healthy autologous T-cells without contamination from malignant cells, which is a technically challenging and risky task. Despite these difficulties, various attempts targeting different antigens have been reported. Some interesting approaches involve the use of CD7 CAR^{CD7-} T-cells, which can be generated either through CD7 depletion using CRISPR/Cas9 technology (Cooper et al., 2018) or by collecting naturally occurring CD7- T-cells and subsequently producing CAR-T cells from them (Freiwan et al., 2022). More recently, allogeneic T-cells have been modified to express a CD7-targeting CAR and to retain only cytoplasmic CD7 expression thus avoiding fratricide. In a phase I trial, these allogeneic donor-derived anti-CD7 CAR T cells were utilized to treat refractory cases of T-ALL. None of the patients experienced dose-limiting toxicities, and 19 out of 20 patients responded positively to the treatment. Notably, the patient who did not respond had lost the expression of CD7 on T-ALL cells (Liu *et al.*, 2023).

4.2.1.4.3. Targeting the TCR complex: a new therapeutic option for T-ALL

TCR-expressing T-ALL represent 50% of pediatric T-ALL cases, 30% of adolescent and 20% of adult cases express TCR (Asnafi et al., 2004). Studies conducted in our research team in different models including 1) mice models, 2) cell lines 3) primary leukemic cells and 4) patient-derived xenografts (PDX) demonstrate that persistent TCR activation in T-ALL results in a profound antileukemic effect.

- 1) A T-ALL mouse model driven by the expression of the *TEL(ETV6)-JAK2* oncogenic fusion protein (TJK2), when crossed with a mouse model for negative selection expressing the transgenic TCR-HY that recognize the male antigen HY, resulted in the inhibition of leukemia development in male recipients but not in females. Additionally, when TEL-JAK2/TCR-HY leukemic cells (isolated from leukemic female mice) were transplanted into syngeneic recipients, either female (HY-negative) or male (HY-positive), fatal leukemia was observed only in female recipients, while most of the male recipients

- failed to develop leukemia, suggesting that TCR activation counteracts T-ALL development and expansion (dos Santos *et al.*, 2006; Trinquand *et al.*, 2016).
- 2) Experiments in vitro using the T-ALL cell line SIL-ALL expressing the transgenic TCR-HY show that upon their exposure with both α CD3 / α CD28 coated beads or with the HY cognate antigen (DBY), cells are induced to cell death as measured by Annexin V/PI and caspase 3/7 activation (Trinquand *et al.*, 2016). Similar results were obtained using primary leukemic cells that died in response to α CD3 treatment.
 - 3) Preclinical studies demonstrate that the use of the α hCD3 monoclonal antibody (mAb) OKT3/Muromonab results in the induction of leukemic cell death and in a robust anti-leukemic effect observed in 12/12 independent Patient-Derived Xenografts (PDX). Additionally, the non-Fc γ R-binding α hCD3 mAbs Foralumab, a fully humanized IgG1 α CD3 mAb, and the clinically relevant humanized version of OKT3, hOKT3g1[Ala/Ala]/Teplizumab, exhibit an antileukemic activity that is as efficient as OKT3 in the same in vivo preclinical model (Trinquand *et al.*, 2016; Tran Quang *et al.*, 2020). As these T-ALL TCR+ independent diagnostic cases harbor different combinations of mutations, this shows that α CD3 mAbs have potent anti-leukemic properties independent of the mutational landscape of T-ALL cases. These findings provide robust evidence that α CD3 mAbs can be used as a new therapeutic tool for T-ALL treatment.

5. Cancer and cell death resistance: a close relationship

The health and homeostasis of organisms depend on the delicate balance between cell proliferation and cell death. Throughout our lives, we undergo dynamic processes that occur in light of this balance, from embryonic development to adult tissue homeostasis. Therefore, any disruption of this balance can lead to various pathologies, with cancer being a suitable example. In physiological conditions the intrinsic ability of cells to self-destruct in response to abnormal stimuli serves as a critical tumor suppressor mechanism (Delbridge, Valente and Strasser, 2012). Therefore, the dysfunction of regulated cell death conducting to its evasion contributes to cell transformation and is considered a hallmark of cancer (Hanahan and Weinberg, 2011). Based on this understanding, cancer therapies, including the widely used chemotherapies and new generation treatments, aim to restore the balance by inhibiting cancer cell survival and/or inducing cell death through varied strategies. Despite the current state of the art regarding the diverse forms of regulated cell death that have been described in both healthy and diseased states, this section will focus on intrinsic apoptosis and TNFRSF-induced cell death pathways, including extrinsic apoptosis within a physiological context. Additionally, specific disorders that promote resistance to cell death in cancer will be addressed, along with pharmacological strategies utilized to restore cell death and ultimately overcome resistance.

5.1. Intrinsic apoptosis

Intrinsic apoptosis is a type of regulated cell death that is initiated among others by DNA damage, endoplasmic reticulum (ER) stress, oxidative stress, microtubular alterations, and growth factor withdrawal. A key irreversible step to intrinsic apoptosis to occur is the mitochondrial outer membrane permeabilization (MOMP), that is tightly regulated by the pro-apoptotic and anti-apoptotic BCL-2

family members (described below). MOMP leads to the release, from the mitochondria to the cytoplasm, of other pro-apoptotic proteins, including cytochrome C (CYT C) and the second mitochondria-derived activator of caspases (SMAC). In the cytoplasm, CYT C associates with apoptosis protease-activating factor-1 (APAF1) and pro-CASP9, forming the apoptosome complex (illustrated in **Figure 17**). The apoptosome activates the initiator caspase 9, which in turn activates the executor caspase 3 and caspase 7, responsible for the ultimate cellular proteolysis (Garrido et al., 2006). Concomitantly, SMAC release facilitates caspase activation by neutralizing endogenous caspase inhibitors, such as XIAP, a member of the Inhibitor of Apoptosis (IAP) family (Du et al., 2000).

Figure 17. Molecular machinery of apoptosis: intrinsic and extrinsic pathways.

Right: Apoptotic stimuli result in the activation of the apoptotic activators BAX and BAK by the BH3-only protein sensors. The activation of BAX and BAK leads to their oligomerization at the mitochondrial outer membrane (MOM). Mitochondrial outer membrane permeabilization (MOMP) is considered a point of no return in the intrinsic apoptosis pathway as it results in the release of pro-apoptotic proteins such as cytochrome C (CYT C) and SMAC. This release ultimately promotes the activation of the proteolytic effector caspases 3 and 7. **Left:** Extrinsic apoptosis is initiated by the binding of TNFR with their ligands. Upon stimulation, FADD associates with the cytoplasmic tails of the TNF receptors and with pro-caspase 8 through death domain (DD) and death effector domain (DED) interactions. The formation of this complex prompts auto-cleavage and activation of caspase 8. Ultimately, activated caspase 8 mediates the activation of the effectors CASP3 and CASP7. Additionally, CASP8 can indirectly induce MOMP through the cleavage and activation of the BH3-only protein BID. Scheme from (Ichim and Tait, 2016).

5.1.1. Bcl-2 proteins: regulators of apoptosis

The Bcl-2 family comprises proteins expressing a sequence homology in a conserved α -helical regions called BCL-2 homology (BH) domains. Based on their structure and role in apoptosis, the family members are classified into three groups: 1) The multidomain anti-apoptotic members, including BCL-2, BCLXL, BCLW, MCL1, and BFL1; 2) The multidomain pro-apoptotic pore-formers/ effectors, such as BAX,

BAK, and BOK; and 3) The BH3-only pro-apoptotic members which are subclassified as activators (BID and BIM) and sensitizers (NOXA, PUMA, BAD, BIK, BMF, HRK) (illustrated in **Figure 18**).

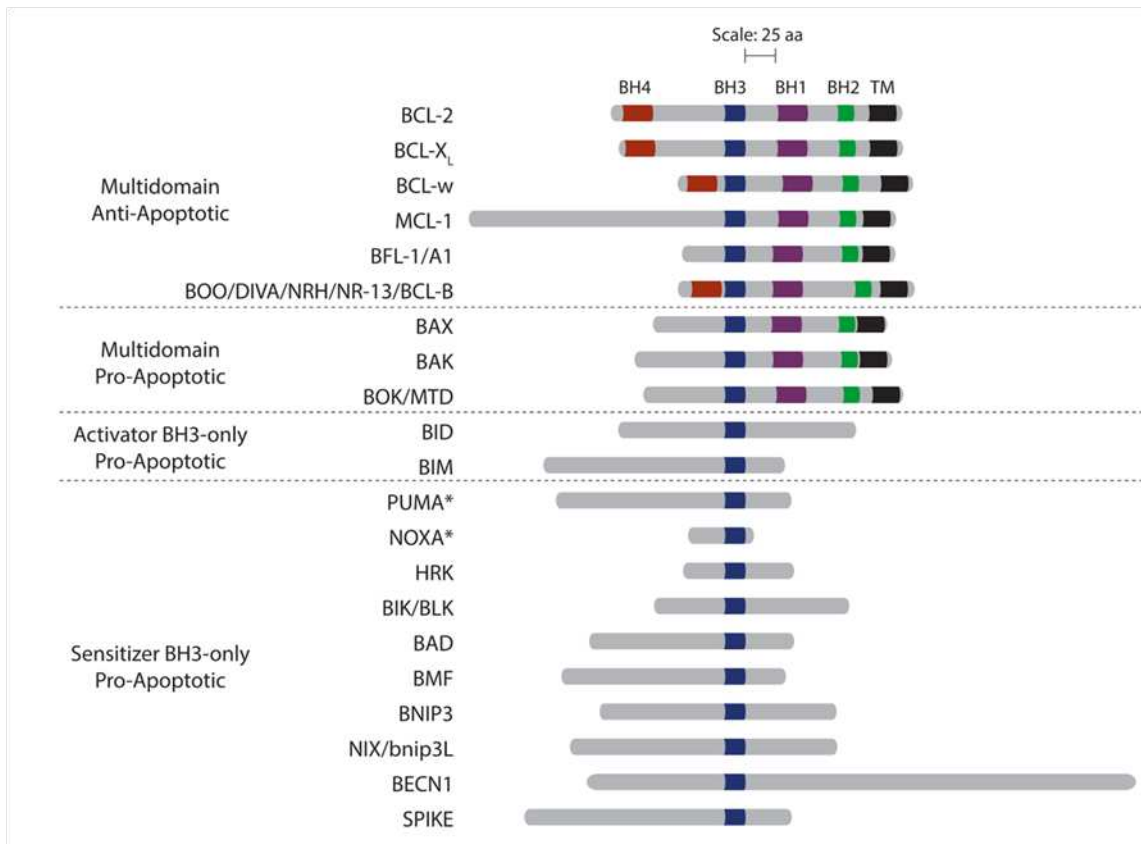


Figure 18. Bcl-2 family members classification.

On the left: Classification of the Bcl-2 family members according to their function. On the right: the Bcl-2 family members and their conserved BCL-2 homology (BH) domains and Transmembrane domain (TM). Scheme modified from (Gross and Katz, 2017).

BCL-2 family members are critical regulators of the intrinsic apoptosis pathway. This regulation is based on their ability to form homo- and heterodimers between anti- and pro-apoptotic proteins, in which cell fate is decided by the net interactions among the BCL-2 family proteins (Kale, Osterlund and Andrews, 2018). Upon apoptotic stimuli, different factors affecting such interactions are altered. These

include transcriptional regulation inducing the expression of pro-apoptotic BCL-2 family members (Miyashita and Reed, 1995; Burlacu, 2003), and posttranslational modifications (mainly phosphorylation), changing the conformational status of BCL-2 proteins and consequently their interactions (Zha *et al.*, 1997).

As observed in **Figure 18** BCL-2 family members can have a hydrophobic C-terminal transmembrane domain (TM) that allow the protein bearers their tethering to intracellular membranes including those from mitochondria (Kale, Osterlund and Andrews, 2018). Importantly, BCL-2 family members possess BH domains that are essential for their interaction with other members of the family. All the anti-apoptotic and some pro-apoptotic members are BH multidomain bearers, containing from 3 to 4 BH domains. The BH1, BH2, and BH3 domains can fold and form a hydrophobic groove that interacts with high affinity with BH3-peptides, which can adopt an amphipathic α -helical conformation that fits into this groove.

Under steady-state conditions, the effector pore formers BAX and BAK remain inactive due to their interaction/sequestration with the anti-apoptotic members like BCL-2, BCLXL and MCL1 (**Table 5**). In contrast, under apoptotic stimuli, the expression of BH3-only proteins is induced, leading to their direct interaction with the anti-apoptotic Bcl-2 family members, which in turn leads to the release of BAX and BAK from their inactivating association. Free BAX and BAK then oligomerize at the mitochondrial outer membrane, eventually leading to its permeabilization (MOMP) (Youle and Strasser, 2008). Given the BH3 sequence homology, the BH3-only proteins can exhibit redundant roles. Reports studying their affinities to bind with multidomain members show that all the BH3-only proteins can bind with at least one Bcl-2 multidomain protein. Only selected BH3-only proteins like BIM and PUMA can bind with high affinity to all BCL-2 anti-apoptotic proteins, which defines their potent capacity as apoptotic inducers (Chen *et al.*, 2005) (**Table 5**).

Multi-domain BCL2-family member	Role in apoptosis	Main functions in apoptosis	BH3-only interacting proteins	Multi-domain BCL2 interacting proteins
BAK	Pro-apoptotic	When activated permeabilizes MOM	BIM, PUMA, tBID, NOXA	BCL2, BCLXL, MCL1
BAX	Pro-apoptotic	When activated permeabilizes MOM	BIM, PUMA, tBID	BCL2, BCLXL, MCL1
BCL2	Anti-apoptotic	Inhibits apoptotic effectors and BH3-only proteins	BIM, PUMA, tBID, BAD	BAX, BAK
BCLXL	Anti-apoptotic	Inhibits apoptotic effectors and BH3-only proteins	BIM, PUMA, tBID, BAD, BIK, HRK	BAX, BAK
MCL1	Anti-apoptotic	Inhibits apoptotic effectors and BH3-only proteins	BIM, PUMA, tBID, NOXA	BAX, BAK, BOK

Table 5. BCL2-family members interactions. Modified from (Dai, Meng and Kaufmann, 2016).

5.1.2. Aberrant expression *BCL-2* family genes in cancer

Decades of studies have shown that cancer cells frequently employ mechanisms to evade cell death, which involves abnormal gene expression at the transcriptional level of anti-apoptotic/pro-survival and pro-apoptotic members of the BCL-2 family. These mechanisms include:

Chromosomal alterations, such as the translocation (14;18) in B-cell lymphomas, which led to the discovery of the founder member of the family, BCL-2. This translocation results in the fusion of the *BCL-2* gene located on chromosome 18, under the regulation of the immunoglobulin heavy-chain enhancer, leading to increased expression of BCL-2 (Bakhshi *et al.*, 1985; Tsujimoto *et al.*, 1985). Another example is the upregulated expression of MCL-1 in multiple myeloma (MM), caused by a frequent chromosomal aberration occurring in 40% of MM patients. This aberration involves the amplification of chromosome arm 1q21 (1q21+), which contains the *MCL-1* gene, of note, patients harboring this abnormality have been associated with lower overall survival (Hanamura, 2021).

Altered gene regulation of members of the BCL-2 family. Anti-apoptotic BCL-2 members can exhibit increased expression levels caused by the deletion or loss-of-function mutations of genes encoding their endogenous negative gene regulators.

Some examples include microRNAs (miRs) found in different leukemias negatively regulating the expression of BCL-2 (Cimmino *et al.*, 2005) and MCL-1 (Garzon *et al.*, 2007). Moreover, aberrant gene regulation can also lead to poor levels of BCL-2 pro-apoptotic members. An example of this are mutations leading to the inactivation or decreased activity of the master transcription factor p53, which occurs in nearly all cancers. p53 is well-known for its tumor suppressor gene activities, primarily exerted through the induction of apoptosis by regulating many target genes among which, BAX, BID, PUMA, and NOXA (Miyashita and Reed, 1995; Yip and Reed, 2008). Consequently, p53-mutated cells are less susceptible to undergo apoptosis. In T-ALL, specifically within the ETP-ALL subgroup, which is often associated with chemotherapy resistance and classified as a high-risk subgroup (Maude *et al.*, 2015), increased levels of BCL-2 expression have been observed. Additionally, BH3-profiling in ETP-ALL cell lines and primary samples demonstrates their anti-apoptotic dependency on the BCL-2 protein (Chonghaile *et al.*, 2014). Regulatory mechanisms behind the specific upregulation of BCL-2 in this T-ALL subgroup are not completely solved. Physiologically, IL-7 exerts pro-survival functions by regulating BCL-2 expression. In T-ALL, activating mutations in IL-7 are frequently observed in the ETP ALL subgroup. Furthermore, IL-7 pro-survival signaling is found to be concurrent with BCL-2 up-regulation (Barata *et al.*, 2001), strongly suggesting that the overexpression of BCL-2 in ETP-ALL may be attributed to IL-7 signaling. This hypothesis is supported by comparative analysis in T-ALL mouse models, which shows that increased levels of BCL-2 expression are only observed in *TEL-JAK2*-expressing leukemic T cells, and not in intracellular NOTCH-1 (ICN1)-driven T cell leukemia (Waibel *et al.*, 2013).

5.2. TNFR-mediated cell death

5.2.1. TNF dead receptors

Extrinsic apoptosis is a regulated form of cell death initiated by alterations in the extracellular microenvironment, triggered by specific receptors of the Tumor Necrosis Factor Super Family (TNFSF). Within this family, a subset of receptors, known as Death Receptors (DR) **Table 6**, are categorized based on their structure, which is characterized by bearing an 80-amino acid sequence in their cytoplasmic tails, called the Death Domain (DD) (Riley et al., 2015).

Receptor Name (Alternative Names)	Ligand Name (Alternative Names)
TNFR1 (TNFRSF1A, DR1)	TNF α (TNFSF2), LT- α (TNFSF1)
Fas (TNFRSF6, CD95, Apo1, DR2)	FasL (TNFSF6, CD95L, Apo1L)
TRAILR1 (TNFRSF10A, DR4, Apo2)	TRAIL (TNFSF10, Apo2L)
TRAILR2 (TNFRSF10B, DR5)	TRAIL (TNFSF10, Apo2L)

Table 6. Principal Death Receptors members of the TNF superfamily and their cognate ligands. Modified from (Dostert *et al.*, 2019).

Despite the critical role of the DDs in DR-induced apoptosis, various multiprotein complexes can be formed through cytoplasmic DD interactions, leading to different cell fates. The hallmark DRs associated with extrinsic apoptosis are FAS, TRAILR1, and TRAILR2, which assemble the death-inducing signaling complex (DISC). In contrast, upon stimulation, TNFR1's DD usually leads to the assembly of a multiprotein complex resulting in cytokine generation, inflammation, and cell survival, unless the TNFR1 cytotoxic potential is triggered by inhibiting these survival

pathways (Varfolomeev *et al.*, 2005; Yamada *et al.*, 2017). The following will describe 1) DR-induced extrinsic apoptosis, and 2) Focus on TNFR1 signaling.

5.2.2. Extrinsic apoptosis

DRs binding with their cognate ligands form hexameric complexes that promote the formation of the intracellular death-inducing signaling complex (DISC) at close proximity of the plasma membrane (Locksley, Killeen and Lenardo, 2001). DISC formation is facilitated by two homotypic interactions: 1) through DD between DR's cytoplasmic tails and cytosolic FADD, and 2) through the death effector domain (DED) between FADD and procaspase-8. Procaspase-8 recruitment to the DISC facilitates its dimerization, leading to conformational changes that promote its autoproteolytic cleavage and activation. Active caspase 8 then mediates the cleavage and activation of the executioner caspases 3 and caspase 7, ultimately leading to cellular proteolysis and cell death (Galluzzi *et al.*, 2018) **(Figure 17)**. Moreover, the extrinsic apoptosis signaling can be amplified through cross-talk with the intrinsic apoptosis pathway. This occurs through BID, a specific substrate of caspase 8. Caspase-8-mediated proteolysis generates truncated BID (tBID), promoting its translocation to the mitochondrial outer membrane (MOM) where it activates BAX and BAK, thereby promoting intrinsic apoptosis. (Li *et al.*, 1998). The significance of mitochondria-mediated apoptosis in the extrinsic apoptosis pathway is cell type-dependent. Type I cells, namely thymocytes and mature lymphocytes, exhibit high levels of DISC formation and high caspase 8 activity, leading to the activation of downstream effectors caspase 3 and 7 sufficient to commit cell apoptosis. In contrast, Type II cells, comprising hepatocytes and most cancer cells, show less DISC formation and, thus, poorer caspase 8 activity. Therefore, it is in this cell type where tBID-mediated apoptosis amplification plays an important role (Vitale *et al.*, 2023).

5.2.3. TNFR1-mediated cell survival signaling and cell death

Upon ligand binding, TNFR1 preferentially assembles the so-called complex I, which involves TNFR1 association through homotypic interactions with the DD-bearing adaptor protein TRADD (TNFR-associated death domain) at the plasma membrane. TRADD, in turn, recruits two additional adaptors: the DD-containing kinase RIPK1 and the TNF receptor-associated factor 2 (TRAF2), the latter serving as a recruiter for two E3 ubiquitin ligases, the cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2. cIAP1 and cIAP2 mediate the conjugation of non-degradative Lys-63 (K63) and K48-linked ubiquitin chains to RIPK1 (Varfolomeev *et al.*, 2008). Polyubiquitinated RIPK1 serves as a scaffold for the recruitment of two protein assemblies: 1) TAK1 kinase associated with its adaptor proteins (TABs) and 2) I κ B kinase (IKK) complex, which comprises two serine-threonine kinases IKK1/IKK α and IKK2/IKK β , as well as the regulatory subunit NF- κ B essential modulator NEMO/IKK γ (Dumétier, Zadoroznyj and Dubrez, 2020). The recruitment of these kinases to RIPK-K63-linked polyubiquitin chains places them in close proximity, facilitating either IKK auto-phosphorylation or TAK-1-mediated activation (**Figure 19**). IKK2/IKK β is best known for mediating the activation of NF- κ B signaling by phosphorylating the Inhibitor of NF- κ B (I κ B), which is a signal for I κ B ubiquitylation-dependent proteasomal degradation. I κ B degradation releases p50/p65(RelA) NF- κ B subunits, thus enabling their nuclear translocation and subsequent regulation of NF- κ B target genes (listed in **Table 1**) (Scheidereit, 2006).

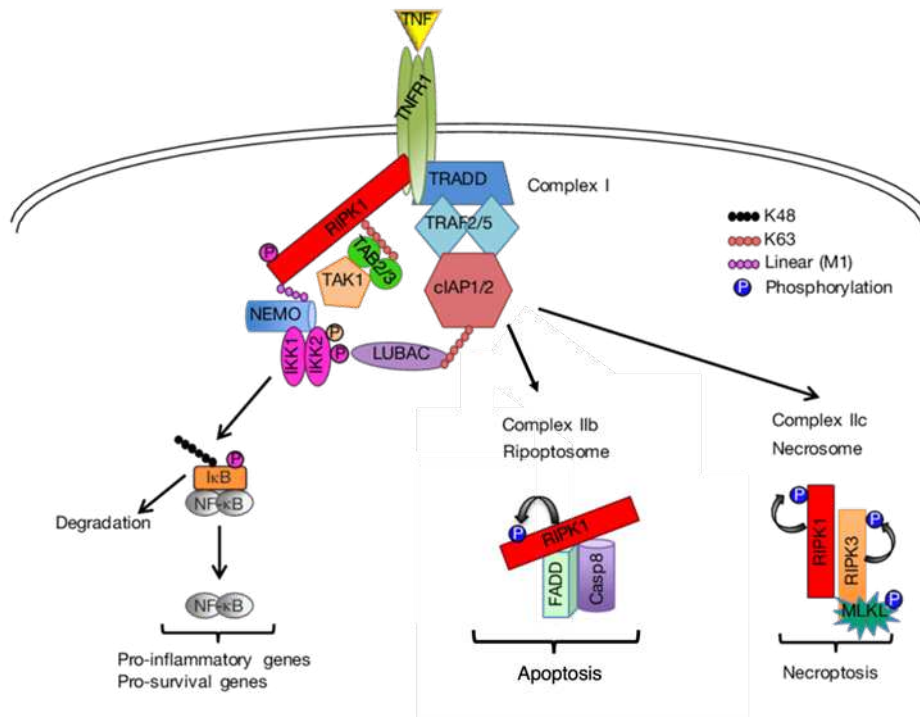


Figure 19. TNFR1 downstream signaling complexes.

TNF binding to TNFR1 initiates the assembly of a receptor proximal complex known as complex I. c-IAP-mediated ubiquitination of RIPK1, TRAF2, and the c-IAPs themselves, promote the recruitment of proteins essential for NF- κ B activation. Alternately, TNFR1 can trigger apoptosis through RIPK1-association with FADD-pro-caspase 8 (complex IIb/riposome). Also, TNFR1 can induce necroptosis through the formation of complex IIc/necrosome, which contains RIPK1, RIPK3, and MLKL. Scheme modified from (Kondylis, 2017)

Conversely, in cellular circumstances in which TNFR1-mediated NF- κ B signaling is inhibited, TNFR1 can induce the assembly of alternative signaling complexes that lead to strong cell death signaling, including apoptosis. Such conditions involve the ubiquitination status of RIPK1 that is dependent on expression and recruitment of cIAP1/cIAP2 to the complex I. Absence of cIAP1/cIAP2 can occur through induced degradation promoted by cIAP1 ubiquitination (Yang *et al.*, 2000) or by its natural binder-inhibitor, SMAC (Deng *et al.*, 2003). The deficit of cIAP1/2-mediated polyubiquitination of RIPK1 destabilizes RIPK1 association to complex I, thus promoting its release (Peltzer, Darding and Walczak, 2016). As depicted in

Figure 19, non-ubiquitylated RIPK1 can form cytosolic signaling complexes that ultimately lead to cell death induction. Specifically, complex IIb, also known as the ripoptosome, emerges when translocated unmodified RIPK1 associates with FADD, pro-caspase 8, and FLIPL. The formation of pro-caspase 8 homodimers and pro-caspase 8 - FLIPL heterodimers mediates RIPK1 inactivation, which is necessary for inducing classical caspase 8-mediated extrinsic apoptosis signaling (Brenner, Blaser and Mak, 2015). Further TNFR1-induced alternative complex assemblies leading to cell death include the complex IIb or necrosome. Necrosome formation relies on the kinase activity of RIPK1 and RIPK3. Given that caspase 8 negatively regulates RIPK1 kinase activity, necrosome assembly can only occur under conditions of depletion or inactivity of caspase 8. RIPK1 and RIPK3 associate through their RIP homotypic interaction motif (RHIM) domains. RIPK3 activation, which is thought to be RIPK1-mediated, promotes recruitment and phosphorylation of the activation loop of the mixed lineage kinase domain-like (MLKL). MLKL activation result in conformational changes leading to the exposure of its N-terminal DED also called 4 helical bundle domain, 4HBD. This exposure is essential for the culmination of necroptosis, as it allows MLKL translocation to the plasma membrane, ultimately leading to cell permeabilization and death (**Figure 19**) (Weber *et al.*, 2018).

5.2.4. Modulators of DR-mediated cell death

Modulation of DR-mediated cell death can occur at many levels of the signaling pathway, ranging from the regulation of the expression of ligands, receptors, adaptors, and executors of the pathway. Additionally, post-translational modifications can also regulate their expression, their conformational state, and subcellular localization. Despite the diversity of regulations that can occur, this section is only focused on two important regulators: c-FLIP and cIAP protein.

5.2.4.1. c-FLIP regulation

The cellular FLICE inhibitory protein (c-FLIP) is a major suppressor of the extrinsic apoptosis pathway expressed in response to NF- κ B activation. NF- κ B-mediated protection from pro-apoptotic signaling is an important mechanism for tissue homeostasis. This has been observed in mice with homozygous deletion of *Cflar*, encoding c-FLIP, which results in embryonic lethality. Additionally, conditional *Cflar* deletion in fully developed mouse epidermis leads to severe inflammation. Furthermore, while *Ripk3* ablation rescues the phenotype of *caspase-8* $-/-$ mice, it does not rescue embryonic lethality in *Cflar* $-/-$ mice, which is accompanied by high caspase-8 activation and apoptosis (Humphreys, Espona-Fiedler and Longley, 2018). These observations suggest c-FLIP as a negative regulator of caspase-8 activity.

Human cells mainly express two c-FLIP splice variants: one long (c-FLIP_L) and one short (c-FLIP_S). Structurally, both c-FLIPs bear two tandem DEDs that enable protein-protein interactions with pro-caspase. In the C-terminal part, only c-FLIP_L contains a caspase-like domain, exhibiting great structural similarities with caspase 8 but lacking caspase activity. High expression levels of c-FLIP promote caspase inhibition at the DISC by associating with pro-caspase 8 via DED interactions and assemble pro-caspase 8/cFLIP heterodimers. c-FLIP_S association with pro-caspase-8 results in competitive inhibition, impeding pro-caspase-8 homodimerization and thus preventing its proteolytic self-activation (Ivanisenko *et al.*, 2022). In contrast, c-FLIP_L association with pro-caspase 8 results in the assembly of an active enzymatic complex capable of cleaving both pro-caspase 8 homodimers and pro-caspase 8/c-FLIP_L heterodimers. This results in a regulatory mechanism that leads to caspase 8 activation or inhibition reliant on c-FLIP_L stoichiometry. Moreover, pro-caspase 8/c-FLIP_L heterodimers can also cleave RIPK1 resulting in a negative regulation of the necrosome formation (**Figure 20**) (Humphreys, Espona-Fiedler and Longley, 2018)

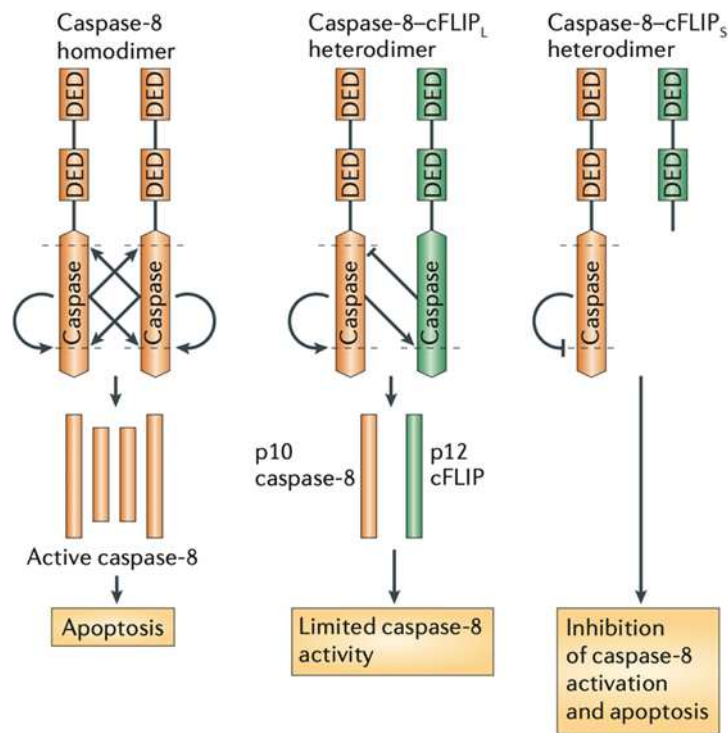


Figure 20. c-FLIP regulates activation of caspase-8. Pro-caspase-8 homodimerization results in its autoproteolytic cleavage, activation, and ultimately apoptosis. Heterodimerization of pro-caspase-8 with cFLIP_L results in partial processing of pro-caspase-8 and non-apoptotic enzymatic activity. Heterodimerization with cFLIP_S, which lacks the activation loop of cFLIP_L, leads to the inhibition of pro-caspase 8 activity and apoptosis. Scheme modified from (Budd, Yeh and Tschopp, 2006).

5.2.4.2. Inhibitor of apoptosis (IAP) family

There are eight IAP proteins in humans: neuronal apoptosis inhibitory protein (NAIP), encoded by *BIRC1*, cellular IAP1 (cIAP1) encoded by *BIRC2*, cellular IAP2 (c-IAP2) encoded by *BIRC3*, X chromosome linked IAP (XIAP) encoded by *BIRC4*, survivin encoded by *BIRC5*, ubiquitin-conjugating BIR domain enzyme apollon encoded by *BIRC6*, melanoma IAP (ML-IAP) encoded by *BIRC7*, and IAP-like protein 2 (ILP2) encoded by *BIRC8* (**Figure 21**). Some IAP family members are well

known for their ability to bind and inhibit caspases. However, among them, cIAP1 and cIAP2 play a prominent regulatory role in TNFR-mediated survival and cell death. Therefore, this section will focus on the regulatory activities of these family members.

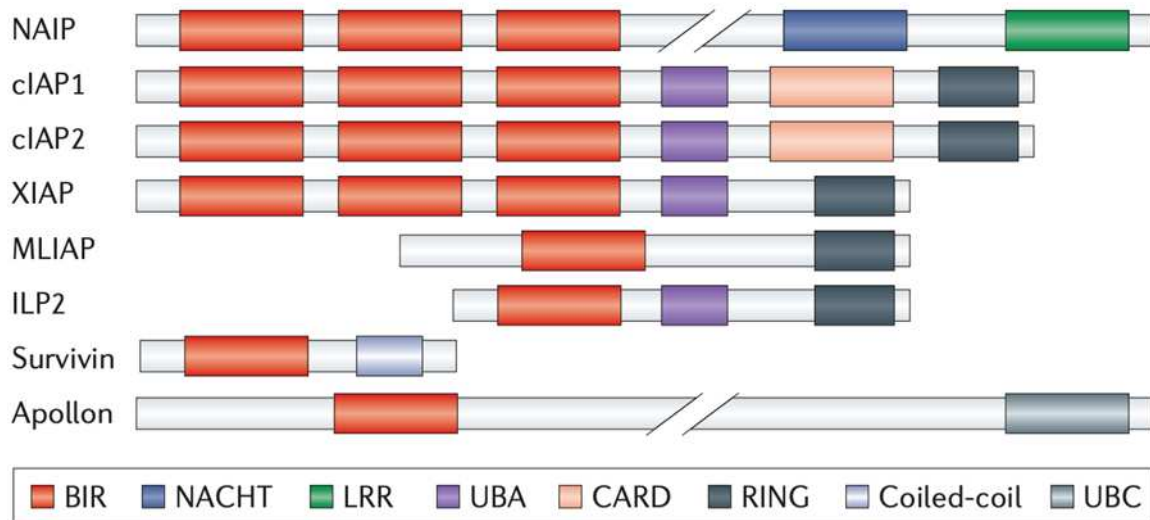


Figure 21. Protein structure of human IAPs. The common feature of all IAP proteins is their expression baculoviral IAP repeat (BIR) domain BIR that mediate IAP's protein-protein interactions. Common domains in cIAP1, cIAP2, XIAP and ILP2 include RING and ubiquitin-associated domain (UBA). RING is located at the carboxy-terminal that provide IAP proteins a E3 ubiquitin ligase activity meanwhile UBA allow IAP proteins to bind to monoubiquitin and polyubiquitin chains. Additional domains include caspase recruitment domain (CARD) in cIAP1 and cIAP2, the ubiquitin-conjugating (UBC) domain in apollon, the coiled-coil region in survivin, and the nucleotide-binding and oligomerization domain as well as the leucine-rich repeat (LRR) domains present in NAIP12–16. Scheme from (Fulda and Vucic, 2012).

cIAP1 and cIAP2 were identified in a search for cytoplasmic proteins associated downstream the TNF receptors. They were found to be recruited via the TNFR adaptor protein TRAF (Rothe *et al.*, 1995). Genetic studies in mice to identify cIAP1 and cIAP2 roles on the TNFR signaling exhibited absence of phenotype in single cIAP1 and cIAP2 knockouts. Due to considerable structural similarities between cIAP1 and cIAP2 these observations are indicative of cIAP1 and cIAP2

compensatory functions. Meanwhile, double knockout mice *clap1*^{-/-} and *clap2*^{-/-} are embryonic lethal, this phenotype is partially rescued by *Tnfr1* gene deletion which result in prolonged embryonic survival until birth, demonstrating that cIAP1 and cIAP2 can regulate TNF-R1 signaling pathway (Moulin *et al.*, 2012). In line with this, overexpression of cIAP1 (Samuel *et al.*, 2006) and cIAP2 (Chu *et al.*, 1997) in mammalian cells show that they mediate NF- κ B activation and suppress TNF cytotoxicity.

Key feature of cIAP1 and cIAP2 to mediate activation of the canonical NF- κ B signaling pathway is their E3 ubiquitin ligase activity. cIAP1 and cIAP2 have been found to mediate the polymerization non-degradative Lys-63 (K63) chains in the critical adapter in the canonical NF- κ B signaling pathway, RIPK1. Experimental conditions that induce cIAP1 and cIAP2 deficiency including genetic ablation (Varfolomeev *et al.*, 2008) or pharmacological induced degradation (Varfolomeev *et al.*, 2007) strongly prevented TNF α -induced NF- κ B activation and decline cell viability. These observations unveiling critical role of cIAP1 and cIAP2 in protection cells against TNF α -induced cell death.

5.2.5. Aberrancies in cancer leading to abnormal extrinsic apoptosis pathway

Defects in DR-dependent apoptosis have been associated with several types of cancer. These include: 1) reduced activity of dead receptor-ligand axis, and 2) aberrant expression of regulators of the pathway.

1) Reduced activity of dead receptor-ligand axis: Hampered activity of FAS-FASL axis related to malignancy can be caused by germline and somatic mutations leading to aberrant loss of FAS. The first *FAS* gene mutations identified in mice (*lpr*) results in lymphadenopathy and splenomegaly (Adachi *et al.*, 1995). In humans, weakened Fas-induced signal transduction caused by germline point mutations occurring in the *FAS* gene is associated with lymphoproliferation, including T and B-cell lymphomas (Lim *et al.*, 1998; Peters *et al.*, 1999). In T-ALL mutations affecting

the *FAS* gene result in *FAS* decreased expression resulting in decreased sensitivity to *FASL* induced apoptosis (Beltinger *et al.*, 1998; Tamiya *et al.*, 1998).

2) Aberrant expression of regulators of the pathway: c-FLIP expression is frequently increased in a wide variety of cancer types, including lymphomas and B-cell leukemias (Olsson *et al.*, 2001; Mathas *et al.*, 2004). c-FLIP upregulation in cancer cells is concurrent with increased resistance to cell death mediated by *FAS* (Korkolopoulou *et al.*, 2004) and *TRAIL* (Zhang *et al.*, 2004), a feature associated with tumor aggressiveness and poor prognosis for patients (Djerbi *et al.*, 1999). Anomalous expression of IAP proteins is often caused by chromosomal amplification in the 11q21-22 region (Bhosale *et al.*, 2017). This amplification leads to high expression of cIAP1 and cIAP2 in various cancer types (Dubrez, Berthelet and Glorian, 2013). In patients with B-cell lymphomas elevated expression levels of cIAP1 and IAP2 has been found (73% and 48% respectively). In B-cell chronic lymphocytic leukemia overexpression of cIAP1 and cIAP2 is even concurrent with low expression of *SMAC*. Of note, a patient showing high levels of cIAP1 and cIAP2 have been associated with treatment resistance and, poor overall survival (Grzybowska-Izydorczyk *et al.*, 2010). Given the significance of apoptosis regulators and their frequent alteration in cancer, they become attractive targets for therapeutic intervention. Representative therapeutic strategies will be described below.

5.3. Reinstalling cell death as anti-cancer therapeutic strategy

5.3.1. Targeting BCL-2 family members

Strategies to interfere with the pro-survival properties of the BCL-2 family members include the use of compounds that regulate gene expression or target the mRNA of the anti-apoptotic BCL-2 family members. Other drugs induce the activation of endogenous antagonists of BCL2 proteins, while others directly inhibit the physiological functions of the anti-apoptotic BCL-2 proteins (Reed and Pellecchia, 2005). The most advanced nowadays are the BH3 mimetic drugs which

sequester anti-apoptotic members of BCL-2 family enabling the release and activation of BAX and BAK, thereby promoting apoptosis without the need for prior apoptotic stimuli (Delbridge and Strasser, 2015).

The first designed BH3 mimetic was ABT-737, followed by ABT263 (Navitoclax), which binds with high affinity to BCL-2, BCL-XL, and BCL-W but not MCL-1 (Delft *et al.*, 2006; Tse *et al.*, 2008). Clinical trials for hematological malignancies and solid tumors showed that Navitoclax causes toxicity by affecting mature platelets, which exhibit increased drug sensitivity due to their physiological high BCL-XL expression levels (Wilson *et al.*, 2010). Consequently, ABT-199 (Venetoclax) was developed as a selective and potent inhibitor of BCL-2 (Souers *et al.*, 2013). In 2015, Venetoclax was approved by the FDA as the first-in-class BCL-2 inhibitor for the treatment of patients with AML and CLL (Yosifov *et al.*, 2019). Specifically in T-ALL, as described in the "**Targeted therapies for relapse cases**" section, relapsed T-ALL patients have benefited from Venetoclax in combination with standard chemotherapy, achieving remission in 60% of the patients (Richard-Carpentier *et al.*, 2020). Nevertheless, despite the on-target off-tumor toxicities of BCL-XL inhibitors, BCL-XL is still an important validated cancer target. Recent studies have developed a BCL-XL proteolysis-targeting chimera (PROTAC) called DT2216 that targets BCL-XL for VHL E3 ligase-mediated degradation. Because VHL is poorly expressed in platelets, this considerably reduces toxicity effects in platelets while conserving its antitumor effects. It has been found to inhibit the growth of different xenograft tumors as a single agent or in combination with chemotherapy (Khan *et al.*, 2019).

Inhibitors targeting MCL-1 have also been generated; however, their clinical development has been delayed. The main cause of this delay is the challenge in producing anti-MCL-1 compounds, given that the hydrophobic BH3 binding groove is large and surface-exposed. As a result, MCL-1 inhibitors often exhibit poor selectivity and/or poor affinity (Day *et al.*, 2005). Despite this difficulty, some MCL-1 antagonists have been designed and synthesized with proven high affinity at low nanomolar concentrations and potent inhibitory activity towards MCL-1. Compounds

with these characteristics include S63845, AZD5991, and the first-in-class orally bioavailable MCL-1 inhibitor AZD5991. These compounds are currently being studied in phase I clinical trials for the treatment of different leukemias, including multiple myeloma (MM), AML, and lymphomas (Bolomsky *et al.*, 2020).

5.3.2. Targeting IAP family members

The AVPI (Ala-Val-Pro-Leu) peptide motif of SMAC binds to and neutralizes IAP functions, thereby promoting pro-death signaling (Fulda and Vucic, 2012). Synthetic molecules that mimic the antagonist interaction of the SMAC AVPI motif with IAP proteins have been generated to interfere with IAP functions. Peptides derived from the N-terminus of active SMAC were the first to be used to show an effective mimicking activity of SMAC. The use of SMAC-derived peptides demonstrated the potential pro-apoptotic stimuli by targeting IAP proteins both *in vitro*, sensitizing cancer cells to chemotherapy agents (Vucic *et al.*, 2002); and *in vivo*, (Fulda *et al.*, 2002). Despite their experimental efficacy, SMAC-derived peptides exhibited poor pharmacological properties.

Monovalent or bivalent small-molecule SMAC mimetic drugs (SM) presented improved pharmacological features including stability, high affinity, and cell permeability (Fulda and Vucic, 2012). Monovalent compounds were designed to mimic the binding of a single AVPI motif, while bivalent compounds consist of two chemically linked AVPI motifs. Similar to SMAC, whose biological functions are mainly active when dimerized, the bivalent compounds display higher affinity to IAP proteins with 2–3 orders of magnitude higher than monovalent antagonist (Sun *et al.*, 2007). One of the most clinically advanced compound is Birinapant, a bivalent SM antagonist that exhibits higher affinity for the BIR3 domain of cIAP1 ($K_i \sim 1$ nM) compared to cIAP2 (36 nM) and XIAP (50 ± 23 nM). Large-scale analysis in 111 different cancer types showed that 16% of the variety of cells had sensitivity to Birinapant as a single agent. Also, Birinapant administration *in vivo* was assessed in

50 different PDX models showing an inhibition of the tumor progression in ~30% of the cases with monotherapy regimens (Moulin *et al.*, 2012). Phase I trials involving the use of Birinapant reported the safety and good tolerability of compound administration in patients . Phase II trials have demonstrated anti-tumor effects of Birinapant monotherapy, resulting in partial remission in patients with various types of tumors, including patients with AML (Frey *et al.*, 2014). Furthermore, trials evaluating the combination of Birinapant with standard chemotherapy have revealed a clinical benefit from this combination in relapse/refractory patients across a range of tumor types (Amaravadi *et al.*, 2013).

Interfering with specific cancer cell survival mechanisms is a pertinent approach to improving cancer treatments. The "**Results**" chapter of my thesis will describe the identification of active survival pathways induced in T-ALL upon α CD3 mAbs administration. Blocking these pathways has shown the potential to strengthen the T-ALL vulnerability to the therapeutic effects of α CD3 mAbs.

Chapter II.

Research objectives

1. Research objectives

Acute lymphoblastic leukemia (ALL) is a group of leukemias defined by the proliferation of lymphoblast blocked at different stages of differentiation, which represent the most common pediatric malignancy. ALL can affect either B or T lineages with T-ALL accounting for 10-15% of pediatric ALL and 25% of adult cases. Although intensive chemotherapy leads to remission, 20% of pediatric T-ALL cases and 50% of adult cases ultimately relapse with few therapeutic options and poor survival outcome. T-ALL form a heterogeneous group of malignancies that are classified into different molecular subtypes on the basis of aberrant expression of specific, driver oncogenic transcription factors and global transcriptomic signatures. However, this impressive knowledge has so far not translated clinically into efficient targeted therapies.

While recent immune-therapeutic approaches have considerably improved the therapeutic arsenal in B-cell acute lymphoblastic leukemia, such therapies are under-developed in T-ALL. My host laboratory demonstrated that sustained activation of TCR signaling in T-ALL cells by anti-CD3 monoclonal antibodies (α CD3 mAbs), including the murine anti-hCD3 mAb OKT3 and the clinically relevant α CD3 mAbs Teplizumab and Foralumab, induce leukemic cell death and show strong anti-leukemic effect in 12/12 Patient-Derived Xenografts (PDX) established in NSG mice from T-ALL TCR+ diagnostic cases. Although use of α CD3 mAbs opens a new immune-therapeutic option in T-ALL, in particular when associated with chemotherapy, anti-CD3 monotherapy results in relapse in T-ALL PDX. It is thus essential to identify the molecular mechanisms underlying the anti-leukemic properties of α CD3 mAbs and target these mechanisms to increase T-ALL vulnerability to α CD3 immuno-therapy.

Transcriptomic analysis identified the molecular signature induced upon α CD3 mAbs treatment in T-ALL PDX. Top up-regulated genes identified by GO/KEGG pathways analyses include: (i) Genes essential to physiological thymic negative selection, including BIM and NR4A1. Accordingly, constitutive expression

of BIM and NR4A1 were found deleterious to the maintenance of T-ALL cells (B Zaniboni, PhD thesis, 2020). (ii) TNF α signaling via NF- κ B, (iii) PDCD1, encoding PD1, a physiological inhibitor of TCR signaling and its ligand PDL1 and (iv) the TNF receptors 4-1BB and OX-40 which are identified as TCR co-receptors. Thus, the aim of the present thesis is to investigate the implication of the identified signaling pathways and individual players on the intrinsic T-ALL cell death and anti-leukemic effects induced by α CD3 mAbs in T-ALL PDXs.

Specifically, the objectives are to:

- (i) Validate and extend the understanding of deregulation of these pathways to a range of T-ALL PDX models harboring different combinations of oncogenic mutations
- (ii) Employ molecular and pharmacological approaches to assess the significance of the deregulation of these pathways in α CD3-induced anti-leukemic effects, as well as identify strategies to enhance T-ALL vulnerability to the α CD3 therapy.

Chapter III.

Material and Methods

1. Material and Methods

This chapter includes the materials and methods that were not described in the manuscript under submission but were implemented in the additional results presented in this Thesis.

1.1. Cell culture conditions and in vitro α CD3 stimulation

CEM and Jurkat TCR+ T-ALL cell lines were cultured in RPMI-1640 medium Supplemented with 50 μ g/ml streptomycin, 50 IU penicillin, and 15% fetal bovine serum (Gibco, Life Technology), maintained at 37°C with 5% CO₂ incubation. α CD3 stimulation was induced in freshly cultured cells using 3 beads per cell (Dynabeads Human Activator CD3/CD28, Invitrogen).

1.2. Lentiviral production and transduction

Human 293T cells were seeded at 3×10^6 cells in a 10cm-dish. 24h after cells were co-transfected with the lentiviral plasmid containing the shRNA of interest along with pSPAX2 (packaging) and pVSVG (envelope) vectors. 48h post-transfection the viral particles contained on the supernatant were harvested and used to infect freshly cultured CEM and Jurkat cells. After 48h of viral particles exposure, successfully transduced cells were selected with puromycin (3 μ g/mL) kept over at least one week before using for experimental assessments. Human PD-1 knockdown plasmids containing shRNAs sequences cloned into the pLKO.1-puro vector shRNA #10 (TRCN0000083510) and shRNA #12 (TRCN0000083512) were obtained from MISSION shRNA Bacterial Glycerol stock from Sigma-Aldrich.

1.3. Pharmacological treatment in vivo

The antagonistic antibody targeting PD-1 Pembrolizumab was co-administered with OKT3 intravenously at the following doses: 200ug of Pembrolizumab and 4ug of OKT3 per mice. PD1 staining for FACS analysis was performed to monitor the length of Pembrolizumab binding on T-ALL cells. anti-PD-1 (EH12.2H7) obtained by BioLegend. The agonistic antibody targeting 4-1BB Urelumab was co-administered with OKT3 intravenously. Doses used were 200ug of Urelumab and 4ug of OKT3 per mice. Urelumab mAb was obtained from Creative Biolabs. Control unstimulated mice were administered with isotypic control antibodies. IgG4 was used as isotypic for Pembrolizumab and Urelumab and IgG2a for OKT3, both isotypic antibodies were obtained from Bio X Cell.

Chapter IV.

Results Part I

Results Part I contains an original manuscript submitted in *Blood* on September 7th, 2023.

Targeting the TNF/IAP pathway synergizes with anti-CD3 immunotherapy in T-Cell Acute Lymphoblastic Leukemia

Short Title (50 characters max comprising space):

- cIAP inhibition synergizes with anti-CD3 in T-ALL

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52

Word count for text: 3997

Word count for abstract: 221

6 Figures, 4 Supplemental Figures, 5 Supplemental Tables

32 references (796 words)

Key points

- TNF via NF κ B pathway is induced in response to anti-CD3 immunotherapy in T-ALL PDXs
- TNF neutralization and the SMAC mimetic Birinapant synergize with anti-CD3-induced cell death and anti-leukemic properties

ABSTRACT

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological malignancy. Current treatments, based on intensive chemotherapy regimens provide overall survival rates of ~85% in children and <50% in adults, calling the search of new therapeutic options. We previously reported that targeting the T cell receptor (TCR) in T-ALL with anti-CD3 (α CD3) mAbs enforces a molecular program akin to thymic negative selection, a major developmental checkpoint in normal T-cell development, induces leukemic cell death and impairs leukemia progression to ultimately improve host survival. However, α CD3 monotherapy resulted in relapse. To find out actionable targets able to re-enforce leukemic cells vulnerability to α CD3 mAbs, including the clinically relevant Teplizumab, we identified the molecular program induced by α CD3 mAbs in PDXs-derived from T-ALL cases. Using large-scale transcriptomic analysis, we found prominent expression of TNF α , LT α and multiple components of the “TNF α via NF κ B signaling” pathway in anti-CD3-treated T-ALL. We show *in vivo* that Etanercept, a sink for TNF α /LT α , enhances α CD3 anti-

leukemic properties, indicating that TNF/TNFR survival pathways interferes with TCR-induced leukemic cell death. However, suppression of TNF-mediated survival and switch to TNFR-mediated cell death through inhibition of c-IAP1/2 with the SMAC mimetic Birinapant synergized with α -CD3 to impair leukemia expansion in a RIPK1-dependent manner and improve mice survival. Thus, our results advocate the use of Birinapant or other SMAC mimetics to improve anti-CD3 immunotherapy in T-ALL.

INTRODUCTION

T-Cell lineage Acute Lymphoblastic Leukemia (T-ALL) derive from the transformation of thymic T cell progenitors and is an aggressive disease characterized by the proliferation and accumulation in blood, bone marrow and lymphoid organs of T-cell precursors abnormally arrested in differentiation. Current first-line chemotherapy regimens provide overall survival rates of approximately 85-90% in children and about 50% in adults. Refractory and relapse cases (r/r T-ALL) have a dismal prognosis that is improved by use of nelarabine either as single agent or combined with chemotherapy ¹. Immunotherapy, including CAR-T cell therapies, which have proven their efficacy in B cell leukemias, lags behind in T-ALL as targetable surface antigens are shared between leukemic cells and normal T cells, rendering specific targeting of T-ALL difficult. For example, CAR-T cells approaches in T-ALL are limited by CAR-T fratricide activity, depletion of host T cells and risk of graft-versus-host disease (GvHD) when performed in an allogeneic setting, that need to be corrected by multiple editing of the CAR-T cell genome that may turn out clinically problematic. It is thus essential to identify new, alternative therapeutic tools to offer more variety of options to treat T-ALL patients.

Our previous genetic studies using a mouse T Cell Receptor (TCR)-positive T-ALL model driven by an activated Janus kinase (JAK) allele show that signaling from a negative-selecting mouse TCR – but not from a positively-selecting TCR- severely interferes with T-ALL development ² and progression ³. We further extended the anti-leukemic properties of TCR activation to T-ALL patient-derived xenografts

(PDXs) by showing that engagement of leukemic cells TCR by either the well characterized murine monoclonal antibody (mAb) to human CD3 ϵ OKT3³ or Teplizumab⁴, a humanized and non Fc γ R-binding version of OKT3, both induced rapid T-ALL cell death and a strong delay in leukemia progression. Furthermore, OKT3 and Teplizumab synergized with chemotherapy to improve leukemic mice survival, although most cases eventually relapse⁴. Anti-CD3 mAbs thus represent a potential therapeutic option for T-ALL patients but it is essential to understand the molecular basis of their anti-leukemic properties to identify potential new vulnerabilities to reinforce their therapeutic potential. We report here the molecular signature induced *in vivo* in T-ALL cells in response to OKT3 and Teplizumab identify the TNF via NF κ B signaling pathway as the most deregulated pathway in Gene Set Enrichment Analysis (GSEA). Importantly, we demonstrate that both anti-CD3 mAbs induce TNF-mediated survival of T-ALL PDXs *in vivo*, a program that can be switched toward cell death and synergy in anti-leukemic properties upon administration of Birinapant, a SMAC mimetic that leads to cIAP1/2 ubiquitin ligases degradation. We also demonstrate both *in vitro* and *in vivo* that the anti-CD3/Birinapant synergy relies upon RIPK1 kinase activity, a key player of cell death pathways downstream of TNFR1. These results advocate that the combination of anti-CD3 mAbs with inhibitors of IAP1/2 proteins will offer new therapeutic options in T-ALL.

METHODS

Patients. T-ALL diagnostic samples were included in the [GRAALL-2005 study](#) or in the [FRALLE protocol](#) and informed consent was obtained from all patients at trial entry (trial registration ID: NCT00327678). Studies were conducted in accordance with the Declaration of Helsinki and approved by local and multicenter research ethical committees.

Mice. NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ* (NSG) female mice (2 month-old; Charles River Laboratories) were maintained under specific pathogen-free conditions. Experiments were carried out in accordance with the European Union and French National Committee recommendations, under agreement APAFIS #32902-20210908816114788 v1

Mice were tail vein injected (10^6 cells/mouse) with leukemic cells (defined as FSC^{hi}; hCD45⁺; hCD7⁺) obtained from the bone marrow of NSG mice xenografted with T-ALL diagnostic cases. Leukemia expansion in blood was followed as previously described ⁴. For long-term survival experiments, treatments were started when tumor burden reach around 1% in blood meaning over 10% in bone marrow. For short-term experiments, treatments were started at high tumor burden (around 10% in blood, meaning >50% leukemic cells in bone marrow). Control mice were always treated with the respective solvents or control antibodies.

***In vivo* antibodies and inhibitors treatments.** Antibodies are listed in Supplemental Methods. Mice were tail vein-intravenously injected with the quantity of antibody indicated in figure legends. Etanercept (10mg/kg) was injected intraperitoneally 6h before anti-CD3 treatment. Birinapant (MedChem Tronica, 20mg/kg) was administrated intraperitoneally every 3-4 days, 2h after anti-CD3 injection. End-point for survival experiment was defined as previously described ⁴.

Transcriptomic analysis

Affymetrix

Total RNA was isolated from flow-cytometry-sorted UPNT776 leukemic cells (hCD45⁺/hCD7⁺) 6h following treatment with either 40 μ g IgG2a (n=3), OKT3 (n=3) or Teplizumab (n=3), using the Rneasy kit (QIAGEN). cRNA synthesis and hybridization of Affymetrix GeneChip Human Gene 2.0 ST array were according to the manufacturer's instructions, as described (<http://www-microarrays.u-strasbg.fr>). Raw data were processed as described in Supplemental Methods. All microarray data have been submitted to the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>) under the accession number GSE240261. (reviewer token: shuhaesoptoljmf).

RNAseq

Total RNA was isolated from flow-cytometry-sorted UPNT420 leukemic cells (hCD45⁺/hCD7⁺) 6h following treatment of leukemic mice with either 40 μ g IgG2a (n=6) or OKT3 (n=6) using the RNeasy mini kit (QIAGEN). 1 μ g of total RNA was

used for the construction of sequencing libraries. RNA libraries were prepared using the Illumina Stranded mRNA Prep Ligation kit following manufacturer's protocols. Sequencing was carried out on a NovaSeq 6000 instrument from Illumina (S1-PE100, paired-end reads). Detailed description of analysis is reported in Supplemental Methods. RNA sequencing data are available under Gene Expression Omnibus series accession number GSE240296 (reviewer token: ojsluyqslbkvlyd). Counts were normalized using TMM normalization from EdgeR (v 3.32.1). Differential gene expression was assessed with the Limma voom framework (v 3.46.0). Genes with an absolute fold-change ≥ 1.5 and an adjusted p-value < 0.05 were labeled significant.

Functional Analyses

Gene Sets Enrichment Analyses (GSEA, <http://software.broadinstitute.org/gsea>) were run using signal-to-noise for the ranking gene metric and 1000 permutations. The negative selection signature used as gene set reference is described in ⁵ and unpublished information by Dr T Baldwin. Enrichment analysis from differentially expressed genes has been performed with KEGG and illustrated by using the KEGG mapper tool.

Nanostring

8 independent PDX xenografts (See **Supplemental Figure 5** for their characteristics) were engrafted into NSG mice. When tumor burden reached 10% in blood, mice were either treated with 40 μ g IgG2a (n=3) or OKT3 (n=3) for 12h. For the kinetic experiment, UPNT776-engrafted mice were treated with either 40 μ g

IgG2a (n=3) or 40 μ g OKT3 (n=3 for each time point) and mice sacrificed after at different time after treatment. Total RNA was isolated from flow-cytometry-sorted leukemic cells (hCD45⁺/hCD7⁺) using the RNeasy kit (QIAGEN) and RNA from each group of 3 mice pooled for further analysis. Nanostring custom panel of 97 genes notably encompassing TNFR superfamily receptors, ligands and effectors and comprising 5 housekeeping genes (see supplemental Methods for exhaustive list of genes) was constructed and hybridized as recommended by manufacturer's instructions.

Cell Culture

The CEM T-ALL cell line was cultured in RPMI-1640 medium supplemented with 50 μ g/ml streptomycin, 50 IU penicillin, and 15% fetal bovine serum (Gibco, Life Technology). TCR stimulation was induced using 2 Dynabeads/cell (Dynabeads Human Activator CD3/CD28, Invitrogen). Necrostatin 2 racemate (10mM) and Birinapant (both obtained from MedChem Tronica) were dissolved in DMSO and added to cells with concentrations as indicated in figure's legends. Control cells were treated with DMSO solvent. To knock-down RIPK1 expression, CEM T-ALL cells were transduced by a lentiviral vector encoding a small hairpin (sh) RNA to human RIPK1 (Vector Builder). Stably transduced cells were selected by flow-cytometry sorting mCherry-positive cells.

Western blot

Cells were washed in cold PBS and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor

Cocktail, ThermoScientific). Proteins were separated by SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). Antibodies are listed in Supplemental Methods.

Statistics. Leukemic load statistical analysis was performed using Prism (version 5.0; GraphPad Software). The data are expressed as mean +/- SD of n = 3 or more determinations. In short-term *in vivo* and *in vitro* assays, we used ANOVA and Tukey's Multiple Comparison Test or Student's T Test. Kaplan Meier survival curves were compared using the log-rank test. Differences were considered statistically significant at * p<0,05, ** p < 0.01, or ***p < 0.001.

RESULTS

We previously found that anti-CD3s antibodies (CD3mAbs) are strongly anti-leukemic in TCR+ T-ALL mouse models and T-ALL PDX in immune-deficient NSG mice. While combination of anti-CD3mAbs with chemotherapy can result in eradication of leukemic cells in T-ALL PDXs, anti-CD3 monotherapy ultimately resulted in relapse in all cases tested ⁴, calling for search of actionable vulnerabilities able to re-enforce anti-CD3 anti-leukemic potential.

To this end, we identified the *in vivo* molecular response of T-ALL cells to anti-CD3 mAbs. For this, groups of NSG mice xenografted with T-ALL PDX UPNT776 were treated at high tumor burden with either anti-CD3 mAbs OKT3 or the clinically-relevant Teplizumab, or with the respective control antibodies. Mice were followed over time for leukemia burden, induction of caspase activity and cell death (see

Supplemental Fig 1A for a scheme of the experiment). In line with our published results ⁴ anti-CD3 administration induced a fast decrease in leukemia burden which becomes significant at 12h (Supplemental Fig. 1B) associated with significant increase of apoptosis at 8h, as illustrated both by increased Annexin V staining (Supplemental Fig 1C) and increased activity of Caspase3 and 8 (Supplemental Fig 1D, E). To identify the molecular programs associated with cell death induction, T-ALL UPNT776-xenografted leukemic mice were treated for 6h with either 40 μ g control antibody, or anti-CD3 mAbs OKT3 or Teplizumab, time after which mice were killed, leukemic cells flow cytometry-sorted and analyzed for gene expression using Affymetrix GeneChip Human Gene 2.0 ST array (see Figure 1A for a scheme of the experiment). As shown in Figure 1B, both OKT3 and Teplizumab induced a clear molecular signature with 1632 and 1349 differentially expressed genes, respectively (FC >1.5, p<0.05, see **Supplemental Figure 1** and **Supplemental Figure 2** for an exhaustive list of differentially expressed genes). Of these, 932 genes were found commonly deregulated, as shown in the Venn diagram and heatmap of Figure 2C, the latter illustrating how the common OKT3/Teplizumab signature clusters away from the transcriptomic signature of control cells. In line with our previous results ³, Gene Set Enrichment Analysis (GSEA) highlighted a very strong and significant enrichment for TCR-induced genes related to clonal deletion/negative selection of normal thymic progenitors ⁵ in both OKT3- and Teplizumab-treated leukemic cells (Fig. 1D) consistent with their induction toward apoptosis. Of note, NR4A1 and BCL2L11, the only genes so far demonstrated essential for thymic negative selection

⁶ were found strongly up-regulated in response to OKT3 and Teplizumab (Fig 1B). A similar anti-CD3-induced gene expression signature was also found by RNAseq in another independent T-ALL case, UPNT 420 (Supplemental Fig 2A, B) in which GSEA also highlighted negative selection enrichment in anti-CD3 stimulated leukemic cells (Supplemental Fig 2C). Interestingly, when investigating the anti-CD3-induced gene expression signature among 50 GSEA Hallmarks to identify which other pathways could be significantly enriched, the “TNF α Signaling via NF- κ B” hallmark consistently ranked first (Fig 1E, Supplemental Fig 2D; Supplemental Tables 3, 4). This enrichment was found profound and significant in both UPNT776 leukemic cells collected from OKT3- or Teplizumab-treated mice (Fig. 1E) and UPNT420 leukemic cells collected from OKT3-treated mice (Supplemental Fig. 2D). When analysis was performed using Human MSigDB collection C2-CP: (Canonical pathways that comprises 3090 gene sets from Biocarta, KEGG, PID subset of CP, Reactome and WikiPathways subset of CP), “TNF α signaling pathway” signature was also significantly enriched in OKT3- (NES=2.57, FDR=0) or Teplizumab (NES=2.55, FDR=0)-treated cells. As illustrated in the schematic representation of TNF/TNFR signaling in Figure 2A and Supplemental Figure 2E, genes encoding ligands, adaptors, signaling proteins, transcription factors and regulators of TNF/TNFR pathway were found induced in T-ALL cells coming from anti-CD3 treated mice.

Using multiplex gene expression technology (ncounter Nanostring), we confirmed anti-CD3-mediated up-regulation of these genes in a panel of 8

independent T-ALL PDXs (Supplemental Fig 3). We also analyzed the induction kinetics of the TNF α , LT α ligands and of the adapters/effectors TRAF1, TRAF3, BIRC3/C-IAP2, NFKBIA, NFKBID, NFKBIE, NFKB1, NFKB2, c-REL, REL-B (Fig 2B), most of them being known NF κ B targets (<https://www.bu.edu/nf-kb/gene-resources/target-genes/>). Of note, TNF α was rapidly and transiently induced while LT α expression was more sustained (Fig. 2B) Taken together, these results show that critical players in TNF α /LT α signaling are up-regulated in T-ALL cells in response to anti-CD3 mAbs, suggesting a possible impact of TNF/TNFR signaling on the anti-leukemic potential of these antibodies.

To investigate the role of TNF α /LT α in the anti-leukemic properties of anti-CD3 mAbs, we thought to interfere with this pathway using Etanercept, a recombinant, dimeric fusion protein consisting of two molecules of the extracellular ligand-binding portion of human TNFR2 linked to the Fc portion of human IgG₁, that binds TNF and lymphotoxin- α , thus acting as a soluble decoy receptor and sink for TNF α /LT α ⁷. This allows investigating the consequences of TNF α /LT α neutralization on the response of T-ALL cells to anti-CD3 mAbs *in vivo*. Thus, groups of leukemic mice xenografted with independent T-ALL cases were pre-treated with Etanercept and, 6h later, treated or not with OKT3. Leukemic load was then compared over time in all groups. As shown in Figure 3, Etanercept did not significantly affect tumor load while, as expected, OKT3 treatment resulted in leukemic load inhibition over time. Importantly, Etanercept administration further enhanced the anti-leukemic properties of OKT3 as shown by decreased tumor burden in Etanercept/OKT3 treated mice as

compared to leukemic mice treated with OKT3 alone (Fig. 3A). Expectedly, Etanercept/OKT3 combinatorial treatment resulted in improved leukemic mice survival as compared to mice treated with OKT3 as single agent (Fig. 3B). These data indicate that $\text{TNF}\alpha/\text{LT}\alpha$, which are induced in T-ALL cells in response to OKT3, interferes with implementation of anti-CD3-induced cell death in T-ALL cells and that $\text{TNF}\alpha/\text{LT}\alpha$ inhibition improves anti-CD3 anti-leukemic potential *in vivo*.

Engagement of TNFR1 by $\text{TNF}\alpha/\text{LT}\alpha$ triggers the formation of different signaling complexes, each resulting in distinct cellular responses. Complex I is assembled at the plasma membrane through recruitment via the TRADD adapter to the TNFR1 death domain followed by other components, including TNFR-associated factor 2 (TRAF2), receptor-interacting serine/threonine-protein kinase 1 (RIPK1), the ubiquitin E3 ligases cellular inhibitor of apoptosis protein 1 or 2 (cIAP1/2) and linear ubiquitin chain assembly complex (LUBAC). Multiple types of ubiquitination events in the TNFR1 complex ultimately lead to I κ B kinase (IKK) and NF κ B activation to promote survival. Disruption of this complex, for example by SMAC-mediated inactivation of the ubiquitin E3 ligases cIAP1/2, results in the formation of several cytoplasmic complexes that mediate RIPK1-dependent and independent cell death (for review see ⁸). Given that TNFR1 and IAP1 are expressed in T-ALL PDXs (Fig.4A, Supplemental. Figure 4A and B and data not shown) and IAP2 is induced upon anti-CD3 treatment (Fig. 2B, Fig. 4A, Supplemental. Fig 3 and 4B), we next investigated whether inhibition of cIAP1/2 could indeed synergize with anti-CD3 mAbs to induce T-ALL cell apoptosis *in vivo*. Groups of leukemic mice xenografted

with either T-ALL case UPTN760 (Fig 4) or UPNT420 (Supplemental Fig 5) were treated for 6 hours with either OKT3, or the SMAC mimetic Birinapant, or the combination of OKT3 and Birinapant, or left untreated as control. Birinapant binds to c-IAP1 and c-IAP2 and induces their auto/trans-ubiquitination followed by their proteasomal degradation⁹. As expected, Birinapant administration suppressed IAP1 expression in T-ALL cells and blunted the OKT3-induced up-regulation of c-IAP2 (Fig 4A). As shown in Fig 4B, while administration of Birinapant as single agent had little effect on leukemia burden, induction of caspase 3/8 activity and apoptosis of leukemic cells, the co-administration of OKT3 + Birinapant induced higher levels of caspase activity and apoptosis, of T-ALL cells as compared to the level induced following OKT3-only administration, that resulted in further inhibition of leukemic load (Fig 4B; Supplemental Fig 5). We conclude from these results that anti-CD3-induced cell death and anti-leukemic properties can be re-enforced by impairing c-IAP1/2 expression using SMAC mimetics.

TNFR1-mediated cell death relies – at least in part- upon the protein kinase activity of RIPK1. The only substrate known so far for RIPK1 is RIPK1 itself on Ser166 in its activation loop¹⁰. We thus investigated whether increased apoptosis induced in response to anti-CD3/Birinapant is associated with increased RIPK1 activity. For this, the phosphorylation status of Ser166 of RIPK1 was assessed in CEM T-ALL cells treated with either anti-CD3, or Birinapant or the combination of both compounds. As shown in Figure 5A, while anti-CD3 treatment failed to induce RIPK1 activation, its combination with Birinapant treatment strongly enhanced

Ser166 RIPK1 phosphorylation. In line with our *in vivo* data, treatment with Birinapant enhanced anti-CD3-induced cell death of CEM cells *in vitro* (Fig 5B). Pharmacological inhibition of RIPK1 activity by Necrostatin 2 (Fig 5B) or knock-down of RIPK1 expression by small-hairpin RNA-mediated silencing (Fig 5C, D) both reduced anti-CD3/Birinapant-induced cell death. Importantly, pharmacological inhibition of RIPK1 activity also impaired the *in vivo* synergy of the anti-CD3 + Birinapant combination in T-ALL PDX as shown by decreased inhibition of leukemia burden and decreased Casp3/7 and Casp8 activities upon RIPK1 inhibition *in vivo* (Fig 5E). We conclude from these results that T-ALL cell death induction by anti-CD3/Birinapant treatment relies on RIPK1 kinase activity.

Since we found increased leukemic cell apoptosis in response to anti-CD3/Birinapant treatment, we next investigated whether this could translate into improved anti-CD3 therapeutic effect. For this, groups of leukemic mice xenografted with either a TCR $\delta\gamma$ + T-ALL or 6 independent T-ALL TCR $\alpha\beta$ + cases (**Supplemental Figure 5**) were treated with OKT3, or the SMAC mimetic Birinapant, or the combination of OKT3 and Birinapant, or left untreated as control. As shown in Figure 6A and consistent with our previous results (Fig. 5), Birinapant alone hardly affected tumor expansion, while, as expected OKT3 treatment resulted in profound inhibition of leukemic load in all of the 7 T-ALL analyzed. Combining OKT3 with Birinapant clearly further reduced leukemic load in all cases (Fig. 6A, compare pink with red bars). Treatment of PDXs derived from T-ALL UPNT420 and UPNT757 with either OKT3 or the OKT3 + Birinapant combination resulted in leukemia eradication at the

time control, non-treated mice were terminally-ill and had to be killed (Figure 6A). Relapse was observed to occur from about 10 weeks in the mice of the OKT3-treated arm (Fig 6A, right panel) whereas mice from the OKT3 + Birinapant treated group remained leukemia-free (Fig 6A). Expectedly, this synergy translated into a strongly improved survival of leukemic mice co-treated with OKT3 and Birinapant (Fig 6B, pink line) as compared to OKT3-treated mice (red line). In case of T-ALL UPTN420, OKT3/Birinapant combination even resulted in the cure of mice (Fig 6B). Importantly, synergy between anti-CD3 and Birinapant was also observed when using the clinically relevant anti-CD3 Teplizumab instead of OKT3 (Fig 6C-D). These results demonstrate the immunotherapeutic properties of anti-CD3 antibodies can be improved by SMAC mimetics such as Birinapant.

DISCUSSION

To improve the pro-apoptotic and anti-leukemic activity of anti-CD3 mAbs in T-ALL, we identified the molecular programs induced *in vivo* by these antibodies, the aim being to unravel pharmacologically actionable targets able to further enhance leukemic cells vulnerability to anti-CD3 immunotherapy. RNAseq and gene chip array expression profiling in PDXs generated from 2 distinct T-ALL cases extended our previous *in vitro* observation that both anti-CD3 OKT3 and Teplizumab induce the expression of actors of physiological clonal deletion, central to negative selection of developing thymocytes, notably *NR4A1/Nur77*^{6,11,12} and *BCL2L1/BIM*^{6,13,14}. *BCL2L1/BIM* repression is a known survival signal in T-ALL¹⁵ whereas constitutive

expression of NR4A1 is antagonistic to T-ALL PDX expansion *in vivo* (our unpublished data).

Interestingly, our profiling analyses also show prominent activation in anti-CD3-treated T-ALL of expression of TNF α , LT α and multiple components of the “TNF α via NF κ B signaling” pathway, an observation that was extended to 6/6 independent T-ALL cases.

Binding of TNF α /LT α to TNFR1 triggers the formation of a signaling complex, which recruits receptor-interacting protein 1 (RIPK1), TNFR-associated factor 2 (TRAF2) and c-IAP1 and c-IAP2, leading to c-IAP-dependent RIPK1 ubiquitination. Ubiquitinated RIPK1 serves as a platform for the ultimate recruitment of the scaffold protein NF- κ B essential modulator (NEMO), and the I κ B kinase (IKK) complex, leading to NF κ B activation^{16,17}. However, in the absence of c-IAP proteins, RIPK1 cannot be ubiquitinated, becomes active as a protein kinase and allows the formation of RIPK1/FADD/caspase8 and RIPK1/RIPK3 cytoplasmic complexes that result in cell death^{10,16,17}. Therefore, IAP antagonist binding to c-IAP1/2 and their subsequent proteolytic degradation both prevents c-IAP-mediated ubiquitination leading to NF κ B activation, and acts like a switch to transform pro-survival TNF signaling into RIPK1-dependent cell death (reviewed in¹⁷).

In T-ALL PDXs, we found the net activity of the induction of the TNF α / LT α ligands in response to anti-CD3 is to interfere with anti-CD3-induced cell death, associated with up-regulation of of NF κ B target genes. Indeed, neutralization of

TNF α /LT α by Etanercept synergized with anti-CD3 mAb to further impair leukemia progression and extend survival of T-ALL PDX mice. Activation of NF κ B has previously been reported to support leukemia progression in mouse models of T-ALL¹⁸ and AML¹⁹. Of note, increased TNF α serum levels appear to correlate with decreased patient survival in acute leukemias²⁰.

A number of small molecules acting as mimic of the mitochondrial cell death effector SMAC/Diablo (SMAC mimetics) have been developed that bind to the E3-ubiquitin ligases c-IAP1 and c-IAP2, activate their ubiquitin ligase activity resulting in their proteolytic degradation. Unlike its cell killing activity as single agent in a subset of B-ALL cases^{21–23} and in solid tumors⁹, we found that the SMAC mimetic Birinapant administered alone has no or only mild anti-leukemic activity in 7/7 T-ALL PDX tested, suggesting that cIAP1/2 have a limited role in cell death regulation in T-ALL. This is in line with the fact that cIAP1/2, in contrast to XIAP, are poor direct inhibitors of caspases²⁴ but are critical to TNF/TNFR extrinsic cell death signaling^{8,16}. This contrasts with the high dependance of T-ALL on central actors of the mitochondrial/intrinsic cell death pathway linked to high levels of BCL2 (immature T-ALL) or BCLX (mature T-ALL) expression/activity and the anti-leukemic effects provided by BH3 mimetics targeting these anti-apoptotic effectors *in vitro* and in T-ALL PDXs^{25,26}. Accordingly, available clinical data indicate promising activity of BH3 mimetics combined with chemotherapy in the treatment of resistant/relapse T-ALL cases (reviewed in²⁷).

Interestingly, while weakly anti-leukemic on its own, we found that administration of Birinapant strongly synergizes with anti-CD3 treatment, inducing rapid leukemic cell death in T-ALL PDXs *in vivo* and T-ALL cell lines *in vitro* through a mechanism associated with and reliant upon the activation of RIPK1 protein kinase activity. This synergy resulted in increased anti-leukemic potential and in near doubling in host survival time in the anti-CD3 + Birinapant arm as compared to anti-CD3 treatment alone in the 5/5 T-ALL PDXs investigated. The anti-leukemic synergy of anti-CD3 mAbs and Birinapant was observed with both anti-CD3 OKT3 and Teplizumab.

Unlike OKT3 (Muromonab), the first mouse monoclonal antibody generated to control acute transplant rejection, which is associated with sometimes severe side effects linked to its immunogenicity and mitogenic potential, Teplizumab is a IgG1-humanized version of OKT3 that carries 2 mutations (L234A;L235A) in its Fc domain and is thereby inefficient for binding FcR+ myeloid cells to induce CDC/ADCC and is poorly mitogenic for normal T cells (for review see ²⁸). Clinical trials using Teplizumab in various auto-immune conditions show efficacy and low level adverse events ^{29,30} and has been recently FDA-approved to delay stage 3 type 1 diabetes (T1D) in adults and pediatric patients. A number of SMAC mimetics have been developed that promote cell death, notably of cancer cells. SMAC mimetics are presently evaluated in phase I/II clinical trials mostly in solid tumor cancer patients. These compounds show efficient on-target activity, are well tolerated but appear to display limited anti-tumor activity as single agent except in a subset of B-ALL cases

²¹⁻²³. However, anti-cancer activity of SMAC mimetics can be improved when combined with several chemotherapies, in particular those leading to TNF α production by tumor cells (for review see ^{31,32})

Our observations in T-ALL PDXs that Birinapant-induced RIPK1 activation strongly enhances the anti-leukemic potential of anti-CD3 mAbs advocates the use of Birinapant, or other SMAC mimetics to improve anti-CD3 immunotherapy in relapse/refractory T-ALL.

ACKNOWLEDGMENTS

This work was supported by funds from Institut Curie, Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (INSERM), Institut National du Cancer (INCa grants 2018 PRTK18-071 and PLBIO-04-ICR1), Ligue contre le Cancer (Programme de Recherche Enfants, Adolescents et Cancer et Ligue de l'Essonne) and the European Union (EU ITN2020 ARCH consortium (grant agreement 813091). AAA was supported by pre-doctoral fellowship from EU ITN2020-ARCH and la Fondation ARC pour la Recherche sur le Cancer, BZ by pre-doctoral fellowships from the University Paris-Diderot and Ligue Nationale Contre le Cancer. The authors thank C Lasgi (Institut Curie cytometry platform), E Belloir, C Alberti and P. Dubreuil (Institut Curie animal facility) for expert technical assistance, V. Skory and Christelle Thibaud from the "Plateforme Biopuces et Séquençage de l'IGBMC", Strasbourg, P. de la Grange from Genosplice for Affymetrix micro-array performance and help in analyses, D. Gentien (Institut Curie Sequencing platform) for performing RNAseq and Nanostring analyses, Dr Baldwin and Dr Hogquist for sharing unpublished data on the transcriptome associated with the negative selection signature of thymocytes ⁵

AUTHORSHIP CONTRIBUTIONS

AAA, CTQ designed the study, carried out the experiments, data analysis and interpretation, organized the data and wrote the manuscript; KN, MdL, BZ, SD

participated to the experimental work and data analysis; VA provided primary patient material and associated immuno-phenotypes and molecular data, carried out data analysis; JG designed the study, carried out data analysis and interpretation, organized the data and wrote the manuscript.

CONFLICT-OF-INTEREST DISCLOSURE

CTQ and JG have research agreements with Servier and Autolus Ltd. The remaining authors declare no competing financial interests.

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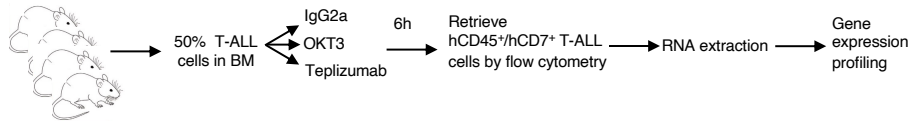
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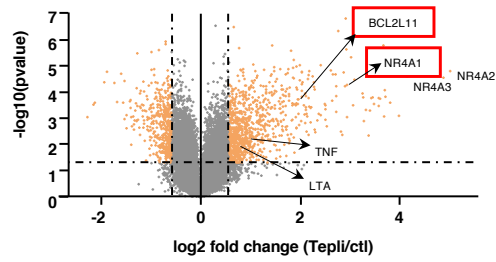
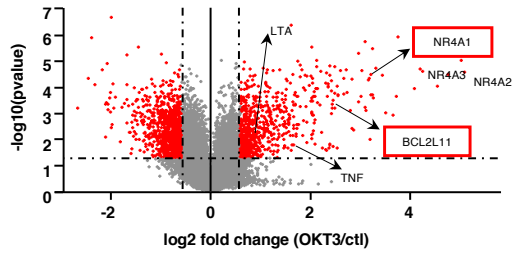
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Figure 1

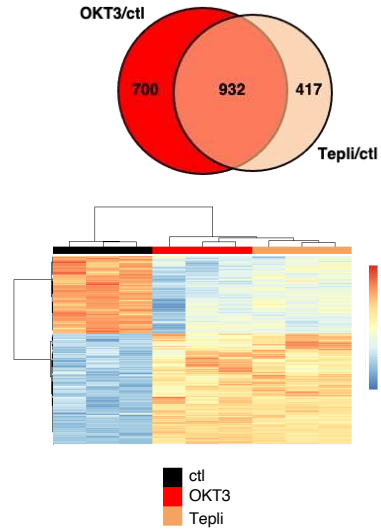
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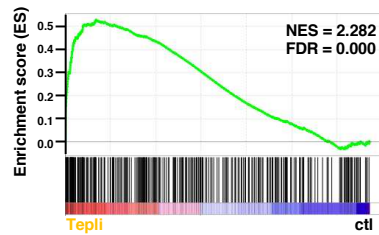
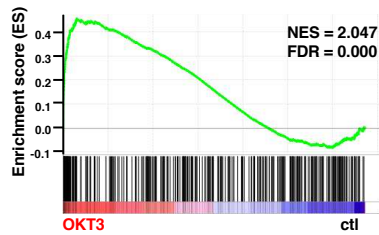


C



D

Enrichment plot : Negative selection signature, up-regulated genes



E

Enrichment plot : Hallmark TNFA signaling via NFKB

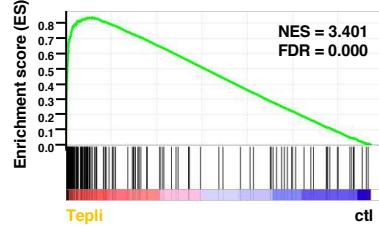
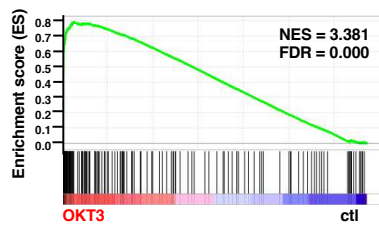


Figure 2

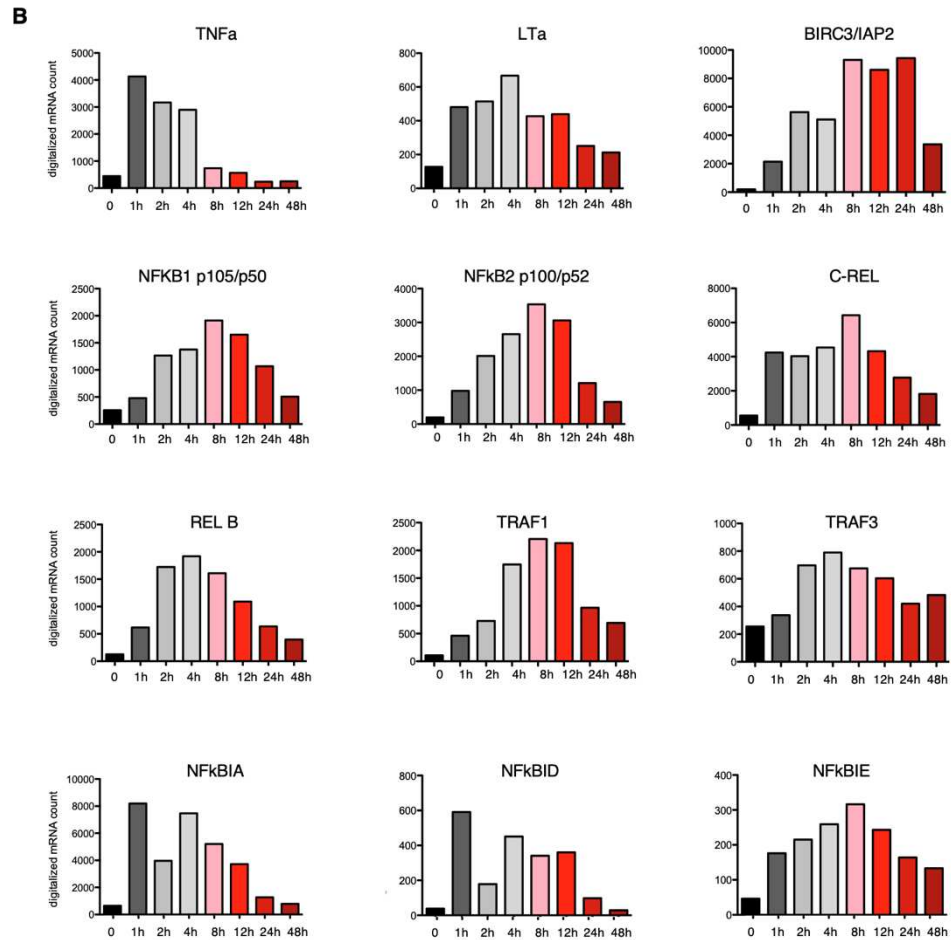
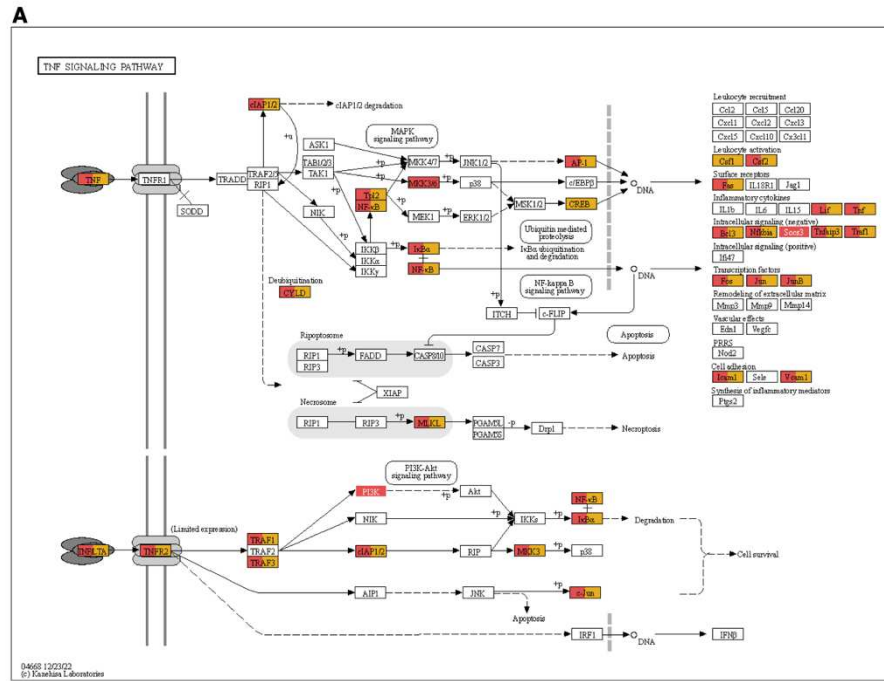


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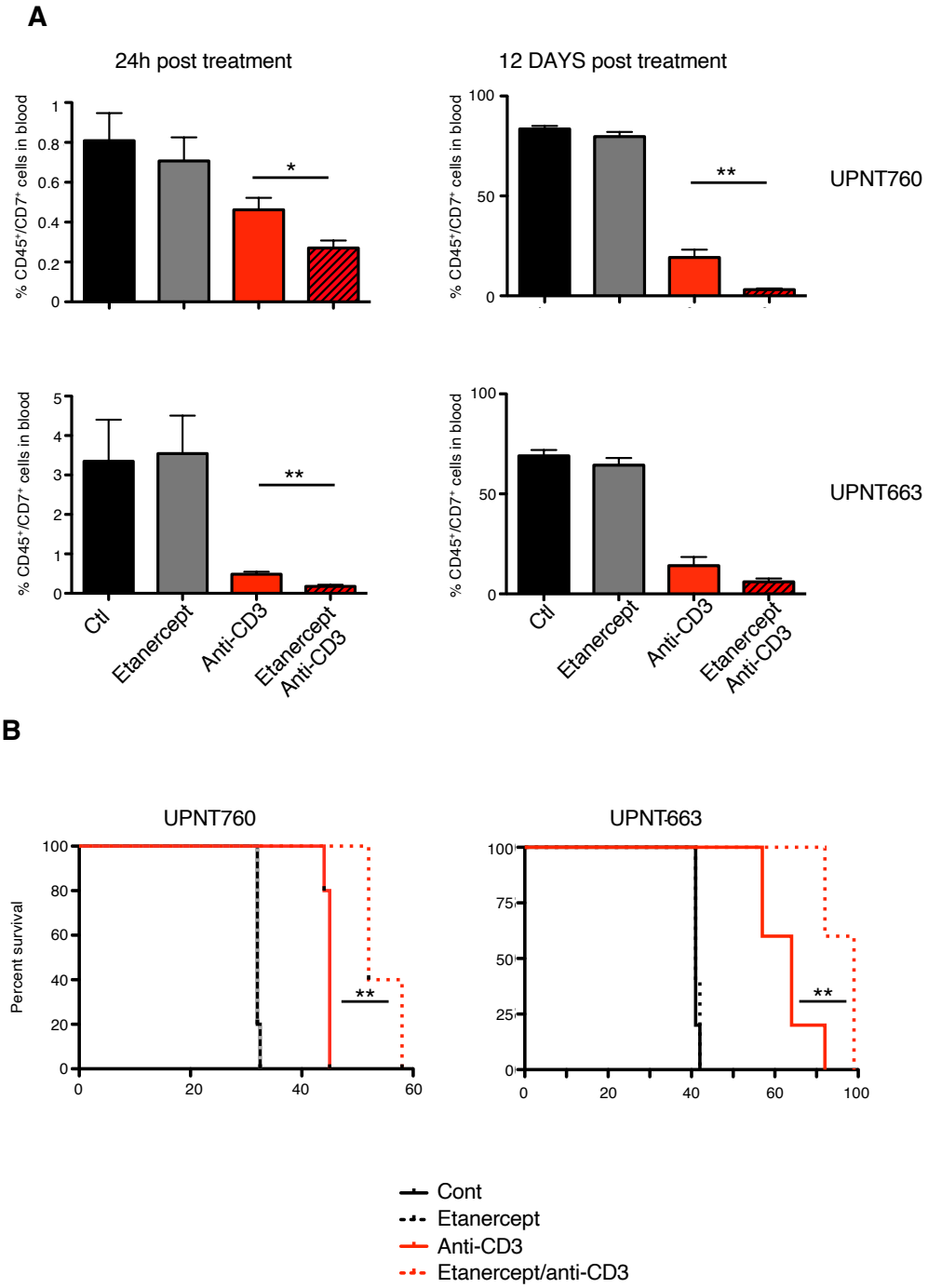
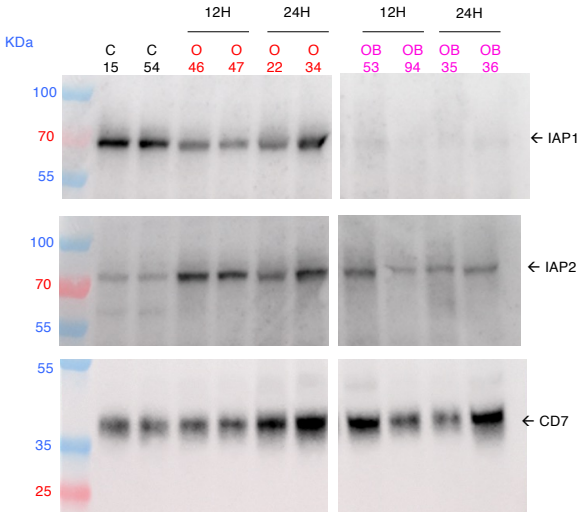


Figure 4

A



B

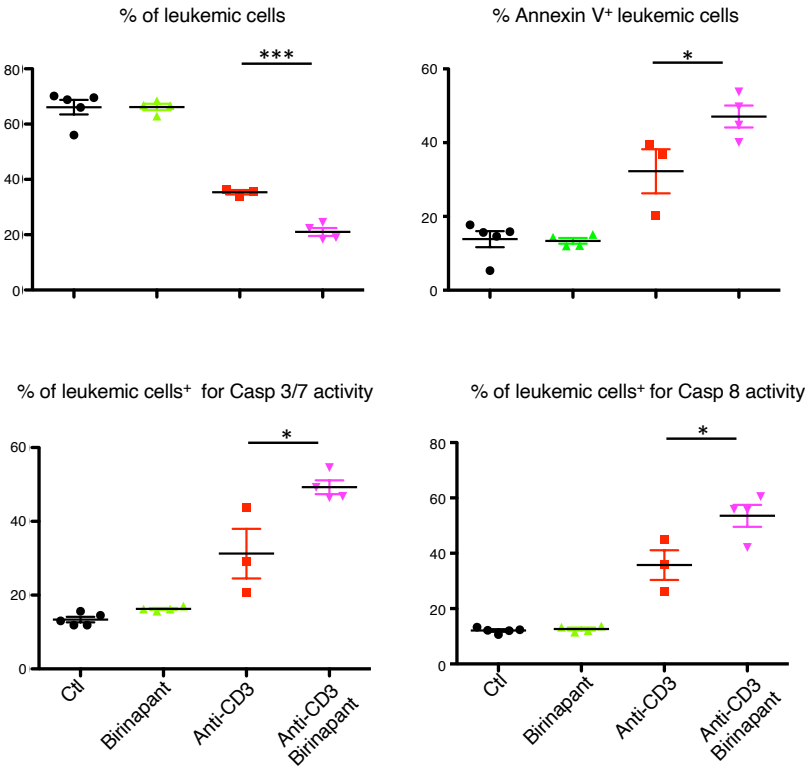


Figure 5

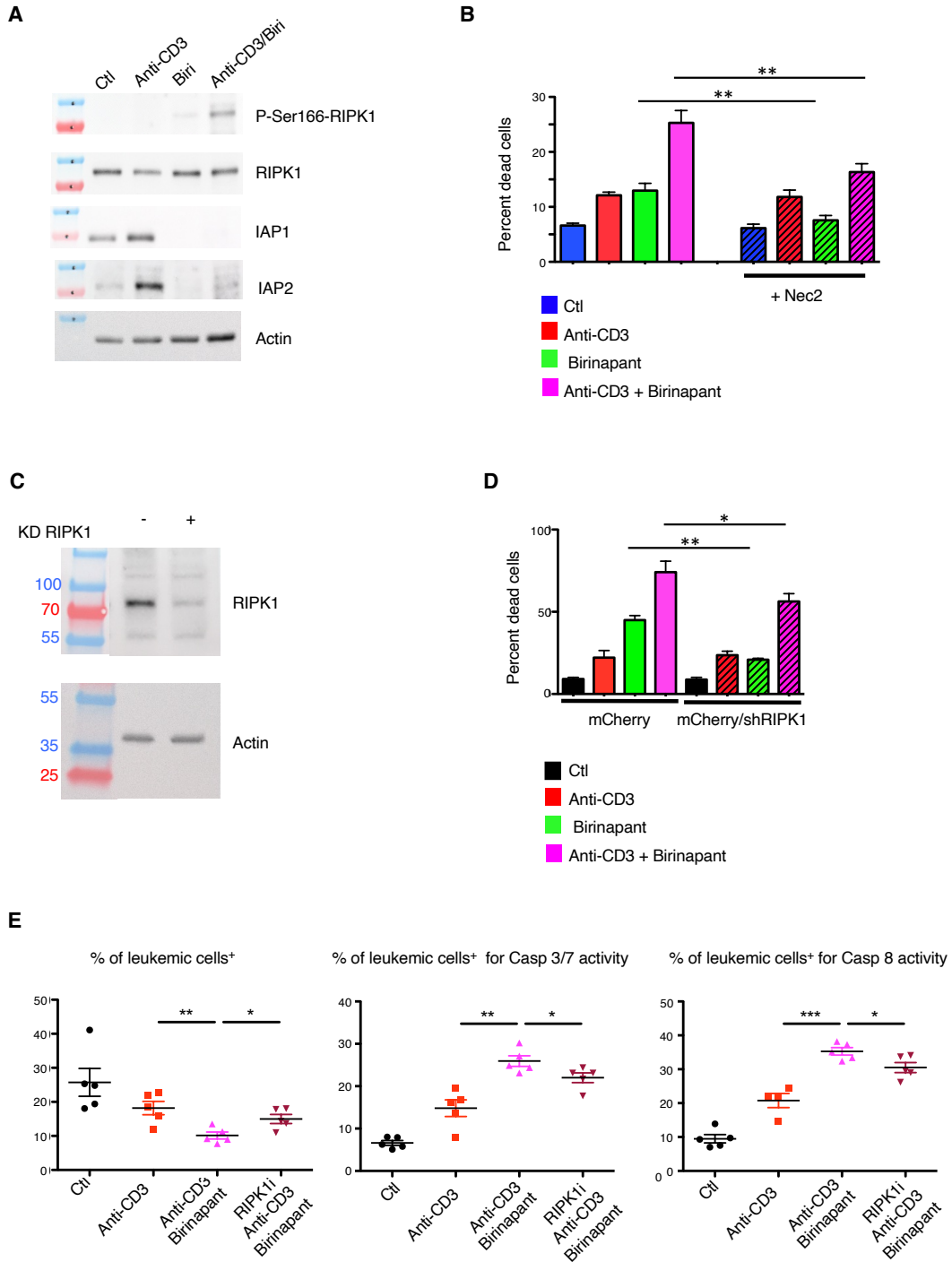


Figure 6

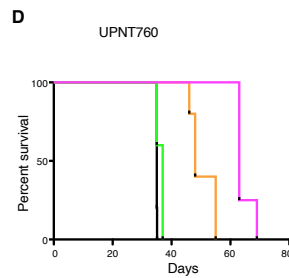
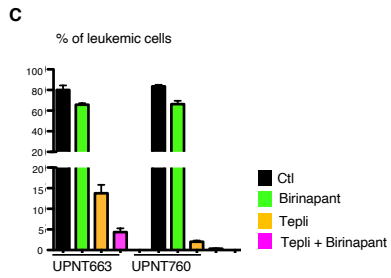
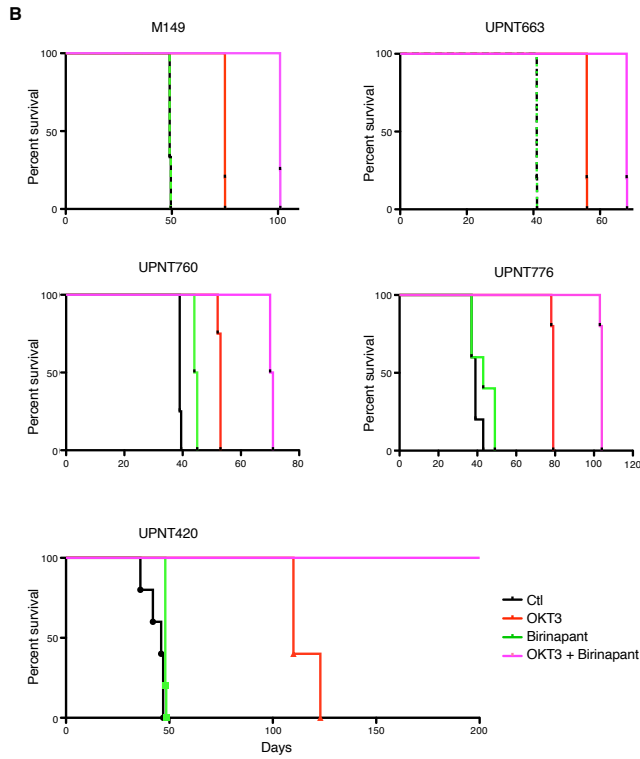
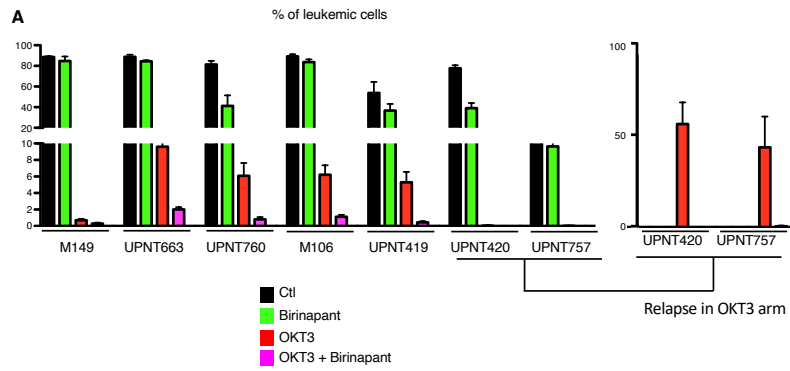


Figure Legends

Figure 1. Anti-CD3 monoclonal antibodies OKT3 and Teplizumab share a commonly-induced transcriptomic signature in T-ALL. (A) Schematic representation of the experiment. NSG mice infused with 10^6 leukemic cells from T-ALL UPNT776, were monitored for leukemia expansion. Once leukemic (23 days post-injection), mice (n=3 per group) were treated with $40\mu\text{g}$ of either control mIgG2a, or OKT3 or Teplizumab. 6h after, mice were killed, hCD45+/hCD7+ leukemic cells sorted from the bone marrow by flow cytometry, RNA extracted and analyzed using Affymetrix GeneChip Human Gene 2.0 ST array. (B) Volcano plot of probe sets differentially expressed between control and either OKT3 (top panel) or Teplizumab (bottom panel)-treated leukemic cells described in (A). Fold change (x axis) is plotted against statistical significance (y axis) for each probe set. Genes with a fold change ≥ 1.5 and $p < 0.05$ are highlighted as red (OKT3) or orange (Teplizumab) dots, respectively. (C) Top: Venn diagram of differentially-expressed genes between control, OKT3-treated (red) and Teplizumab-treated (light orange) leukemic cells described in (A). Commonly-deregulated genes are highlighted in dark orange; Bottom: heat map representation of the 932 genes commonly deregulated by OKT3 and Teplizumab as compared to the transcriptomic signature of control cells. (D) GSEA of the negative selection transcriptional signature of mouse thymocytes undergoing negative selection as published by Baldwin and Hogquist⁵ with the transcriptomic signature induced *in vivo* in T-ALL cells in response to OKT3 or Teplizumab, showing a strong and significant enrichment of this thymic signature in anti-CD3-treated samples. (E) GSEA of the “TNF α signaling via NF κ B” signature with that induced in response to OKT3 or Teplizumab in T-ALL cells, showing a strong and significant enrichment of this signature in anti-CD3-treated samples. NES: Normalized Enrichment Score; FDR: False Discovery Rate

Figure 2. TNF signaling components are induced *in vivo* in response to anti-CD3 in T-ALL. (A) KEGG schematic representation of “TNF α signaling via NF κ B” pathway. Genes whose expression is deregulated in T-ALL cells in response to anti-CD3 mAbs as described in Figure 1B-C, are highlighted as red (OKT3), or light orange (Teplizumab) boxes, respectively. Black writing in coloured boxes indicates up-regulation in expression while white writing indicates down-regulation. (B) RNA obtained from leukemic cells (T-ALL UPNT776) of engrafted mice that were either left untreated, or treated for the indicated time periods (hours) with anti-CD3 OKT3 were analyzed using a Nanostring custom panel of 97 genes comprising 5 housekeeping genes. Histograms show digitalized mRNA expression for TNF α , LT α , BIRC3, NF κ B1, NF κ B2, C-REL, RELB, TRAF1, TRAF3, NF κ BIA, NF κ BID, NF κ BIE after normalization with Nsolver software.

Figure 3. Neutralization of TNF α /LT α by Etanercept administration improves the anti-leukemic properties of anti-CD3.(A) Leukemia tumor burden (hCD45⁺/hCD7⁺ at the indicated time point (24h, 12 days) after treatment of leukemic mice with either 10mg/kg Etanercept, or 4 μ g of OKT3 in presence or absence of 10mg/kg Etanercept. Experiments were performed using independent T-ALL PDXs derived from UPNT760 and UPNT663 diagnostic T-ALL cases, as indicated. Black bar: control mice treated with control IgG2a and carrier solvent; Grey bar: Etanercept-treated mice; Red bar: anti-CD3-treated mice; red-dashed bar: anti-CD3/Etanercept-co-treated mice. (B) Kaplan-Meier survival curves of NSG-mice transplanted with T-ALL UPNT-760 and UPNT-663 and treated as in A.

Figure 4. SMAC mimetics Birinapant synergizes with anti-CD3 to induce leukemic cells apoptosis.(A) Western blot analysis of IAP1 (top panel) or IAP2 (middle panel) expression in leukemic cells of UPNT760 T-ALL-xenografted mice at 12 or 24h after administration of either 4 μ g OKT3 (O, red writing) or OKT3/Birinapant (20mg/kg) (OB, pink writing) or control IgG2a/carrier solvent (C, black writing). Results from 2 independent mice are shown. Anti-hCD7 expression is used as loading control (bottom panel). (B) NSG mice were injected with 10⁶ UPNT760 T-ALL cells. At high leukemia burden, mice were treated with either control IgG2a/solvent (black dots), IgG2a/Birinapant (20mg/kg, green triangles), 4 μ g OKT3/solvent (red squares) or combination of OKT3 and Birinapant (pink triangles) for 6 hours, time after which leukemia burden (hCD45/hCD7⁺ cells), Annexin V/PI staining and Caspase 3/7, Caspase 8 activities were analyzed by flow cytometry.

Figure 5. Anti-CD3/Birinapant-induced cell death is dependent on RIPK1.(A) Western blot analysis of IAP1, IAP2 expression and RIPK1 phosphorylation in CEM T-ALL cells stimulated for 4h with anti-CD3, Birinapant (2,5nM) or anti-CD3Birinapant + combination. RIPK1 and actin expression are used as normalizers. (B) CEM T-ALL cells were stimulated for 24h with either anti-CD3 (red), Birinapant (2,5nM, green) or the anti-CD3 + Birinapant combination (purple), either in absence (open columns) or presence (dashed columns) of the RIPK1 inhibitor, Necrostatin2. Cell death (PI⁺ cells) is measured by flow cytometry 24h after start of the treatment. The average of 3 independent experiments is shown. (C) Western blot analysis of RIPK1 expression in control (-) versus CEM T-ALL cells stably expressing a RIPK1 shRNA (+). Actin expression is used as a loading control. (D) Percentage of cell death as measured by PI positivity by flow cytometry in non-treated cells (black), or

24h after treatment with either anti-CD3 (red) or Birinapant (100nM; green) or the combination of anti-CD3 + Birinapant (purple). The average of 3 independent experiments is shown. **(E)** NSG mice were injected with 10^6 UPNT420 T-ALL cells. At high leukemia burden, mice were treated with either control IgG2a/carrier solvent (black dots), or $4\mu\text{g}$ OKT3/carrier solvent (red squares), or the combination of OKT3/Birinapant/carrier solvent (purple triangles) or the combination of RIPK1 inhibitor (50mg/kg), OKT3 and Birinapant (brown triangles) for 12 hours, time after which leukemia burden (hCD45/hCD7+ cells), Caspase 3/7 and Caspase 8 activities were analyzed by flow cytometry.

Figure 6. Birinapant synergizes with anti-CD3 to inhibit leukemia expansion.**(A)** Leukemia load in NSG mice that were injected with the indicated human T-ALL primary PDXs. Once tumor burden reached 1% in blood, mice were treated 6 times with either $4\mu\text{g}$ of control IgG2a/carrier solvent (n=4, black), or OKT3/carrier solvent (n=4, red), or IgG2a/Birinapant (20mg/kg, n=4, green) or OKT3 + Birinapant (pink) every 3-4 days. In the left part of the panel, leukemia load is measured at the end of treatment. For UPNT420- and 757-derived T-ALL PDXs, mice treated with OKT3/carrier solvent were found to be in remission for 70 (UPNT420) and 110 (UPNT757) days after beginning of the treatment but relapsed thereafter. The right part of the panel shows leukemia load at day 102 days (UPNT420) and day 171 (UPNT757) after beginning of treatment. Note that at that points, OKT3 + Birinapant-treated mice are leukemia-free. **(B)** Kaplan-Meier survival curves of NSG-mice transplanted with T-ALL M149, UPNT663, UPNT-760, UPNT776 and UPNT-420 of the cohorts described in (A). **(C)** The same experimental protocol was used in T-ALL PDXs derived from UPNT663 and UPNT760, using the clinically-relevant anti-CD3 Teplizumab. **(D)** Kaplan-Meier survival curves of NSG-mice transplanted with T-ALL UPNT-760 and treated as in (C).

SUPPLEMENTAL METHODS

***In vivo* used antibodies**

The IgG2a mouse OKT3 mAb to human CD3 ϵ and its isotypic control (mIgG2a) were from Bio X Cell (West Lebanon, USA). Teplizumab was obtained from Creative Biolabs. Its isotypic control (hIgG1) was from Bio X Cell.

Apoptosis Analysis

In vivo leukemia cell apoptosis was analyzed with BD Pharmingen PE Annexin V Apoptosis Detection Kit I according to manufacturer instruction. Caspase 3/7 and Caspase 8 activity were determined using FLICA 660 Caspases 3 & 7 Assay Kit or Green FLICA Caspase 8 Assay Kit respectively from EuroBio Scientific, according to manufacturer instructions. Cell death was analyzed by staining cells with 1 μ g/mL of propidium iodide.

Transcriptomic analysis

Affymetrix

Raw data were processed with Affymetrix Expression Console software version 1.1 to calculate probe set signal intensities, using Robust Multi-array Average (RMA) algorithms with default settings. A paired Student's t-test was performed to compare gene intensities in the different biological replicates (n=3).

Genes were considered significantly regulated when absolute fold-change was ≥ 1.5 and *p value* ≤ 0.05 .

RNAseq

Raw sequencing reads were first checked for quality with Fastqc (0.11.9) and trimmed for adapter sequences with cutadapt (1.12) using the TrimGalore (0.6.2) wrapper. Trimmed reads were subsequently aligned to the complete human ribosomal RNA sequence with bowtie (2.4.2). Reads that did not align to rRNA were then mapped to the human reference genome hg19 and read counts per gene were generated with STAR mapper (2.7.6a). The bioinformatics pipelines used for these tasks are available online (RNAseq v4.0.0: <https://gitlab.curie.fr/data-analysis/RNA-seq>, doi: 10.5281/zenodo.7443721).

Detailed list of genes of the Nanostring custom panel.

ABCF1, ACKR3, AHR, AREG, ARHGAP31, ARL5C, BCL11B, BCL2, BCL2A1, BCL2L1, BCL2L11, BHLHE40, BIRC3, CASP8, CCL22, CCND1, CD274, CR2, CRTAM, CSF2, CXCL12, CXCR4, DUSP2, DUSP4, DUSP5, EEF1A2, EGR3, FA2H, FADD, FAS, FASLG, G6PD, GADD45B, GUSB, HCAR1, HIC1, HRH2, IL2RA, IRF4, LT α , MCL1, NFE2, NFKB1, NFKB2, NFKBIA, NFKBID, NFKBIE, NINJ1, NKX3-1, NR4A1, NR4A2, NR4A3, PDCD1, PDCD1LG2, POLR2A, PRDM8, PTCRA, PTPN14, RAG2, RARG, REL, RELA, RELB, RGS16, SDC4, SLC27A2, STX11, TAGAP, THEMIS, THEMIS2, TNF, TNFRSF10A, TNFRSF10B, TNFRSF10C, TNFRSF10D, TNFRSF14, TNFRSF1A, TNFRSF1B, TNFRSF25,

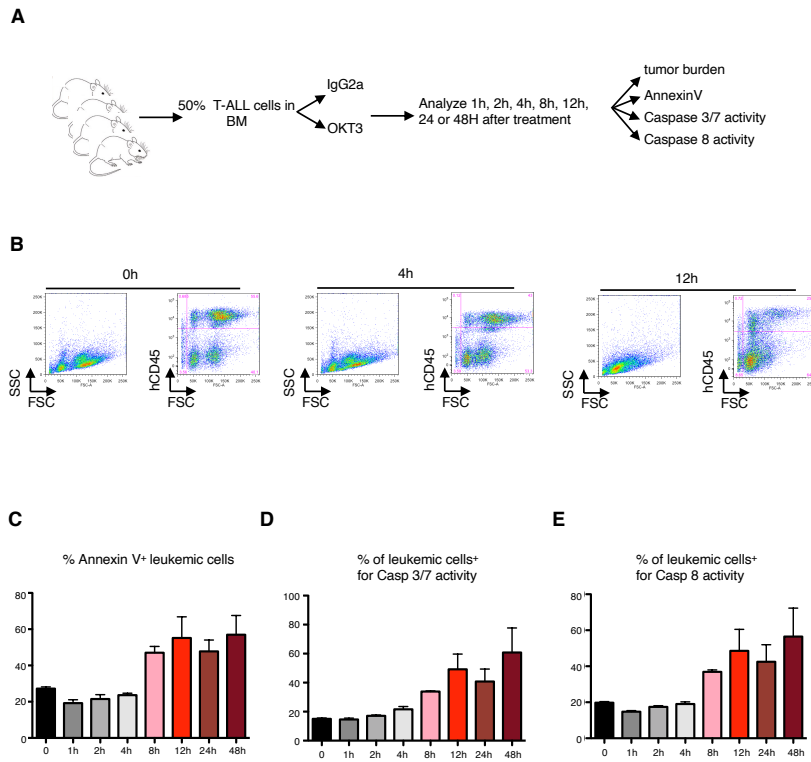
TNFRSF4, TNFRSF8, TNFRSF9, TNFSF10, TNFSF14, TNFSF15, TNFSF4, TNFSF8, TNFSF9, TP53, TRADD, TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, TUBB, XCL2

Antibodies used in western blot.

Anti-P-Ser166 RIPK1 (#65746 from Cell Signaling), RIPK1 (#551042 BD Pharmingen), c-IAP1 (#7065T from Cell Signaling), c-IAP2 (#3130S from Cell Signaling), Actin (A5441 from Sigma), hCD7 (#Sc-59108 from Santa Cruz). Anti-mouse (GENA931) and anti-rabbit (GENA934) IgG-HRP secondary antibodies are from Sigma. Chemiluminescence was detected by ChemiDoc XRS+ Vilber (Bio-Rad) using the West Dura SuperSignal kit (ThermoScientific).

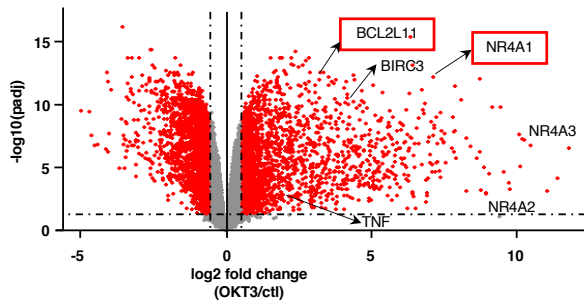
SUPPLEMENTAL FIGURES

Supplemental Figure 1

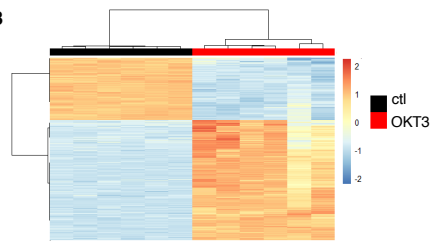


Supplemental Figure 2

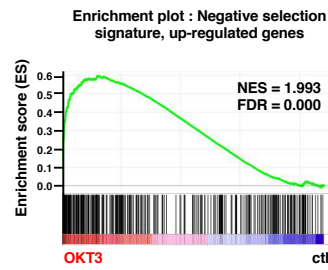
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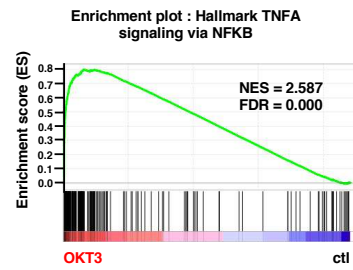
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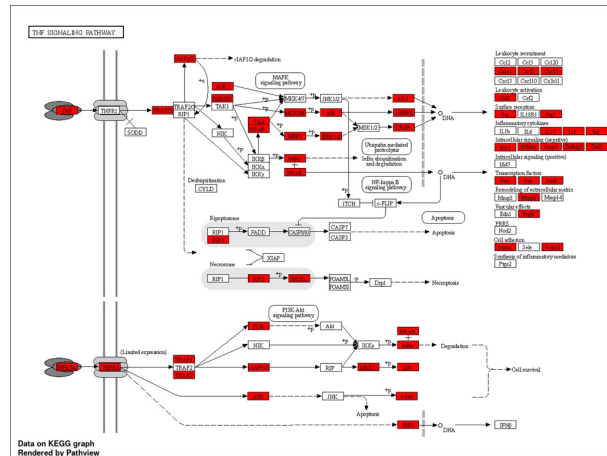
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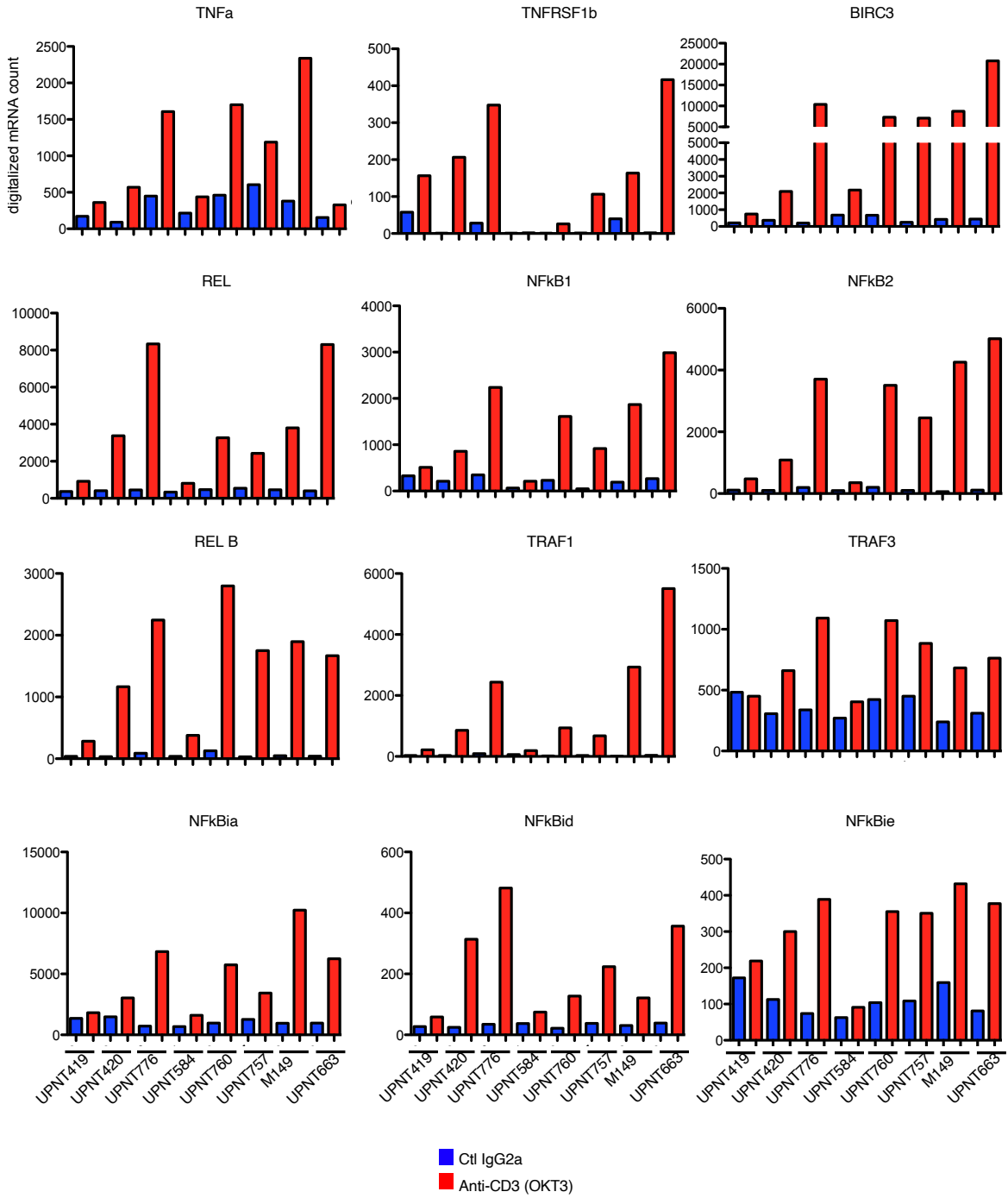
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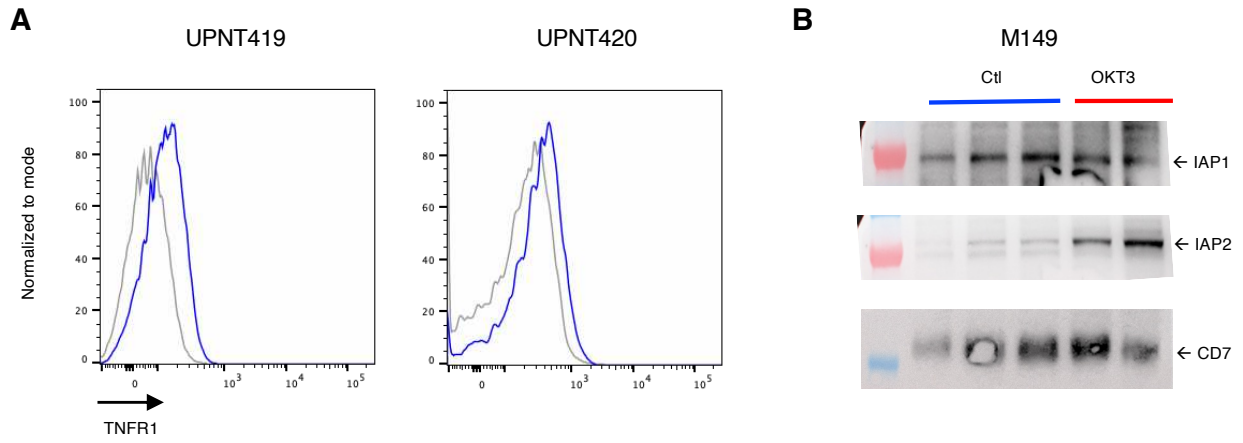
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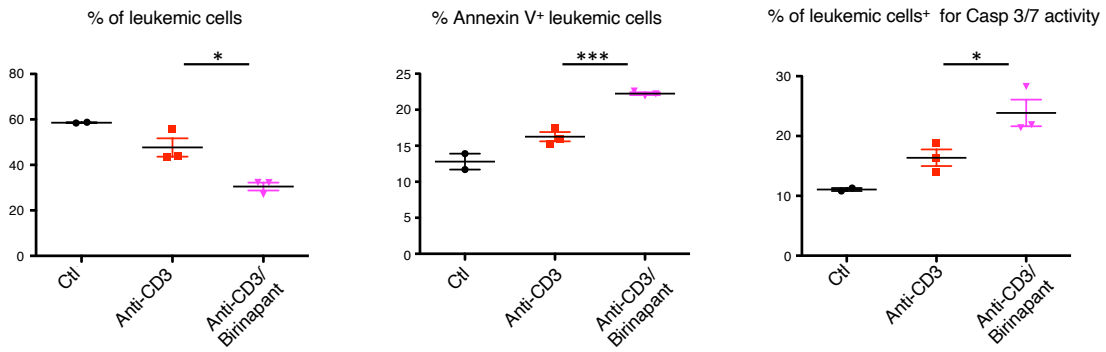
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



SUPPLEMENTAL TABLES

Supplemental Table 1

Not attached

Supplemental Table 2

Not attached

Supplemental Table 3

OKT3

NAME	ES	NES	NOM p-val	FDR q-val
HALLMARK_TNFA_SIGNALING_VIA_NFKB	0.7913771	3.3815684	0.0	0.0
HALLMARK_IL2_STAT5_SIGNALING	0.6793238	2.8800952	0.0	0.0
HALLMARK_INFLAMMATORY_RESPONSE	0.5663274	2.4341187	0.0	0.0
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	0.5654593	2.4190102	0.0	0.0
HALLMARK_P53_PATHWAY	0.5456333	2.3383062	0.0	0.0
HALLMARK_HYPOXIA	0.5102344	2.1791003	0.0	0.0
HALLMARK_APOPTOSIS	0.50786	2.1187844	0.0	0.0
HALLMARK_ALLOGRAFT_REJECTION	0.48966777	2.0968883	0.0	0.0
HALLMARK_TGF_BETA_SIGNALING	0.5925561	2.0511973	0.0	1.25E-4
HALLMARK_IL6_JAK_STAT3_SIGNALING	0.53768986	2.044088	0.0	1.125E-4
HALLMARK_KRAS_SIGNALING_UP	0.4618554	1.9768151	0.0	3.3875366E-4
HALLMARK_ESTROGEN_RESPONSE_LATE	0.41280508	1.7690157	0.0	0.0018441487
HALLMARK_ANGIOGENESIS	0.53813267	1.7641368	0.003875969	0.001797782
HALLMARK_UV_RESPONSE_UP	0.41359904	1.7182806	0.0	0.0036657567
HALLMARK_ESTROGEN_RESPONSE_EARLY	0.3971698	1.7170091	0.0	0.0035013729
HALLMARK_MTORC1_SIGNALING	0.387932	1.6518354	0.0018248175	0.006621785
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	0.41750762	1.6435562	0.0037523452	0.006383151
HALLMARK_INTERFERON_GAMMA_RESPONSE	0.38340065	1.6362987	0.0	0.0067218193
HALLMARK_MYC_TARGETS_V2	0.45465866	1.5632756	0.018761726	0.01339543
HALLMARK_UV_RESPONSE_DN	0.37040976	1.480812	0.017921148	0.026930213

Teplizumab

NAME	ES	NES	NOM p-val	FDR q-val
HALLMARK_TNFA_SIGNALING_VIA_NFKB	0.8367997	3.4006908	0.0	0.0
HALLMARK_IL2_STAT5_SIGNALING	0.7146334	2.933821	0.0	0.0
HALLMARK_P53_PATHWAY	0.6700407	2.7582648	0.0	0.0
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	0.6517824	2.727807	0.0	0.0
HALLMARK_APOPTOSIS	0.6682332	2.6611037	0.0	0.0
HALLMARK_INFLAMMATORY_RESPONSE	0.6366209	2.6189947	0.0	0.0
HALLMARK_HYPOXIA	0.6044763	2.485195	0.0	0.0
HALLMARK_UV_RESPONSE_UP	0.5983955	2.3364267	0.0	0.0
HALLMARK_ALLOGRAFT_REJECTION	0.5634604	2.304701	0.0	0.0
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	0.5902581	2.2476516	0.0	0.0
HALLMARK_UV_RESPONSE_DN	0.5729121	2.247188	0.0	0.0
HALLMARK_TGF_BETA_SIGNALING	0.67230004	2.2278433	0.0	0.0
HALLMARK_IL6_JAK_STAT3_SIGNALING	0.59880906	2.1536038	0.0	0.0
HALLMARK_MTORC1_SIGNALING	0.51261014	2.1136465	0.0	0.0
HALLMARK_ESTROGEN_RESPONSE_LATE	0.5086977	2.0971665	0.0	0.0
HALLMARK_KRAS_SIGNALING_UP	0.50762355	2.0866518	0.0	0.0
HALLMARK_CHOLESTEROL_HOMEOSTASIS	0.5562497	1.9340333	0.0	1.6806723E-4
HALLMARK_WNT_BETA_CATENIN_SIGNALING	0.6012654	1.9253628	0.0	1.5873017E-4
HALLMARK_ESTROGEN_RESPONSE_EARLY	0.46465212	1.9069116	0.0	4.7447399E-4
HALLMARK_INTERFERON_GAMMA_RESPONSE	0.45858556	1.8791467	0.0	6.443736E-4

Supplemental Table 4

NAME	ES	NES	NOM p-val	FDR q-val
HALLMARK_TNFA_SIGNALING_VIA_NFKB	0.80116934	2.5867467	0.0	0.0
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	0.7056657	2.2774	0.0	0.0
HALLMARK_IL2_STAT5_SIGNALING	0.7109388	2.2761087	0.0	0.0
HALLMARK_INFLAMMATORY_RESPONSE	0.6689844	2.1429615	0.0	0.0
HALLMARK_ALLOGRAFT_REJECTION	0.6387669	2.0460603	0.0	0.0
HALLMARK_APOPTOSIS	0.6297285	1.9913967	0.0	0.0
HALLMARK_HYPOXIA	0.6058847	1.9523258	0.0	0.0
HALLMARK_ANGIOGENESIS	0.776062	1.9412025	0.0	1.3392857E-4
HALLMARK_P53_PATHWAY	0.59656745	1.9354947	0.0	1.1904762E-4
HALLMARK_KRAS_SIGNALING_UP	0.5998679	1.9350984	0.0	1.07142856E-4
HALLMARK_ESTROGEN_RESPONSE_LATE	0.5638948	1.8100545	0.0	7.7219267E-4
HALLMARK_IL6_JAK_STAT3_SIGNALING	0.61610544	1.7886325	0.0	8.015933E-4
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	0.5834855	1.7752318	0.0	8.168553E-4
HALLMARK_UV_RESPONSE_UP	0.5615924	1.7635199	0.0	9.0979E-4
HALLMARK_MYOGENESIS	0.54513025	1.7347195	0.0	0.001217957
HALLMARK_TGF_BETA_SIGNALING	0.6156664	1.6848884	0.003931848	0.0028517842
HALLMARK_MYC_TARGETS_V2	0.5952265	1.6754792	0.0026737968	0.0030138637
HALLMARK_UV_RESPONSE_DN	0.5216361	1.6552831	0.0	0.003934733
HALLMARK_COMPLEMENT	0.5077009	1.6054299	0.0	0.006707115
HALLMARK_MTORC1_SIGNALING	0.49673793	1.6052531	0.0	0.0063717593

Supplemental Table 5

PATIENTS	Phenotype	Chromosomal alteration	Mutations or Indels detected by sequencing
UPNT419	TCRab	CALM-AF10	PTEN del/mut
UPNT420	TCRab	SIL-TAL1	PTEN mut
UPNT776	TCRab	SIL-TAL1	PTEN mut, ATXN mut
UPNT584	TCRab	SIL-TAL1	CTCF mut, NOTCH mut
UPNT760	TCRab	SIL-TAL1	PTEN del
UPNT757	TCRab	SIL-TAL1	POT1 mut
M149	TCRgd	TLX3	WT1 mut, SH2B3 mut, TET2 mut, STAT5b mut, NOTCH1 mut
UPNT663	TCRab	unknown	PTEN mut

LEGENDS SUPPLEMENTAL FIGURES

Supplemental Figure 1. Anti-CD3 OKT3 antibody induces rapid apoptosis of leukemic cells in vivo. (A) Schematic representation of the experiment. NSG mice infused with 10^6 leukemic cells from T-ALL UPNT776, were monitored for leukemia expansion. Once leukemic (22 days post-injection), mice (n=3 per group) were treated with $40\mu\text{g}$ of either control mIgG2a or OKT3 and killed at the indicated time points after anti-CD3 treatment; (B) Leukemic burden is shown before treatment (0h) and at 4h and 12h post-treatment with OKT3. Live leukemic cells are $\text{FSC}^{\text{hi}}/\text{hCD45}^+$; note the change in the FSC/SSC profile in response to OKT3 concomitant with decreased leukemia burden. One representative mouse of each group is shown. (C) Annexin V staining, (D) Caspase 3/7 activity and (E) Caspase 8 activity were analyzed by flow cytometry in leukemic cells from control mice (0h) and at the different time points following anti-CD3 administration (n=3 mice/group).

Supplemental Figure 2. Identification of OKT3-induced transcriptomic signature in UPNT420 T-ALL PDX. NSG mice infused with 10^6 leukemic cells from T-ALL UPNT420, were monitored for leukemia expansion. Once leukemic, mice (n=6 per group) were treated with $4\mu\text{g}$ of either control mIgG2a or OKT3. 6h later, mice were killed, $\text{hCD45}^+/\text{hCD7}^+$ leukemic sorted from the bone marrow by flow cytometry, RNA extracted and analyzed by RNAseq (A) Volcano plot of genes differentially expressed between control and OKT3-treated cells. Fold change (x axis) is plotted against statistical significance (y axis) for gene. Genes with a fold change ≥ 1.5 and $p < 0.05$ are highlighted in red. (B) Heat map representation of OKT3 (red) deregulated genes as compared to control (black) (C) GSEA of the thymic negative selection gene signature⁵ with the transcriptomic signature induced in T-ALL cells in response to OKT3 administration, showing a strong and significant enrichment of this negative selection signature in anti-CD3-treated leukemic cells. NES: Normalized Enrichment Score; FDR: False Discovery Rate. (D) GSEA of “TNF signaling via NF κ B” with transcriptomic signature induced in response to OKT3, showing a strong and significant enrichment of this signature in OKT3-treated samples. (E). KEGG schematic representation of “TNF α signaling via NF κ B” pathway. Genes whose expression is significantly deregulated in response to OKT3 as described above (Supplemental. Fig 2A, B) are highlighted in red.

Supplemental Figure 3. TNF signaling components induced *in vivo* in response to anti-CD3 in T-ALL in a panel of PDXs derived from 8 independent T-ALL cases. NSG mice infused with 10^6 leukemic cells from either T-ALL UPNT419, UPNT420, UPNT776, UPNT584, UPNT760, UPNT757, M149 or UPNT663, were monitored for leukemia expansion. Once leukemic (>50% leukemic cells in BM), mice (n=3 per group) were treated with $40\mu\text{g}$ of either control mlgG2a or OKT3. 12h later, mice were killed, hCD45+/hCD7+ leukemic cells were sorted from the bone marrow by flow cytometry, RNA extracted and analyzed using a Nanostring custom panel of 97 genes (see Materials and methods) comprising 5 housekeeping genes. Histograms show digitalized mRNA expression for TNF α , TNFRSF1b, BIRC3, C-REL, NFKB1, NFKB2, RELB, TRAF1, TRAF3, NFKBIA, NFKBID, NFKBIE after normalization with Nsolver software.

Supplemental Figure 4. TNFR1, IAP1 and IAP2 expression in control and anti-CD3-treated leukemic cells. (A) Flow cytometry analysis of TNFR1 cell surface expression in PDXs derived from T-ALL UPNT419, UPNT420. Leukemic cells (hCD45+) were stained with either an anti-TNFR1 antibody (blue line) or the isotypic control antibody (grey line). One representative mouse is shown. (B) Western blot analysis of IAP1 and IAP2 expression in leukemic cells from xenografted M149 T-ALL treated as in Supplemental Fig. 3. Results from 2 to 3 independent mice are shown. Analysis of hCD7 expression is used as loading control.

Supplemental Figure 5. SMAC mimetic Birinapant synergizes with anti-CD3 to induce leukemic cells apoptosis. NSG mice were injected with 10^6 UPNT420 T-ALL cells. At high leukemia burden, mice were treated for 12 hours with either control IgG2a/solvent (black dots), $4\mu\text{g}$ OKT3/solvent (red squares) or with the combination of OKT3 and Birinapant (purple triangles), time after which leukemia burden (hCD45/hCD7+ cells), Annexin V/7AAD and Caspase 3/7 activities were analyzed by flow cytometry. Student T-test was used to compare anti-CD3 *versus* anti-CD3/Birinapant-treated cells data.

LEGENDS SUPPLEMENTAL TABLES

Supplemental Table 1. List of the 1632 differentially expressed genes in OKT3-treated leukemic cells as compared to control antibody-treated mice. FC >1,5, p<0.05

Supplemental Table 2. List of the 1349 differentially expressed genes in Teplizumab-treated leukemic cells as compared to control antibody-treated mice. FC >1,5, p<0.05

Supplemental Table 3. List of the top first 20 pathways, the signature of which is significantly enriched in OKT3(Top)- or Teplizumab (bottom)-treated leukemic mice xenografted with T-ALL UPNT776 as compared to leukemic mice treated with the control antibody.

Supplemental Table 4. List of the top first 20 pathways, the signature of which is significantly enriched in OKT3(Top)- or Teplizumab (bottom)-treated leukemic mice xenografted with T-ALL UPNT420 as compared to leukemic mice treated with the control antibody.

Supplemental Table 5. Identification, TCR status and oncogenetic characteristics of T-ALL diagnostic samples.

Chapter V.

Results Part II

1. Additional targetable candidates to enhance the α CD3 antileukemic properties

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Figure 1. Identification of potential candidates to improve α CD3 antileukemic properties.

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2. Validation and generalization of induction of PD1, 4-1BB and OX40 in T-ALL PDX in response to anti-CD3

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Figure 2. PD-1, 4-1BB and OX-40 are consistently induced in T-ALL PDXs in response to the α CD3 OKT3.

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Figure 3. Increased PD-1, 4-1BB and OX-40 RNA levels translate into upregulation at the protein levels

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3. Effect of PD-1 inhibition to the α CD3 anti-leukemic properties

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Figure 4. PD-1 downregulation enhances α CD3-induced leukemic cell death in vitro.

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Figure 5. PD-1 is a targetable player to improve the α CD3 anti-leukemic properties

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4. Transcriptomic profiling of the anti-leukemic α CD3 mAbs and Pembrolizumab combination

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Figure 6. PD-1 inhibition upon the α CD3 treatment is not associated with a distinctive molecular pathway.

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Figure 7. Pembrolizumab-mediated PD-1 inhibition amplifies the anti-CD3-induced molecular signature.

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5. Enhancing the synergistic anti-leukemic effect of α CD3 mAbs and Birinapant through PD-1 inhibition

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Figure 8. PD-1 inhibition enhances the anti-leukemic effect of α CD3 and Birinapant combination.

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6. Effect of the activation of the TCR co-receptor 4-1BB to the α CD3 anti-leukemic affect

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**Figure 9. 4-1BB activation enhance the anti-leukemic properties of the α CD3
OKT3**

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Chapter VI.

Discussion

1. Antibody-based therapies in T-ALL

The recent clinical use of immunotherapies in the last few decades has revolutionized cancer treatment, including that of Acute Lymphoblastic Leukemia (ALL). B-ALL patients, for example, have had significant benefits from the identification of CD20 and CD19 as enriched antigens for B-ALL cells (Maury *et al.*, 2016; Cappell and Kochenderfer, 2023). However, a drawback associated with antibody- and cellular-based immunotherapies is that the shared antigen expression between healthy and malignant cells can lead to on-target off-tumor cytotoxicity, resulting in lymphocyte aplasia. B-cell aplasia can be tolerable in the context of B-ALL treatment with immunoglobulin administrations; however, T-cell aplasia leads to severe immunosuppression (Halim and Maher, 2020). Due to this challenge, T-ALL patients have lagged behind in obtaining the benefits of immunotherapy. Highly expressed antigens in malignant T-lymphoblasts, such as transmembrane receptors CD1a (Coventry and Heinzl, 2004), CD5 (Fleischer, Spencer and Raikar, 2019), CD7 (Campana *et al.*, 1991), and CD38 (Tembhare *et al.*, 2020), have been identified. However, attempts to target T-ALL cells using these antigens have been associated with T-cell aplasia, Cytokine Release Syndrome (CRS), and T-cell fratricide when using these targets for CAR-T cell production (Caracciolo *et al.*, 2023).

Research conducted in our laboratory has shown that targeting the TCR complex in T-ALL cells with the mouse mAb, OKT3, which targets human CD3 ϵ , leads to a profound clearance of leukemic cells in T-ALL patient-derived xenograft (PDX) that translates into enhanced survival of the recipient mice. Indeed, OKT3 was the first antibody to receive approval for human use as a treatment for organ transplant rejection in 1986. However, its application in patients was restricted to acute cases due to severe secondary effects, including CRS (Chatenoud *et al.*, 1990). Such side effects are associated with Fc γ receptors (Fc γ R)-mediated immune responses against the murine antibody, leading to a transient acute release

of pro-inflammatory cytokines. This situation prompted the development of new generations of safer α CD3 mAbs (Kuhn and Weiner, 2016).

The current generations of α CD3 monoclonal antibodies (mAbs) in clinical development include humanized antibodies, notably including the hOKT3 γ 1(Ala-Ala) Teplizumab, which carry two point mutations replacing two leucine amino acid residues with alanine, L234A and L235A, in the Fc CH2 domain. This alteration reduces mechanisms related to Fc receptor binding, resulting in significantly decreased cytokine release and negligible immunogenicity. Clinical trials have been conducted using Teplizumab to prevent rejection of allograft transplantation. However, the most clinically advanced applications of Teplizumab are in autoimmune diseases, particularly in the treatment of type 1 diabetes (T1D). Teplizumab has shown the ability to protect insulin-producing pancreatic-beta cells from autoreactive T-cells while proving to be safe for patients (Herold *et al.*, 2019). During its use in T1D patients, only mild adverse events of limited duration were observed. Consequently, Teplizumab obtained FDA approval in 2022 for its use as a treatment for T1D patients (Misra and Shukla, 2023).

It is in this historical context that our laboratory has also demonstrated that the experimental administration of non-Fc γ R-binding anti-CD3 mAbs, including Teplizumab, results in efficient anti-leukemic effect in vivo in T-ALL preclinical models, evidencing a potentially safe, clinically relevant therapeutic option for the treatment of T-ALL (Tran Quang *et al.*, 2020). Additional therapeutic strategies exploiting the TCR complex as a target include anti-TRBC1 CAR T cells, which are designed to target the constant TCRB chain. The use of anti-TRBC1 CAR T cells has been demonstrated as a strategy to target leukemic cells while avoiding CAR-T fratricide and pan T-cell aplasia in T-ALL mouse models (Maciocia *et al.*, 2017). While the antileukemic effects of anti-TRBC1 CAR T cells have been attributed to classical cytokine-based T cell cytotoxicity, in light of our findings, the binding of CAR-T cells to the TCRB chain of T-ALL cells could also result in an intrinsic anti-leukemic effect induced by the engagement of the TCR. Biological and molecular features induced by the anti-CD3 therapy described in this study would also help to

compare the intracellular anti-leukemic effect of the anti-TRBC1 CAR T. The anti-TRBC1 CAR T, inducing an intrinsic cell death mechanism in T-ALL cells, would represent a potent double-edged weapon, inhibiting leukemia with two different powerful anti-tumoral mechanisms.

Overall, targeting the TCR complex with α CD3 antibodies (including one approved by the FDA) induces a strong, relatively specific, and potentially safe anti-leukemic response. Therefore, the results of the present thesis, in accordance with other data generated so far, support the use of this therapeutic target for its application in the clinic against T-ALL.

2. Antileukemic cooperation of α CD3 mAbs with chemotherapy in T-ALL

Patients diagnosed with T-ALL are treated with risk-based intensive chemotherapy regimens. While these therapeutic strategies have led to event-free survival (EFS) rates above 85%, salvage therapy remains challenging for relapse/refractory patients, with EFS rates of less than 25% (Raetz and Teachey, 2016). Due to the complexity of chemotherapy regimens, relapsed T-ALL exhibits significant genetic heterogeneity (Ferrando and López-Otín, 2017).

The mechanisms associated with chemotherapy resistance in T-ALL remain largely unknown. Recent studies conducting whole sequencing analyses on matched pairs of initial and relapsed T-ALL samples reported that driver mutations and oncogenic fusion genes are highly conserved from the time of diagnosis until relapse. Additionally, clonal evolution of relapsed T-ALL was found to arise from pre-existing leukemic clones (Richter-Pechańska *et al.*, 2022; Sentís *et al.*, 2020). Mutations frequently found in r/r T-ALL include PTEN alterations, leading to activation of the PI3K/AKT signaling pathway (Beesley *et al.*, 2009; Piovan *et al.*, 2013), as well as mutations leading to constitutive activation of IL7R-JAK-STAT(Li *et al.*, 2016; Richter-Pechańska *et al.*, 2022) and NOTCH signaling (Sentís *et al.*,

2020). On this regard, previous studies in our laboratory have shown that while administration of conventional chemotherapy resulted in relapsed T-ALL PDX, combining chemotherapy with α CD3 mAbs resulted in a cooperative antileukemic effect in all T-ALL PDX tested, independently of their mutational footprint (Tran Quang *et al.*, 2020)

The mechanisms behind the improved anti-leukemic effect of the combination of chemotherapy with α CD3 were not addressed in the present study, but the identification of the α CD3-induced molecular signature presented here could be cross-compared with transcriptomic profiles induced by conventional chemotherapies to analyze whether α CD3 and chemotherapy share common molecular players that can be targeted or that should be avoided. Similar to what was identified with the TNF α signaling pathway, these features could be exploited to enhance the anti-leukemic molecular program induced by the co-administration of α CD3 mAbs with chemotherapy.

Another aspect that could explain the cooperation between α CD3 and chemotherapy is the differential sensitivity of leukemic clones to each of the treatments. Single-cell analysis of the response of T-ALL PDX to α CD3 treatment, both alone and in combination with chemotherapy, could help identify molecular characteristics of i) Clones sensitive and resistant to chemotherapy, ii) Clones sensitive and resistant to anti-CD3, and iii) Clones sensitive and resistant to the combination of the treatments. The data generated from such experiments would help find strategies to overcome resistance to the treatment. Additionally, the identification of markers of resistance could assist in determining prognostic markers for the use of α CD3 therapy in relapsed/refractory T-ALL cases that have undergone chemotherapy regimens.

3. TNF α signaling in response to α CD3 mAbs in T-ALL

The present study shows that the TNF α signaling via NF- κ B is the top deregulated pathway in the transcriptomic profiling of a T-ALL PDX in response to α CD3 treatment. Consequently, we proceeded to validate the upregulation of

various members of this signaling pathway, including the receptors TNFR1 and TNFR2, their cognate ligands TNF α and LT α , as well as the adaptors and effectors of the pathway. Analysis of seven independent T-ALL PDX models following α CD3 treatment confirmed the induction of TNF α and LT α transcription, along with other pathway members, indicative of an active signaling pathway. These results are consistent with previous observations described in an old case report of a patient with r/r T-ALL treated with a total of 500 mg of OKT3, administered in multiple doses not exceeding 50 mg/day, who showed transient decrease of leukemia load in peripheral blood, accompanied by a strong increase in serum protein levels of TNF α a few hours after each OKT3 administration (Gramatzki *et al.*, 1995).

TNF α was first described as a molecule that induced tumor cell death in vitro (Carswell *et al.*, 1975) and soon demonstrated anti-tumor effects in vivo in murine and human cancers (Haranaka, Satomi and Sakurai, 1984). Indeed, its use as an anti-cancer treatment rapidly escalated to clinical studies (Feinberg *et al.*, 1988; Zamkoff *et al.*, 1989), but failed in 18 phase-I clinical trials due to dose-dependent acute toxicities (Roberts, 2011). This could be clarified by the discovery of the essential role of TNF α as a promoter of inflammation (Beutler, Milsark and Cerami, 1985). Nowadays, the pleiotropic role of TNF α is well established. TNF α and its associated signaling pathways regulate several processes that are context dependent, including not only cell death and pro-inflammatory responses, but also cell survival, cell differentiation, cell proliferation and migration, all converging in NF- κ B activation (Bradley, 2008; Brenner, Blaser and Mak, 2015). TNF α is in this way a paradoxical player in cancer development and treatment.

In the present study, to unravel the role TNF α signaling induced by α CD3 mAbs in T-ALL we used etanercept, a decoy receptor drug that quenches TNF α and prevents its binding with its receptors. Inhibition of TNF α signaling enhanced the anti-leukemic effects of α CD3, and ultimately resulted in improved survival of mice carrying T-ALL PDX. Based on these findings, we conclude that TNF α signaling in the context of α CD3-treated T-ALL cells function as a pro-survival mechanism that limits α CD3 toxicity. In this way, interfering with this break potentiates the anti-

leukemic process of α CD3. In line with our data, a recent study has also highlighted the association between TNF α production and the promotion of leukemia cell survival (Verma *et al.*, 2022). The study of patients with acute leukemias, including T-ALL, revealed that patients' plasma levels of TNF α were significantly higher compared to those of healthy control donors. These elevated TNF α levels correlated with higher blast counts and worse patient survival outcomes (Verma *et al.*, 2022). In this sense, etanercept, an antibody approved for its clinical use, could represent a therapeutic option against T-ALL under the anti-CD3 treatment setting. However, more research should be conducted on this subject, as etanercept could have pro-leukemic roles (Bakland and Nossent, 2003; Cansu, Teke and Korkmaz, 2017).

The TNF α signaling pathways leading to cell survival rely on the formation of a multi-protein complex involving TRADD-RIPK1-TRAF2-cIAP1/cIAP2, among other proteins at the receptor level. In accordance with this, experimental inhibition of cIAP1/cIAP2 with the Smac Mimetic drug Birinapant demonstrated that TNF α signaling can be regulated and redirected from a pro-survival to a cell death signaling pathway (Varfolomeev *et al.*, 2007). In this way, the α CD3-induced TNF α signaling can be exploited therapeutically. In the present study, we show that, while individual administration of Birinapant in our T-ALL PDX models has a negligible effect on leukemia expansion and survival, combination with α CD3 act synergically. These results align with reports studying the combination of SM drugs with TNF α -inducing therapies such as different chemotherapies (Probst *et al.*, 2010), (Tao *et al.*, 2019), and immune checkpoint inhibitors (ICIs) (Beug *et al.*, 2017) resulting in synergistic anti-tumor effects. Moreover, phase I and II clinical trials are currently evaluating the safety and efficacy of SM drugs in combination with these TNF α -inducing therapies (Morrish, Brumatti and Silke, 2020). The results of these studies contribute to the rationale for evaluating the clinical combination of α CD3 and Birinapant in T-ALL. Furthermore, considering the anti-leukemic cooperation of α CD3 and chemotherapy reported in our laboratory, another pre-clinical step that would be important to validate is the use of the α CD3-Birinapant combination with chemotherapy regimens and other standards of care in our T-ALL PDX models.

4. Amplifying TCR signaling strengthens α CD3 anti-leukemic effects

Previous studies in our laboratory have shown that α CD3 anti-leukemic effects are TCR-signaling dependent (Trinquand *et al.*, 2016). In the present study, we demonstrate a consistent induction of PD-1 and PD-L1 expression in response to α CD3 stimulation in independent T-ALL PDX models. PD-1 is a co-inhibitory receptor of the TCR that, upon engagement with its endogenous ligands, negatively regulates TCR signaling. Therefore, we evaluated whether PD-1 upregulation could act as a brake on the α CD3-mediated cell death. Indeed, we showed that genetic downregulation of PD-1 in T-ALL cell lines or its pharmacological inhibition *in vivo* using Pembrolizumab led to enhanced α CD3-mediated cell death.

Upregulation of PD-1 ligands by tumor cells is a well-documented mechanism leading to evasion of T cell-mediated immune responses. Antibodies that block the PD-1/PD-L1 axis have demonstrated to successfully re-activate anti-tumor T cells and proven to be clinically safe in various settings. By 2020, six different anti-PD-1/PD-L1 mAbs for treating different types of cancers have been approved by the FDA (Twomey and Zhang, 2021). In this way, anti-PD1 antibodies could represent an emerging therapeutic strategy to activate immune T cell mechanisms against T-ALL cells extrinsically (as for other malignancies), but in the context of therapies including anti-CD3 treatment, also intrinsically, by enhancing α CD3-mediated cell death. Future directions for the therapeutic improvement of α CD3 antibodies by targeting PD-1 should include the use of immunocompetent models to assess T cell-cytotoxic effects towards T-ALL. For this end, syngeneic models could be used, but accounting for the great genetic heterogeneity of T-ALL, their preclinical significance would be limited unless several models are used. Therefore, although challenging, a suitable experimental model with high preclinical significance could be PDX engraftment in humanized NSG mice (Saito, Shultz and Ishikawa, 2020)

Our in vivo data show that the individual treatment of the α PD-1 mAb Pembrolizumab had a negligible effect on the leukemia load measured in the blood and the survival of three independent T-ALL cases. Consistently, basal cell death of Jurkat and CEM T-ALL cell lines was not affected upon genetic downregulation of PD-1. These observations contrast with recent studies reporting that PD-1 is a marker of a minor T-ALL subpopulation with leukemic stem cell (LSC) characteristics (Xu *et al.*, 2023). In vivo inhibition of PD-1 signaling in mice xenografted with T-ALL containing this subpopulation result in tumor burden reduction and extended overall survival of the recipient mice. The contrasting therapeutic outcomes could be explained by heterogenic basal expression of PD-1 in T-ALL and the presence or absence of this PD-1 enriched subpopulation.

Finally, in the present work we have documented a robust induction of the TNFR family members 4-1BB and OX40 in response to α CD3 treatment in numerous T-ALL PDX. Similar to other therapeutic approaches aiming to modulate TCR-signaling we made use of an agonistic mAb targeting 4-1BB to amplify the α CD3-induced effect (Chester *et al.*, 2018). Our results showed that the activation of the 4-1BB co-receptor in combination with α CD3 OKT3 resulted in enhanced anti-leukemic properties of α CD3, supporting our hypothesis that stronger TCR signaling leads to the stronger α CD3 therapeutic effects. The combination of 4-1BB agonistic mAbs with PD-1-blocking mAbs in a pre-clinical mouse model results in increased numbers of activated human T lymphocytes (Sanmamed *et al.*, 2015; Tolcher *et al.*, 2017), suggesting that the combination of these immunotherapies can lead to a cooperative amplification of the TCR signaling. Along these lines, the triple combination of α CD3 treatment concurrently with 4-1BB activation and PD-1 inhibition could result in enhanced TCR activation and thus further increase the α CD3 anti-leukemic properties. This could also lead to an optimization of the minimum effective doses of all drugs, thus limiting potential on-target off-tumor toxicities. Finally, activation of TCR co-receptors could also represent an alternative strategy to amplify α CD3 effects for PD-1 negative T-ALL cases.

5. Conclusions and future perspectives

The α CD3 mAbs are new antibody-based therapy with potent antileukemic effects in pre-clinical T-ALL PDX models. The results in this thesis have contributed to understand the molecular mechanisms behind the anti-leukemic effects of the α CD3 mAbs. Analysis of the transcriptomic profiling of the in vivo response to OKT3 and Teplizumab α CD3 mAbs in T-ALL-derived PDX confirmed our in vitro observations that α CD3-induced molecular signature is highly associated to the thymic negative selection, a key physiological checkpoint in T-cell development (Trinquand *et al.*, 2016). Additionally, we identified new molecular cues to enhance the α CD3 efficacy, such as TNF α signaling via NF- κ B, and PD-1 signaling, which, in light of their physiological role, represent new vulnerabilities that can be targeted using clinically relevant agents.

The present study identified promising players focusing only on α CD3-induced upregulated genes, however, other potential players that could participate to anti-leukemic properties of the α CD3 mAbs can be found in the α CD3-induced downregulated genes. As an example of this, data obtained in our laboratory (not shown in the present work) show that upon α CD3 treatment, CXCR4 is strongly downregulated at the cell surface, suggesting that α CD3 could favor a leukemic release from the bone marrow niche affecting thus the leukemic cells survival.

Despite its potent anti-leukemic effect, α CD3 mAbs monotherapy in all TCR+ T-ALL-derived PDX tested resulted in relapse. Thus, future directions of this study should include research on mechanisms of resistance to α CD3 therapy, as mentioned before, by using single-cell sequence analysis. Pharmacological strategies to improve α CD3 therapeutic features could include the coupling of α CD3 mAbs with clinically relevant pharmacological agents. The results obtained in this thesis encourage the manufacture of α CD3 antibody drug-conjugate (ADC) coupled with a smac mimetic small molecule such as Birinapant or the production of bi-specific antibodies targeting CD3 and PD-1 or 4-1BB to optimize α CD3 therapeutic properties.

Taken together, in this work, we generated and validated a transcriptomic profile induced by the α CD3 mAbs that implicated key players in α CD3-mediated T-ALL inhibition. The use of pharmacological agents to regulate TNF α and TCR signaling demonstrated an improvement in the α CD3 anti-leukemic properties in independent T-ALL-derived PDX models. Furthermore, the current clinical use of the therapeutic agents employed in this study strongly supports their consideration for clinical testing in combination with α CD3 as an anti-leukemic treatment for TCR+ T-ALL cases.

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