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Étude du rôle de l'inflammasome et de la kinase Styk1 dans la régulation des lymphocytes cytotoxiques

Sébastien Fauteux-Daniel

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Étude du rôle de l'inflammasome et de la kinase Styk1 dans la régulation des lymphocytes cytotoxiques

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Étude du rôle de l'inflammasome et de la kinase Styk1 dans la régulation des lymphocytes cytotoxiques.

Lors de la reconnaissance d'une cellule cible, les lymphocytes cytotoxiques T CD8 et Natural Killer (NK) opèrent un relargage polarisé de vésicules cytotoxiques contenant des molécules effectrices, notamment la perforine (PRF1) et le granzyme B. Le dysfonctionnement de l'exocytose des granules cytotoxiques est responsable d'une susceptibilité accrue aux pathogènes intracellulaires qui s'accompagne de l'activation continue et anarchique des lymphocytes cytotoxiques et des macrophages. Ce phénomène conduit à la lymphohistiocytose hémophagocytaire (HLH), un syndrome auto-inflammatoire fatal en absence d'intervention thérapeutique. Les mutations des gènes codant pour la perforine (PRF-1) ou pour certaines des protéines impliquées dans la biogénèse ou le transport vésiculaire des granules cytotoxiques sont causales des formes familiales ou primaires de la HLH (FHL). La HLH fait également partie des complications secondaires aux infections à *herpesviridae* et à certains désordres immunologiques importants. En effet, on retrouve celle-ci chez les patients souffrant d'arthrite juvénile idiopathique (SoJIA). Dans ce contexte, l'étiologie demeure toutefois incomprise.

Au moment d'entreprendre les travaux présentés dans ce manuscrit, le premier cas de HLH induite par une mutation menant à l'activation constitutive de la composante NLRC4 de l'inflammasome était décrit. L'inflammasome est une structure multimérique composée d'un récepteur cytosolique, de la protéine échafaud ASC et de la Caspase-1. On le retrouve notamment chez les macrophages et cellules dendritiques, permettant ainsi la reconnaissance de signaux pathogéniques (PAMP) ou de danger (DAMP). L'activation de l'inflammasome mène à la maturation des pro-formes de l'IL-1 β et l'IL-18 ainsi qu'à leur sécrétion. L'activation constitutive de NLRC4 étant suffisante au déclenchement de la HLH, nous avons tenté de comprendre si cette structure y était essentielle dans le cadre des défauts génétiques de cytotoxicité. Avec pour objectif de répondre à cette question, nous avons invalidé l'inflammasome en abrogeant la protéine ASC ou Caspase-1 dans le modèle murin de HLH déficient pour la perforine (PRF1 -/-). Nous avons également testé l'hypothèse qu'un déficit de cytotoxicité pouvait expliquer le développement de la HLH chez les patients souffrant de SoJIA.

Nos résultats montrent que l'inflammasome est nécessaire à la production d'IL-18 lors de la HLH mais qu'il n'est pas essentiel au développement de la maladie dans le cadre des FHL. Par ailleurs, nous montrons que la cytotoxicité des cellules NK semble normale chez les patients atteints de SoJIA, ce qui suggère que les mécanismes immunologiques à l'origine de la HLH dans les FHL et dans les maladies autoinflammatoires comme la SoJIA sont distincts.

Dans la seconde partie de ce manuscrit, nous avons étudié sur le rôle de la sérine/thréonine/tyrosine kinase Styk1 dans la régulation des lymphocytes cytotoxiques NK. Ces derniers sont responsables du contrôle immunitaire précoce des pathogènes intracellulaires et contribuent à l'immunosurveillance des cellules tumorales. Suite à leur activation, ils relâchent de très grandes quantités d'IFN- γ et de TNF- α , faisant notamment le lien entre l'immunité innée et adaptative. La reconnaissance des cellules cibles par les lymphocytes NK est gouvernée par l'expression d'un éventail de récepteurs qui transduisent des signaux, activateurs ou inhibiteurs, et dont la balance se traduit par l'activation ou la tolérance. Ces récepteurs sont codés au sein de deux complexes génétiques très denses, le complexe de cytotoxicité naturelle (NCR) et le complexe des récepteurs des leucocytes (LRC). Au moment de commencer ces

travaux, nous avons révélé que l'expression de la kinase Styk1 fait partie de la signature transcriptionnelle des lymphocytes NK. Sa fonction dans le système immunitaire demeure toutefois inconnue. Néanmoins, la localisation génétique favorable de Styk1, près du NCR, ainsi que son implication dans la voie PI3K-AKT, en faisaient un candidat potentiel de régulation des lymphocytes NK. Afin de connaître le rôle de Styk1 dans le développement et les fonctions effectrices des lymphocytes NK, nous avons donc généré une souris pour laquelle Styk1 est invalidé. Nos résultats confirment que Styk1 est exprimée de façon spécifique par les cellules NK, et n'est pas retrouvée chez les autres types de cellules immunitaires, en particulier les cellules lymphoïdes innées (ILCs). Nous avons également détecté une diminution de l'activité constitutive de la voie AKT/mTOR dans les cellules NK, mais le développement, l'homéostasie et la fonction des cellules NK sont cependant normaux dans les souris déficientes en Styk1.

Role of the inflammasome and of Styk1 kinase in the regulation of cytotoxic lymphocytes

Upon recognition of infected or target cells, CD8+ T and Natural Killer (NK) lymphocytes initiate a polarized degranulation of vesicle storing cytotoxic molecules (perforin: PRF1 and granzyme B). By altering the target cell's cellular membrane integrity, perforin allows granzyme B to translocate to its cytosol. Genetic anomalies may affect normal cytotoxic functions and severely hamper the control of intracellular pathogens. In this context, the pathogenic signal remains uninterrupted and both cytotoxic lymphocytes and macrophages are continuously stimulated. This auto-inflammatory pathological condition is named hemophagocytic lymphohistiocytosis (HLH) and is fatal without therapeutic intervention. HLH can also occur secondary to infection with viruses from the *herpesviridae* family, or be concomitant to important immune alterations such as systemic onset juvenile idiopathic arthritis (SoJIA), with no clear etiological cause identified.

In 2014, a case of HLH mediated by the constitutive activation of the NLRC4 inflammasome receptor was first described. The inflammasome is a multimeric structure involving a cytosolic receptor, a scaffold protein – ASC – and Caspase-1. In the immune system, the inflammasome is expressed in macrophages and dendritic cells and senses pathogenic (PAMP) and danger (DAMP) associated signals. Once activated, inflammasome's protein Caspase-1 catalyzes the maturation of pro-IL-1 β and pro-IL-18 and leads to their secretion. Since NLRC4 constitutive activation appears to be sufficient for triggering HLH, we aimed to understand if the inflammasome structure was essential to the development of the syndrome. In order to address this question, we invalidated the inflammasome through the abrogation of ASC or Caspase-1 in PRF1 $-/-$ HLH mouse model. We also tested the hypothesis that an altered cytotoxic function could explain the high prevalence of HLH in the proinflammatory context of SoJIA.

The results we present here show that the inflammasome is responsible for the elevated levels of IL-18 in the serum of HLH patients. However, the inflammasome is facultative for its development. We also demonstrate that in patients suffering from SoJIA, NK cells show normal cytotoxicity, suggesting that immunological mechanisms involved in FHL and secondary HLH are distinct.

In the second part of this manuscript, we aim at understanding the role of Styk1 serine/threonine/tyrosine kinase in NK cells' regulation. NK cells are in charge of eliminating stressed, virally infected or transformed cells. Upon activation, they secrete large amounts of IFN- γ and TNF- α , thus bridging innate and adaptive immunity. Capabilities for recognition of target cells is endowed by the expression of numerous stochastically expressed activating and inhibitory receptors. The balance between activating and inhibitory signal allows for self-tolerance or activation upon engagement of abnormal cells. Activating and inhibitory receptors are germline encoded in two dense, large complexes, the Natural Killer Complex (NKC) and the Leukocyte Receptor Complex (LRC). At the moment of starting this work, we had recently identified that Styk1 was a signature transcript of NK cells. However, its function in NK cells and more generally in the immune system remains unknown. Nevertheless, its genetic localisation near the NKC and its potential implication in the PI3K-AKT pathway prompt that it may play a role in NK cell development and/or functions. In order to evaluate the role of Styk1 in NK cells' regulation, we generated a mouse model in which its expression is abrogated.

Our data confirms that amongst all immune subsets, Styk1 is specifically expressed by NK cells. Styk1 was also able to discriminate NK cells from other ILCs. In this study, we show that Styk1 invalidation lead to a decrease of activity in the AKT/mTOR pathway. However, NK cells development, homeostasis and function were surprisingly normal in Styk1 ^{-/-} mice.

Discipline / Field

Immunologie / Immunology

Mots-clés

Syndrome d'activation macrophagique, lymphohistiocytose hémophagocytaire, auto-inflammation, lymphocytes cytotoxiques, perforine, inflammasome, ASC, Caspase-1, SoJIA.

Keywords

Macrophage activation syndrome, hemophagocytic lymphohistiocytosis, auto-inflammatory disorders, cytotoxic lymphocytes, perforin, inflammasome, ASC, Caspase-1, SoJIA.

Intitulé et adresse du laboratoire

Immunité innée dans les maladies infectieuses et auto-immunes

Innate immunity in infectious and autoimmune diseases

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Liste des publications

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Article 2: Fauteux-Daniel, S., Faure, F., Geary, C., Marotel, M., Daussy, C., Sun, J.C. and Walzer, T., Styk1 expression is a hallmark of NK cells but is dispensable for their immune function. Ready for submission.

Annexe 1: Zhang, J., Marotel, M., Fauteux-Daniel, S., Mathieu, A-L., Viel, S., Marçais, A. and Walzer, T. (2018) T-bet and Eomes in mouse and human NK cells and ILC1s. *EJI*, 0, 1-13.

Annexe 2 : Marçais, A., Marotel, M., Degouve, S., Koenig, A., Fauteux-Daniel, S., Drouillard, A., Schlums, H., Viel, S., Besson, L., Allatif, O., Bléry, M., Vivier, E., Bryceson, Y., Thauinat, O., Walzer, T. (2017). High mTOR activity is a hallmark of reactive natural killer cells and amplifies early signaling through activating receptors. *eLife*, 6, e26423.

Pré-doctorat

Fauteux-Daniel, S., Larouche, A., Calderon, V., Boulais, J., Béland, C., Ransy, D.G., Boucher, M., Lamarre, V., Lapointe, N., Boucoiran, I., Le Campion, A and Soudeyins, H. (2017) Vertical transmission of hepatitis C virus: variable transmission bottleneck and evidence of mid-gestation in utero infection. *Viruses*, 4(12), 3531,3550. <http://doi:10.1128/JVI.01372-17>

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Le Campion, A., Larouche, A., Fauteux-Daniel, S., & Soudeyins, H. (2012). Pathogenesis of Hepatitis C During Pregnancy and Childhood. *Viruses*, 4(12), 3531–3550. <http://doi.org/10.3390/v4123531>

Liste des abréviations

ADN: Acide désoxyribonucléique

AIM2 : Absent du mélanome 2 / Absent In Melanoma 2

ALAT : ALanine aminotransférase

APC : Cellules présentatrices d'antigènes / Antigen Presenting cells

ARN : Acide ribonucléique

ARNm : ARN messenger

ASAT : Aspartate aminotransférase

ASC : Apoptosis-Associated Speck-Like Protein Containing CARD ou PYCARD

BCR : Récepteur des lymphocytes B / B Cell Receptor

CARD: Domaine de reconnaissance de la caspase / CAspase Recognition Domaine

CMH : Complexe Majeur d'Histocompatibilité

DAMP: Patron moléculaire associé au danger / Danger-Associated Molecular Pattern

DC : Cellule dendritique / Dendritic Cell

EBV : Virus Epstein-Barr / Epstein-Barr Virus

FADD : Domaine de mort associé à FAS / Fas-Associated Death Domain

FGFR : Récepteur du facteur de croissance des fibroblastes / Fibroblast Growth Factor Receptor

FHL : Hémophagocytose lymphohistiocytaire familiale / Familial Hemophagocytic Lymphohistiocytosis

HLH : Lymphohistiocytose hémophagocytaire / Hemophagocytic LymphoHistiocytosis

IFN : Interféron

IL : Interleukine

ILC : Cellules lymphoïdes innées / Innate Lymphoid Cell

ITAM : Récepteur immunitaire activateur à motif tyrosine / Immunoreceptor Tyrosine-based Activating Motif

ITIM : Récepteur immunitaire inhibiteur à motif tyrosine / Immunoreceptor Tyrosine-based Inhibitory Motif

LCMV : Virus de la chorioméningite lymphocytaire / Lymphocytic ChorioMeningitis Virus

LYST: Aussi connue comme CHS1

MCMV : Cytomégalovirus murin / Murine CytoMegaloVirus

Munc13-2: Homologue mammalien de UNC 13-2 / Mammalian homologue of UNC protein 13-2

NK : Lymphocyte Natural Killer

NLR: Récepteur NOD-like / NOD-Like Receptor

NLRC4: Récepteur de type NOD à domaine CARD 4 / NOD-Like Receptor with CARD domain 4

NLRP1: Récepteur de type NOD contenant un domaine pyrine / NOD-Like Receptor containing Pyrin 1

NOD: Récepteur à domaine d'oligomérisation des nucleotides / Nucleotide Oligomerization Domain Receptor

NOK: Nouvelle kinase oncogene / Novel Oncogene Kinase

PAMP : Patron moléculaire associé aux pathogènes / Pathogen Associated Molecular Pattern

PDGFR : Récepteur du facteur de croissance dérivé des plaquettes / Platelet-Derived Growth Factor Receptor

pHLH : HLH primaire

PRF1 : Perforine / Perforin

PRR : Récepteurs reconnaissant les patrons moléculaires / Pattern Recognition Receptor

PYD : Domaine pyrine / PYrin Domain

RAG : Gène activateur de la recombinaison / Recombination Activating Gene

RPTK : Récepteurs Protéine Tyrosine Kinase

SAM : Syndrome d'Activation Macrophagique

SCID : Déficit immunitaire combiné sévère / Severe Combined ImmunoDeficiency

sHLH : HLH secondaire

SoJIA : Arthrite juvénile idiopathique / Systemic onset Juvenile Idiopathic Arthritis

STX11 : Syntaxine 11 / Syntaxin 11

STXBP2 : Protéine liant la syntaxine 2 / SynTaXin Binding Protein 2

Styk1 : Sérine Thréonine Tyrosine Kinase 1

TCR : Récepteur des lymphocytes T / T Cell Receptor

TLR: Récepteur Toll-like / Toll-Like Receptor

UNC : Gène non coordonné / UNCoordinated gene

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Préambule

Le système immunitaire

Le système immunitaire est constitué de deux sous-ensembles, les systèmes immunitaires inné et adaptatif (Figure 1). Le premier est basé sur la reconnaissance de patrons moléculaires associés aux pathogènes ou aux situations de danger, ce qui permet aux cellules de l'immunité innée de mettre en place une réponse généraliste mais rapide. Le second est un mécanisme plus complexe faisant intervenir l'éducation de cellules fonctionnelles suite au réarrangement somatique des gènes codant pour leurs récepteurs. Ce processus est générateur de diversité mais nécessite l'expansion clonale des lymphocytes qui sont spécifiques lors de la réponse immunitaire. En conséquence, le versant adaptatif du système immunitaire est plus lent lors de son déclenchement. Il permet toutefois de cibler une grande variété d'agents pathogéniques.

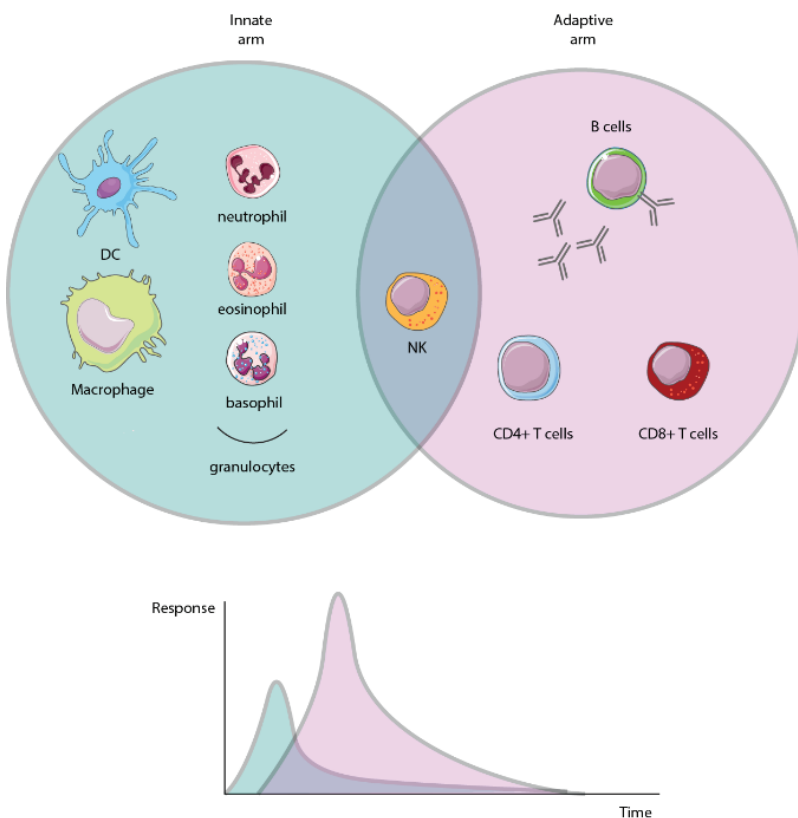


Figure 1 : Représentation simplifiée du système immunitaire et de sa réponse

Les cellules qui composent le bras inné du système immunitaire répondent rapidement mais de façon moins ciblée que les cellules de leur contrepartie adaptative. À l'inverse Les cellules de l'immunité adaptative ciblent très spécifiquement les pathogènes et persistent dans le temps. À l'interface, les lymphocytes Natural Killer, capables de mémoire mais exempts de mécanisme générateur de diversité. Inspiré de [1], tiré et adapté de [2].

L'immunité innée

L'identification des perturbations homéostatiques par les cellules de l'immunité innée est effectuée par des récepteurs reconnaissant des patrons moléculaires, les PRR. Ces derniers reconnaissent les patrons moléculaires qui sont associés aux pathogènes (PAMP) ou aux situations de danger (DAMP). Ces patrons moléculaires sont divers et les macrophages et cellules dendritiques (DC) expriment une myriade de PRR de différentes spécificités qui peuvent être membranaires ou encore cytosoliques [3].

Un des PRR importants de l'immunité innée est l'inflammasome. C'est un complexe multimérique de grand poids moléculaire permettant la reconnaissance de PAMP et de DAMP. Son activation mène à la maturation et sécrétion de l'IL-1 β et de l'IL-18 [4]. La détection de signaux pathogéniques ou non-homéostatiques par l'inflammasome repose sur un répertoire de senseurs cytosoliques de type NOD-like (NLR), notamment constitué de NLRP1, NLRP3 et NLRC4. L'inflammasome fait également intervenir les senseurs AIM2 et Pyrine [5]. Suite à l'activation des NLRs, ce qui a pour effet de révéler leurs domaines d'interaction, ces derniers s'associent avec la protéine échafaud ASC dont la structure contient un domaine pyrine (PYD) ainsi qu'un domaine de reconnaissance de la caspase (CARD). Il en résultera le recrutement éventuel de la pro-Caspase-1. La constitution de ce complexe induit le clivage de la pro-Caspase-1, son activation permettra à son tour de catalyser la maturation de la pro-IL-1 β et de la pro-IL-18, deux cytokines pro-inflammatoires. En parallèle, l'activation de la Caspase-1 catalyse également le clivage de la Gasdermine D, causant ainsi la pyroptose, un type de mort cellulaire spécialisée qui potentialise la réponse immunitaire en permettant notamment la relâche des cytokines (Figure 2).

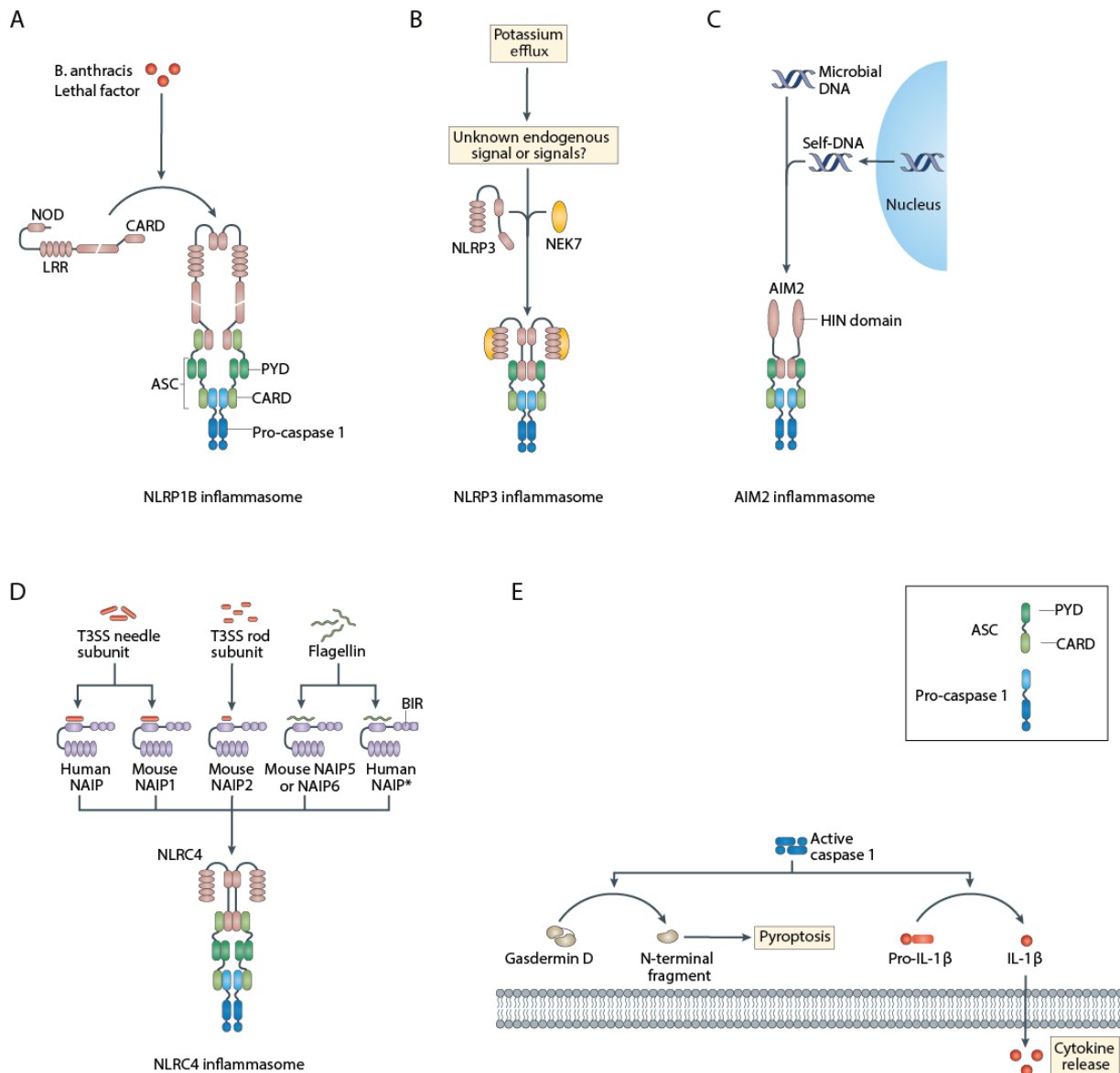


Figure 2 : Les voies de l'activation de l'inflammasome

L'inflammasome est une structure multimérique composée du récepteur A) NLRP1, B) NLRP3, C) AIM2 D) NLRC4 ou pyrine (non-montré) qui contient un domaine pyrine (PYD). Suite à son activation et la révélation du domaine d'interaction, ce récepteur s'associe avec la protéine ASC contenant également un domaine PYD ainsi qu'un domaine de reconnaissance des caspases (CARD). Cette association permet le recrutement de la pro-Caspase-1 et son activation. E) La Caspase-1 activée catalyse la maturation des pro-formes de l'IL-1 β et de l'IL-18. Chacun des récepteurs reconnaît des PAMPs ou DAMPs qui lui sont spécifiques. NLRP1, NLRP3 AIM2 et NLRC4 sont respectivement activés par la toxine de *B. anthracis*, le potentiel potassique, l'ADN microbien ou endogène cytosolique, ou la flagelline et les composantes qui servent à son transport. Tiré et adapté de [5].

L'Immunité adaptative

Les acteurs principaux de l'immunité adaptative sont les lymphocytes B et T. Ces derniers expriment la recombinaison RAG, ce qui leur confère la capacité de réarranger somatiquement les segments de gènes codant pour leurs récepteurs, respectivement le BCR et le TCR [6]. Ce mécanisme générateur de diversité permet la coexistence d'un très vaste répertoire de lymphocytes de spécificité variée [7]. Dans le cas du TCR, la reconnaissance de peptides exogènes se fait dans le contexte de la présentation par le complexe majeur d'histocompatibilité (CMH), que la vaste majorité des cellules exprime [8]. L'activation des lymphocytes T dépend donc de la présentation antigénique, une fonction qui est remplie par les cellules présentatrices d'antigène professionnelles (APC), dont font partie les macrophages et les DC [9]. Suite à la reconnaissance d'un peptide présenté à l'interface du CMH, les lymphocytes T qui en sont spécifiques subissent une prolifération massive. Une phase de contraction du pool de lymphocytes spécifiques suit le contrôle immunitaire et certaines cellules persistent. Une réexposition des lymphocytes persistants au stimulus entraîne une réponse rapide et vigoureuse. Le système immunitaire adaptatif a donc la particularité de générer une mémoire immunologique en pérennisant les cellules spécifiques d'un antigène [10].

La cytotoxicité comme mécanisme effecteur de l'immunité innée et adaptative

Suite à la reconnaissance de cellules anormales ou infectées, les effecteurs du système immunitaire sont en charge de leur élimination. Cette fonction est remplie par les lymphocytes cytotoxiques qui sont capables d'induire la mort cellulaire par apoptose. En effet, les lymphocytes cytotoxiques T CD8 ainsi que les cellules Natural Killer (NK) produisent des granules cytotoxiques contenant les molécules effectrices responsables du déclenchement de l'apoptose, la perforine et les granzymes [11]. Bien que dans une temporalité et avec des modalités d'activation différentes, ces lymphocytes relarguent leurs granules cytotoxiques de façon polarisée, provoquant ainsi la mort de la cellule cible. Ce processus est, nous allons le voir, très finement régulé par un grand nombre d'intervenants protéiques. Ceci, afin de prévenir les dommages importants qu'une dégranulation non-spécifique pourrait engendrer.

Thématique de recherche

Comme ce préambule le laisse deviner, la réponse immunitaire est multifactorielle et engendre une variété de développements biologiques. La déficience de l'une ou l'autre de ses composantes mène parfois à des conséquences sévères qui vont de la susceptibilité aux pathogènes aux phénomènes auto-inflammatoires mortels.

La première partie de cette thèse est consacrée à l'étude des déterminants d'un syndrome auto-inflammatoire, la lymphohistiocytose hémophagocytaire ou HLH. La cause la plus connue de HLH est la déficience de la voie de la dégranulation cytotoxique. Plus précisément, nous évaluons le rôle de l'inflammasome, un PRR de l'immunité innée, dans le déploiement de la HLH. Ce travail est présenté dans la première section de ce manuscrit.

Dans un second temps, nous traiterons de la régulation des lymphocytes NK. Nous avons récemment révélé que l'expression du gène *Styk1* est spécifique de ces lymphocytes de l'immunité innée. Afin de mieux comprendre son rôle, nous avons étudié l'impact de son invalidation sur le maintien homéostatique des lymphocytes NK, de leurs capacités effectrices ainsi que sur l'intégration des signaux environnementaux.

Section I : Abrogation de l'inflammasome dans un modèle murin de lymphohistiocytose hémophagocytaire.

1.1 La lymphohistiocytose hémophagocytaire

L'hémophagocytose fait référence à la phagocytose par les macrophages de composantes sanguines en conditions immunitaires anormales. La lymphohistiocytose hémophagocytaire (HLH) est un syndrome auto-inflammatoire qui est caractérisé par l'activation anarchique des lymphocytes T et des macrophages. Son nom fait référence aux histiocytes hémophagocytaires identifiés lors des premières descriptions du syndrome (Figure 2) [12, 13].

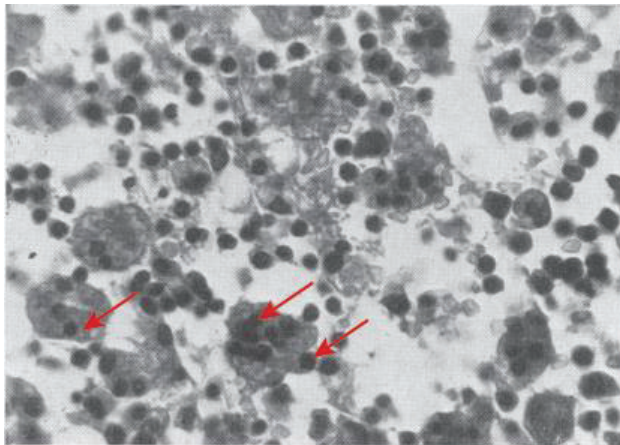


Figure 3 : Identification de macrophages (histiocytes) hémophagocytaires

Macrophages phagocytant des éléments du sang au niveau de la moelle osseuse lors des premières descriptions de la HLH, historiquement appelée réticulose hémophagocytaire familiale. Les flèches pointent des érythrocytes phagocytés. Tiré et adapté de [12].

Traditionnellement, les cas de HLH sont catégorisés en formes primaires (pHLH) et secondaires (sHLH). Dans sa forme primaire, la HLH affecte environ 1 naissance sur 50-100 000 et son déroulement est fatal en absence d'intervention thérapeutique [14, 15]. Ce syndrome est caractérisé par une inflammation systémique importante culminant à la défaillance des organes [14]. Les formes primaires représentent moins de 50% des cas répertoriés de HLH [16]. Dans un contexte secondaire à une infection ou à un désordre immunologique, la nomenclature « syndrome d'activation macrophagique » (SAM) ou simplement HLH acquise remplace parfois l'appellation sHLH. Les formes secondaires sont à ce jour moins bien comprises et se déroulent également en épisodes de fièvre auto-inflammatoire. La sHLH, se présente habituellement en condition d'infection et/ou de façon secondaire à un désordre immunologique important. L'infection au virus Epstein-Barr (EBV) est retrouvée le plus fréquemment lors des épisodes de HLH, ce qui suggère que l'exposition à ses antigènes est un élément déclencheur en contexte

immunologique et/ou génétique favorable. L'infection par d'autres types de virus peut également coïncider avec le déclenchement du syndrome, c'est notamment le cas de l'infection par le virus herpes simplex, un second membre de la famille des *herpesviridae*. Des cas concourants d'infections bactériennes, fongiques ou par des protozoaires ont également été rapportés. Le tableau clinique des formes secondaires et primaires étant chevauchant et la connaissance de l'étiologie de la HLH se précisant, la distinction entre les deux formes tend néanmoins à se confondre, nous en traiterons plus avant.

1.1.1 Tableau clinique

Les signes cliniques cardinaux de la HLH incluent : hépatosplénomégalie, pan-cytopénie, de hauts niveaux de triglycérides et cytokines pro-inflammatoires. En effet, les patients atteints de HLH présentent des niveaux extrêmement élevés d'IL-18, d'IFN- γ , de M-CSF, d'IL-6 et de TNF- α sériques. Des atteintes neurologiques sont également rapportées [17]. En clinique, un diagnostic moléculaire ou la concomitance de 5 des critères présentés au tableau I permettent d'identifier les cas de HLH primaire ou secondaire [14]. Les lignes directrices de prise en charge pharmacologique (protocole HLH-2004) incluent généralement une combinaison de cyclosporine A, dexaméthasone et etoposide [14]. En cas de comorbidité le traitement peut différer. Les HLHs réfractaires au traitement classique peuvent alternativement être traitées avec un anticorps monoclonal ciblant CD52 quoique cette thérapie demeure controversée en raison de possible réactivation de l'infection virale sous-jacente [18, 19].

Tableau I : Paramètres biologiques de la HLH

Fièvre
Splénomégalie
Cytopénie (> 2 lignées cellulaires)
Hémoglobine < 90g/L (enfants < 4 semaines < 100g/L)
Plaquettes < 100x10 ⁹ g/L
Neutrophiles < 10 ⁹ /L
Triglycérides à jeun > 3 mMol/L
Fibrinogène < 1,5 g/L
CD25 soluble (sIL-2R) > 2400 U/ml
Activité NK faible ou absente
Ferritine > 500 µg/L
Hémophagocytose

Tableau tiré et adapté de [14]

1.1.2 Conditions prédisposantes

Déficit de la fonction des lymphocytes cytotoxiques

Lorsque les lymphocytes T CD8 ou NK rencontrent une cellule infectée, ils opèrent un relargage polarisé de vésicules contenant des molécules effectrices, notamment la perforine et le granzyme B. La perforine est présente sous forme de monomères au sein des granules et forme des polymères qui s'insèrent à la membrane de la cellule-cible. Ce phénomène génère ainsi des pores perturbant fortement l'homéostasie cellulaire. Ces derniers permettent au granzyme B, une sérine protéase pro-apoptotique, de passer au cytosol et d'entraîner la mort de la cellule cible. Ce processus est essentiel à la fonction des lymphocytes cytotoxiques et de ce fait, au contrôle des pathogènes intracellulaires. Le relargage des granules cytotoxiques est un processus très finement régulé qui nécessite une profonde réorganisation du cytosquelette et l'intervention d'un ensemble de protéines assurant le transport, l'accostage et la fusion vésiculaire [11]. Ce phénomène se déroule en vase clos à l'interface de la cellule cible et du lymphocyte cytotoxique dans une interaction appelée la synapse immunitaire. Celle-ci est résolue à la mort apoptotique de la cellule cible, un événement qui nécessite la protéolyse de caspases au sein de la cellule cible (Figure 4) [20].

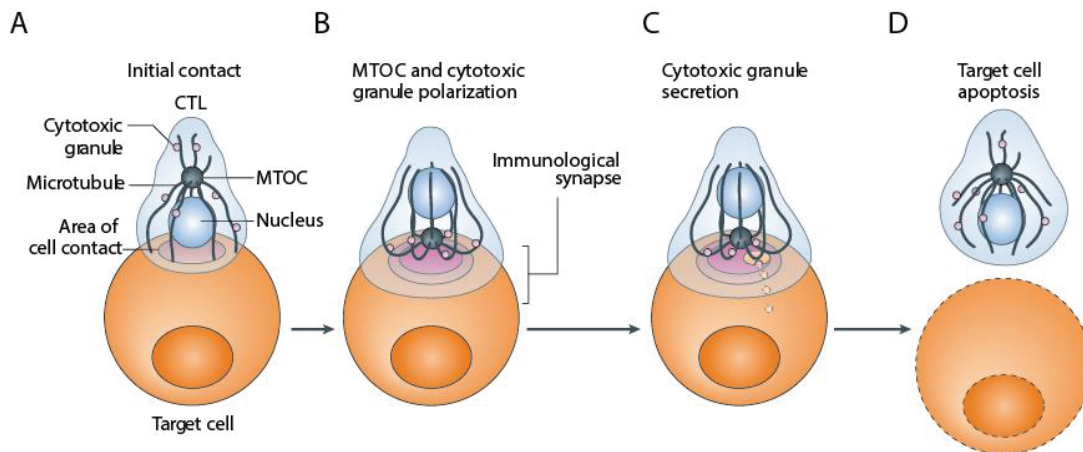


Figure 4 : Processus séquentiel de dégranulation des vésicules cytotoxiques

A) Suite à la reconnaissance d'une cellule-cible, B) les vésicules cytotoxiques se dirigent vers le centre d'organisation des microtubules (MTOC), C) ce qui est suivi par la polarisation de ce dernier vers la cible et la fusion des vésicules cytotoxiques au site de la synapse immunologique. L'interaction se termine à la mort par apoptose de la cellule-cible. Tiré et adapté de [11]

L'étiologie de la pHLH est généralement expliquée par un déficit autosomal récessif des gènes assurant le transport vésiculaire des granules cytotoxiques, ou encore des molécules qu'elles contiennent. En effet, les déficits en perforine (PRF-1), Munc13-4 (UNC13D), Syntaxine 11 (STX11) ou Munc 18-2 (Syntaxine binding protein, STXBP2) sont responsables de la pHLH [21-25]. Ces déficits sont respectivement désignés FHL2, FHL-3, FHL-4 et FHL-5 pour *Familial Hemophagocytic Lymphohistiosis* de type 2-5. Les mutations RAB27A (syndrome Griscelli de type 2) et Lyst (syndrome Chediak-Higashi) sont également impliquées dans le déclenchement de la pHLH [26-28]. La perforine est une protéine en charge de la perforation des cellules cibles lors de la dégranulation cytotoxique. Les protéines des FHL3-5 sont impliquées respectivement dans l'amorçage, l'accostage et la fusion des vésicules cytotoxiques à la synapse immunologique. Les protéines RAB27A et Lyst sont, quant à elles, engagées dans le transport et la maturation des vésicules (Figure 5) [11]. L'hypopigmentation de la peau associée au déficit de ces deux dernières composantes de la voie de la cytotoxicité témoigne du partage fonctionnel de ces protéines avec la voie de sécrétion de la mélanine.

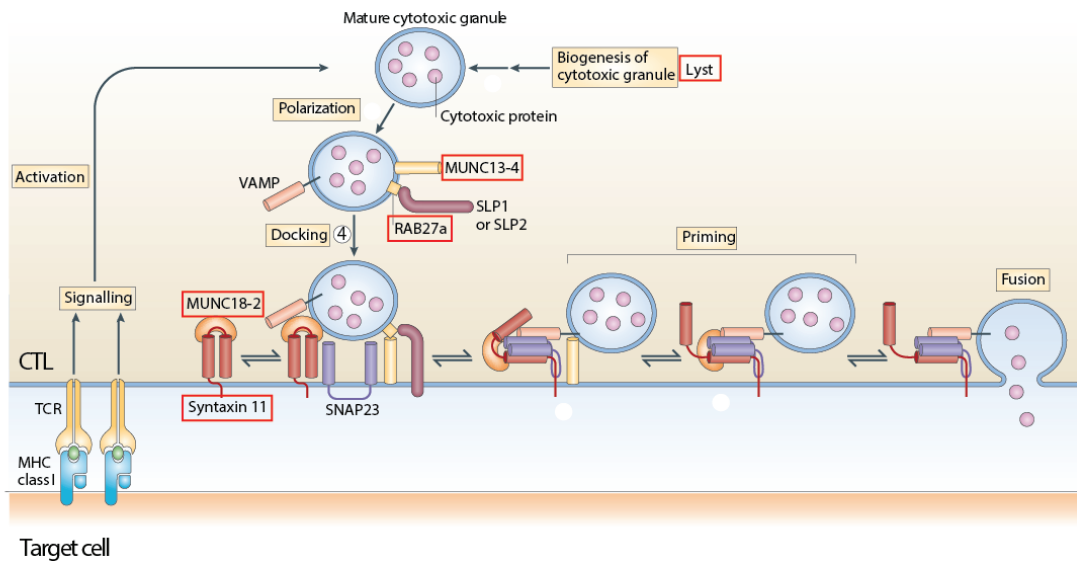
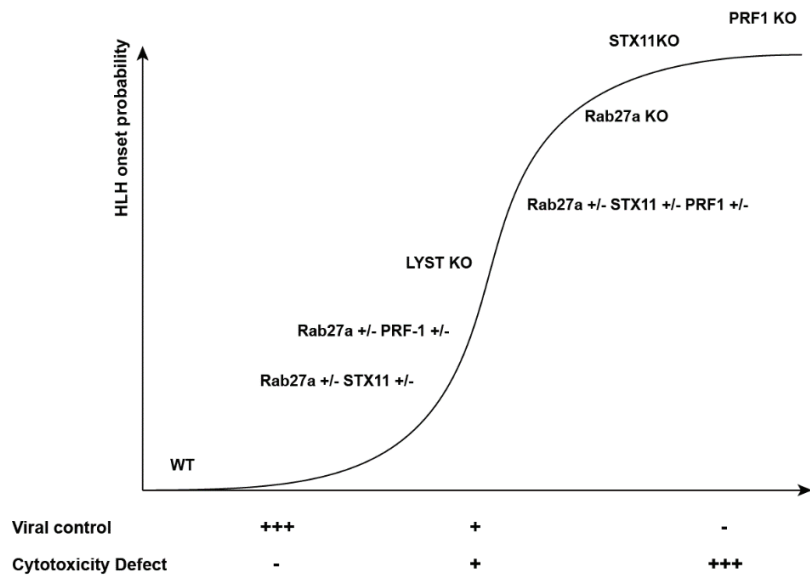


Figure 5 : Étapes importantes de la fusion vésiculaire des granules cytotoxiques

Suite à l'activation du lymphocyte cytotoxique (CTL), les granules cytotoxiques sont sécrétées de façon polarisée vers la cellule cible. Ce processus implique un grand nombre d'intervenants dont : Lyst, un régulateur du trafic lysosomal, Rab27a, une GTPase impliquée dans le trafic vésiculaire, MUNC13-4, une protéine impliquée dans l'amorçage (*priming*) de la fusion de granule cytotoxique. MUNC18-2 (STXBP2), et Syntaxine 11 sont impliquées dans la fusion vésiculaire. Encadrées en rouge, les protéines connues dont la déficience favorise la HLH. Tiré et adapté de [11].

Il existe un gradient de sévérité dans l'invalidation des éléments de la voie de sécrétion des granules cytotoxiques (Figure 5). Ceci est illustré par le développement d'une HLH dont la sévérité est plus ou moins importante et une mise en place plus ou moins tardive en fonction des gènes impliqués (Figure 6) [29]. Cette observation est appuyée par la reconnaissance récente de l'apport des mutations hypomorphes dans le déclenchement d'une HLH tardive, laquelle est fréquemment associée à un contexte infectieux [30, 31]. Par ailleurs, la probabilité de développement du syndrome serait également sous l'influence de l'accumulation de mutations dans la voie de la dégranulation. En effet, la génération de souris doublement ou triplement mutées de façon mono-allélique pour Rab27a, Syntaxine11 et/ou PRF-1 démontre que la présence de 3 mutations est davantage favorable à l'installation d'une HLH en contexte infectieux que 2, et que certaines combinaisons sont plus délétères que d'autres [32].

Figure 6 : Probabilité de développement de la HLH en fonction de la sévérité de l'invalidation de la cytotoxicité.
 Inspiré de [29] et [32].



L'invalidation des gènes impliqués dans la fonction effectrice des lymphocytes cytotoxiques impacte négativement le contrôle des infections à pathogènes intracellulaires. C'est ce qui entraîne une stimulation antigénique dont la durée est prolongée et dispersée dans les différents organes. Éventuellement, l'invalidation des fonctions cytotoxiques entraîne une boucle de rétroaction positive qui se met en place entre les lymphocytes T et les macrophages, provoquant de ce fait le relargage massif de cytokines pro-inflammatoires. La HLH peut donc être vue comme étant un syndrome à double modalité mettant en relation l'importance de l'invalidation de la réponse cytotoxique et la présence d'un contexte pro-inflammatoire : l'infection ou le désordre immunitaire.

Infections

Comme nous l'avons mentionné précédemment, le déclenchement d'un SAM/sHLH est fréquemment concomitant à une infection virale. Bien que l'infection à EBV soit la plus fréquemment associée à la sHLH, des cas d'infections concomitantes par d'autres souches virales (cytomegalovirus, parvovirus B19, virus de l'immunodéficience humaine, herpes virus-6, influenza, etc.) ont également été rapportés. De plus, des cas d'infections bactériennes, fongiques ou à protozoaires, synchrones du développement du SAM ont également été décrits [33]. Notons néanmoins que plusieurs de ces occurrences ont été rapportées en présence de facteurs confondants (état immunologique compromis, contexte de transplantation, tumeurs, etc.). Il convient donc d'envisager la relation causale des souches microbiennes avec précaution.

D'autant plus, que ce ne sont pas tous les virus qui provoquent le déclenchement de la HLH. En effet, chez la souris PRF1^{-/-}, les virus LCMV et MCMV induisent le syndrome, mais pas l'infection au virus respiratoire syncytial ou au virus murin de la pneumonie [29]. De façon comparable, les patients présentant une HLH de façon tardive, à l'adolescence ou à l'âge adulte, sont susceptibles d'avoir rencontré différents virus au cours de leur vie sans dérèglement homéostatique majeur. Mentionnons également que l'ensemble des modèles murins décrits jusqu'à maintenant ne développe pas de HLH de façon spontanée. En parallèle, l'état actuel des connaissances ne permet pas d'exclure une exposition à des antigènes viraux chez les patients souffrant de pHLH, et ce même si aucune source infectieuse n'est détectée en clinique. Finalement, bien que cela ne fasse pas l'objet des travaux présentés dans ce manuscrit, la relation entre l'infection, ses conséquences immunitaires et le déclenchement de la HLH est un sujet qui reste à clarifier.

Désordres immunologiques

Maladies auto-inflammatoires ou auto-immunes

La HLH fait partie des complications d'un large panel de désordres immunologiques. Entre autres, le lupus érythémateux, l'arthrite juvénile idiopathique (SoJIA) ainsi que son versant adulte, la maladie de Still, prédisposent au développement d'une sHLH. L'étiologie de ces trois désordres est encore mal comprise mais ces maladies ont toutes la caractéristique de présenter un déroulement inflammatoire. En effet, ces trois désordres immunologiques sont caractérisés par de hauts niveaux d'IL-6, une cytokine potentialisant la réponse immunitaire [34, 35]. Notons néanmoins que seuls, de hauts niveaux d'IL-6 sont insuffisants pour déclencher le syndrome [35]. Ce type de terrain immunologique semble toutefois propice au déclenchement de la sHLH. En effet, chez les patients souffrant de SoJIA, la HLH se retrouve avec une prévalence d'environ 50 % si l'on considère les formes infra-cliniques [36, 37]. D'ailleurs, un certain pourcentage (30%) de ces patients présente des variations (mutations, polymorphismes, délétions, etc.) dans l'un ou plusieurs des gènes impliqués dans la pHLH [32, 38, 39]. Toutefois, l'impact précis de la plupart de ces variations génétiques n'a pas été étudié. Dans le contexte pro-inflammatoire de la SoJIA, il est donc plausible qu'une déficience, même partielle, de la fonction cytotoxique favorise le déclenchement de la HLH [32, 40].

La reconnaissance récente des variantes génétiques impliquées dans la pHLH chez les patients souffrant de SoJIA confond la catégorisation dichotomique actuelle (pHLH *versus* sHLH). En effet, certains de ces

patients présentent une HLH qu'il conviendrait de catégoriser comme secondaire en raison de son mode de déclenchement. Toutefois, l'occurrence de mutations associées à la pHLH dans le contexte du SoJIA suggère que ces patients peuvent également appartenir à la catégorie de la pHLH. Les causes et mécanismes biologiques sous-jacents au déclenchement du syndrome dans le contexte des maladies immunitaires restent donc à clarifier. Les connaissances acquises à ce jour pointent vers un mécanisme de déclenchement nécessitant un arrière-plan génétique – la déficience de gènes impliqués dans la cytotoxicité – et immunologique – l'inflammation. Dans ce modèle, l'inflammation peut être la conséquence d'une infection, d'une dérégulation immunitaire, ou du cumul des deux.

Déficiences immunitaires primaires

La HLH fait également partie des complications associées aux immunodéficiences primaires, la plupart du temps en contexte infectieux. Le déclenchement, bien qu'exceptionnel, de HLH chez les patients présentant une déficience immunitaire combinée sévère liée à l'X (SCID-X) en est un exemple éloquent [41]. Notons que de façon intrigante, ces patients développent une HLH dans un environnement immunologique exempt de lymphocytes T. Bien que l'absence de contrôle viral puisse expliquer une partie du déclenchement, la source de l'IFN- γ associée à l'auto-inflammation observée chez ces patients est inconnue. À lui seul, cet exemple témoigne de l'hétérogénéité étiologique très probablement comprise dans l'entité clinique : lymphohistiocytose hémophagocytaire.

1.1.3 La mécanique du déclenchement de la HLH

L'IFN- γ sécrété par les lymphocytes T CD8+ est essentiel au déclenchement du syndrome

Dans le cas particulier du déficit en perforine, la synapse immunologique des lymphocytes cytotoxiques est prolongée en raison de l'incapacité de ces derniers à éliminer leur cible. Ceci engendre une forte potentialisation de leur activation [20]. Les lymphocytes T CD8 prolifèrent alors de façon importante et relâchent de très grandes quantités d'IFN- γ (Figure 7A-B). Il s'ensuit une augmentation de l'activité de présentation antigénique par les DC [42]. La sécrétion de grandes quantités d'IFN- γ par les lymphocytes cytotoxiques ainsi que l'activation des cellules présentatrices d'antigènes sont responsables et essentiels au déclenchement du syndrome. En effet, l'IFN- γ occupe un rôle central dans la mise en place de cette

boucle de rétroaction [43]. La déplétion des lymphocytes T CD8+, source principale de l'IFN- γ , ou encore sa neutralisation *in vivo* préviennent ce développement [43, 44]. Par ailleurs, cette approche thérapeutique est nouvellement utilisée avec succès. En effet, l'administration à titre compassionnel de l'anticorps monoclonal NI-0105 (α -IFN- γ) semble améliorer la survie des patients lors du déclenchement de HLHs [45, 46]. L'exposition prolongée des macrophages à l'IFN- γ , quant à elle, potentialise leur activité hémophagocytaire [47].

Les cellules présentatrices d'antigènes

La stimulation des macrophages par l'IFN- γ provoque la sécrétion par ces derniers d'IL-6, une cytokine pro-inflammatoire pléiotropique. Ces derniers sécrètent également le TNF- α et le M-CSF (Figure 7C) [48]. Le rôle des macrophages dans le déclenchement de la HLH est moins bien compris. Il semblerait, comme il a été mentionné précédemment, que la déficience en cytotoxicité entraîne une augmentation de leur activité de présentation antigénique en raison de l'exposition à de hauts niveaux d'IFN- γ [42, 49]. Les macrophages infiltrants expriment alors CD163, un récepteur à hémoglobine-haptoglobine, et phagocytent des éléments du sang (Figure 7D) [50]. L'activité de présentation antigénique des DC occupe un rôle central dans le développement de la HLH et leur déplétion prévient également son déclenchement [49].

Le rôle immunomodulateur des lymphocytes NK

Comme en témoignent les expériences de déplétion des cellules NK chez la souris PRF1 $-/-$, il semblerait que ces lymphocytes ne soient pas essentiels au déclenchement de la HLH [43]. Il s'avère néanmoins que qu'ils sont impliqués dans la régulation des cellules activées (Figure 7). En effet, la présence de NK compétents pour la perforine dans un hôte autrement PRF1 $-/-$, diminue le niveau d'activation des lymphocytes T et des macrophages suite à l'infection à LCMV, ce qui permet la survie lors du premier mois d'infection [51]. Par ailleurs, l'inactivation de la perforine spécifiquement chez les NK mais pas chez les lymphocytes T CD8, entraîne une prolifération plus intense de la part des lymphocytes T CD8 suite à l'infection à LCMV. Ces résultats démontrent que les NK ont un rôle immuno-modulateur qui est non redondant avec la fonction des lymphocytes T CD8 dans le contexte du déclenchement de la HLH, mais

qu'ils sont également facultatifs à sa mise en place. Ce mécanisme impliquerait vraisemblablement la cytotoxicité perforine-dépendante des lymphocytes NK.

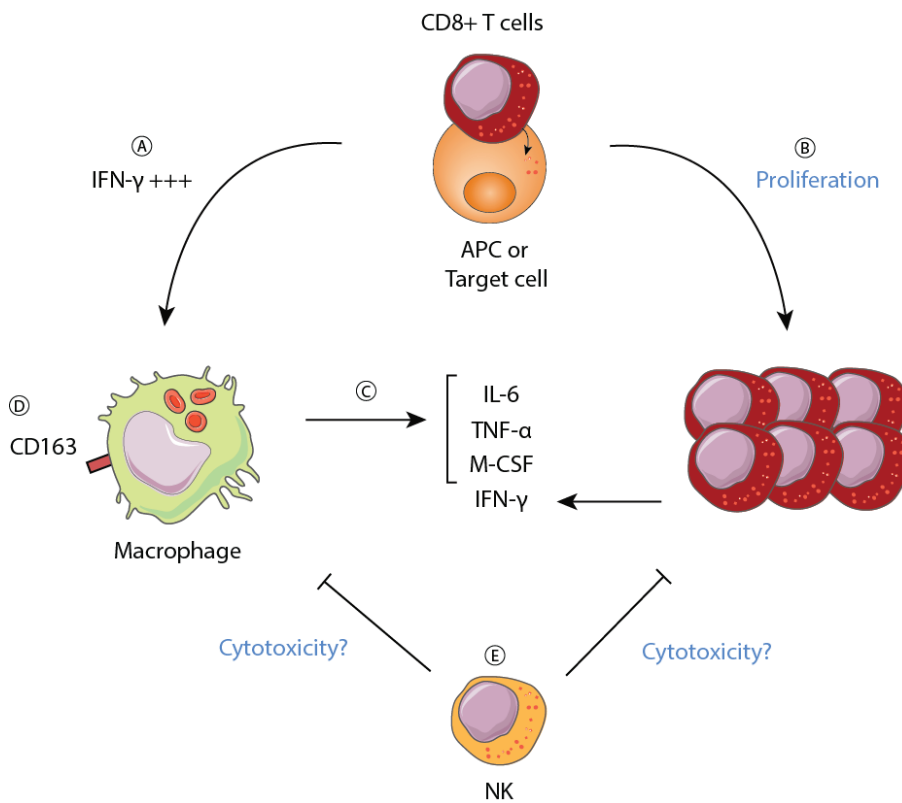


Figure 7 : Principaux intervenants cellulaires et mécanique de déclenchement de la HLH

A et B) Lors de la HLH, les lymphocytes T CD8+ déficients pour la cytotoxicité subissent une prolifération massive en présence de stimulus viral approprié, ce qui est accompagné par le relargage massif d'IFN-γ. **C et D)** Les niveaux importants d'IFN-γ contribuent à l'activation anarchique des macrophages qui phagocytent alors des éléments du sang, augmentent leur activité de présentation antigénique, expriment CD163 et relâchent de l'IL-6, du TNF-α et du M-CSF. **E)** Les lymphocytes NK contrôlent le niveau d'activation des lymphocytes T CD8+ et des macrophages.

Dérégulation de la signalisation de l'immunité innée

Les données expérimentales récentes évoquent l'implication de la signalisation de l'immunité innée dans le déclenchement de la HLH.

En effet, la stimulation répétée du TLR9, un PRR constitutivement exprimée par les cellules présentatrices d'antigènes, mène à un syndrome inflammatoire qui correspond au portrait clinique d'une HLH modérée [52]. À l'instar des cas de HLH rapportés en contexte SCID, où le décompte de lymphocytes T est faible, l'amorçage du syndrome par la stimulation répétée du TLR9 ne nécessite pas la présence des lymphocytes

T CD8. Mentionnons également que la délétion de Myd88, un adaptateur en aval de la plupart des TLRs mais également des récepteurs de l'IL-1b, l'IL-18 et l'IL-33 entrave le développement du syndrome dans le contexte de la déficience en perforine [53]. Sachant que Myd88 est impliqué dans la transcription de l'IL-1b dans sa pro-forme via l'activation de la voie NF- κ B, il est tentant de présenter cette étape initiale comme étant essentielle à l'induction de la HLH.

Les mutations activatrices de plusieurs récepteurs de l'inflammasome, un complexe multiprotéique senseur de l'immunité innée, sont responsables de divers désordres auto-inflammatoires [54-60]. Comme mentionné en préambule, l'activation de cette structure mène à la maturation et la sécrétion de l'IL-1b et de l'IL-18. Par conséquent, ces dérégulations activatrices entraînent toutes une inflammation systémique. Parmi la variété des inflammasomopathies, seule l'induction constitutive du récepteur NLRC4 présente un portrait clinique qui est similaire à la HLH [61]. Dans ce contexte pathologique, les macrophages sont constitutivement activés ou répondent de façon accrue aux stimuli et montrent des signes d'hémophagocytose [55, 62, 63]. À l'instar des patients souffrant de pHLH, ces patients présentent de hauts niveaux d'IL-18 et de ferritine sériques associés à une hépatosplénomégalie [64]. Or, l'exposition des lymphocytes T et des NK à l'IL-18 favorise la production d'IFN- γ [65, 66]. Bien que le blocage de l'IL-18 par l'IL18-BP ne résolve pas la HLH chez la souris, elle permet néanmoins d'amenuiser les dommages hépatiques associés au syndrome [67]. Au moment d'écrire ce manuscrit, un nouveau rapport soulignant l'importance de l'IL-18 dans la HLH était publié. Dans le modèle de HLH induite par la stimulation du TLR9, l'absence de l'IL-18BP, une protéine sérique limitant la biodisponibilité de l'IL-18, accroît fortement la sévérité de la HLH [68]. La présence de hauts niveaux d'IL-18 sériques lors des pHLHs laisse penser que l'inflammasome puisse être sollicité lors de son déclenchement. Le déclenchement du SAM lors de l'activation de NLRC4 se déroule en absence de mutations associées à la pHLH, ce qui incite à penser que l'inflammasome à lui-seul est suffisant pour déclencher le syndrome. Cependant, bien que l'activation constitutive de NLRC4 provoque un SAM, ceci n'est que très rarement le cas pour les mutations activatrices de NLRP3 et pyrine, deux autres senseurs menant à la constitution de l'inflammasome. À ce jour et à notre connaissance, aucun cas de SAM sous-jacent à une activation de NLRP1 n'a été décrit.

Chez la souris, l'invalidation de XIAP mène à l'activation de l'inflammasome NLRP3 et au relargage de grandes quantités d'IL-1 β en condition de stimulation par le TLR [69]. On ne sait pas si la relation XIAP/NLRP3 existe chez l'humain mais les patients déficients pour XIAP présentent une forte probabilité de développer une HLH en contexte d'infection à EBV et ce, en absence de défaut de cytotoxicité [70, 71]. Les causes de cette susceptibilité à EBV demeurent incomprises mais notons de façon anecdotique que

l'inflammasome NLRP3 est une cible de *silencing* du micro ARN miR-BART15 codé par EBV [72]. Par ailleurs, EBV semblerait induire la production d'IL-1b de façon caspase dépendante par un mécanisme qui demeure débattu [73, 74].

L'importance de la voie d'activation de l'inflammasome dans le déroulement de la HLH demeure inconnue. Nous nous sommes donc intéressés à cette question en invalidant l'inflammasome dans deux modèles murins de HLH, déficients pour la PRF1 ainsi que pour la protéine ASC (PRF1 -/- X ASC -/-) ou encore caspase-1 (PRF1 -/- X CASP1 -/-). Comme évoqué précédemment, la souris PRF-1 est un modèle de HLH lors de l'infection à LCMV mais également à MCMV.

Objectif du travail de thèse, Section I

La HLH est favorisée par un environnement pro-inflammatoire ainsi que par la déficience des lymphocytes cytotoxiques. Bien que nous sachions aujourd'hui que les désordres immunitaires représentent un terrain propice au développement de la HLH, que le dérèglement de l'immunité innée favorise également son déroulement et que la déficience de cytotoxicité en soit causale, la relation qu'entretiennent les cellules présentatrices d'antigène avec le déclenchement du syndrome demeure incomprise. La déficience en cytotoxicité engendre une pHLH qui est caractérisée par de hauts niveaux d'IL-18 sérique, ce qui suggère l'activation de l'inflammasome. Au moment de débiter ces travaux, nous savions que l'activation constitutive de NLRC4 mène à un SAM récurrent qui est également caractérisé par de hauts niveaux d'IL-18. Ce phénomène n'est toutefois pas observé chez les patients souffrant d'une activation de NLRP3 mais présentant des niveaux plus modérés d'IL-18. À ce moment, aucune mutation de NLRP1 n'était décrite.

Tenant compte du rôle de l'IL-18 dans l'induction de l'IFN- γ , **l'objectif principal du présent travail était de comprendre quel est le rôle de l'inflammasome dans la mise en place du syndrome.** Nous avons cherché à expliquer si cette structure était impliquée et essentielle au déclenchement de la HLH en abrogeant la protéine échafaud ASC ou encore la sous-unité catalytique Caspase-1 sur le fond génétique PRF1 $-/-$. Pour ce faire, nous avons suivi les paramètres biologiques et de survie des souris PRF1 $-/-$ ASC $-/-$ et PRF1 $-/-$ CASP1 $-/-$ suite à l'infection par LCMV ou MCMV, des virus respectivement non-cytopathique et cytopathique.

La déficience en cytotoxicité demeure néanmoins un bon indicateur de la sévérité de la pHLH. Nous avons donc émis l'hypothèse que les patients présentant une HLH dans le contexte d'un SoJIA pouvaient éventuellement présenter un déficit de fonction cytotoxique. En effet, plusieurs rapports font état de mutations dans la voie de la sécrétion des granules cytotoxiques chez ces patients. Nous avons donc évalué l'intégrité de la fonction cytotoxique des lymphocytes NK chez une cohorte de 14 patients souffrant de SoJIA, dont certains ont déjà vécu au moins épisode de HLH (n=5). Les résultats de ce travail sont présentés dans l'article 1 de la section résultats.

Résultats : Article 1

Macrophage activation syndrome occurring during autoinflammatory diseases or genetic defects of cytotoxicity are caused by distinct immune mechanisms

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Running title: Inflammasome abrogation in HLH mouse models

Character count: 43101, spaces excluded

Abstract:

Macrophage activation syndrome (MAS) occurs in patients with genetic defects of cytotoxicity. Elevated serum levels of IL-18 characterize this syndrome. MAS also often arise in patients with systemic onset juvenile idiopathic arthritis (sJIA). Cases of successful treatment of sJIA by IL-1 blockade suggest inflammasome implication in this disease. The occurrence of MAS in both disease groups suggests common pathophysiological mechanisms that may involve elevated inflammasome activity in the context of reduced cytotoxicity. To address this question we used two complementary strategies. We first tested the role of inflammasome in mouse models of familial hemophagocytic lymphohistiocytosis (FHL) by deleting genes encoding for Caspase-1 or the inflammasome adapter ASC. These deletions did not influence the development of MAS in perforin deficient *prf1*^{-/-} mice infected with LCMV or MCMV and did not reduce serum IFN- γ levels despite reduced production of IL-18. Second, we explored Natural Killer (NK) cell function and phenotype in a cohort of patients with sJIA. We did not detect any consistent defect in NK cell degranulation or perforin expression. These data show that pathophysiological mechanisms underlying MAS in FHL and autoinflammatory syndromes may be distinct and suggest that different therapeutic strategies should be used to treat these diseases.

Introduction

Hemophagocytic syndrome also called macrophage activation syndrome (MAS) or histiolymplocytosis (HLH) is a severe disease caused by abnormal activation and proliferation of macrophages with an increased phagocytic activity (1). The main clinical and biochemical features of MAS include non-remitting high fever, hepato-splenomegaly, cytopenia, hypertriglyceridemia, and hyperferritinemia. This inflammatory syndrome is also characterized by the presence of numerous well-differentiated macrophages phagocytosing red blood cells (hemophagocytosis) in the bone marrow (2).

Known primary causes of MAS are genetic defects of cytotoxicity or mutations of the inflammasome receptor/adaptor NLRC4 (1, 3, 4). Besides, MAS can also occur secondary to infections especially by viruses of the herpes family, malignancies such as non-Hodgkin lymphoma, as well as auto-inflammatory diseases such as systemic onset juvenile idiopathic arthritis (sJIA) and its adult form Still's disease (5). Early diagnosis of MAS and aggressive treatment are mandatory to prevent fatal evolution. Despite improved treatment of MAS, its prognosis remains severe with approximately 50% mortality. First line therapy protocols typically include steroids associated to etoposide (6). A better knowledge of immune mechanisms causing MAS are therefore crucially needed to improve treatment.

Familial histiolymplocytosis (FHL) are caused by mutations affecting the cytolytic effector protein perforin, or proteins involved in the molecular machinery required for the biogenesis or exocytosis of perforin-containing vesicles into the immune synapse. Thus, mutations in perforin (PRF1) are responsible for FHL type 2 (FHL2), UNC13-D for FHL3, STX11 for FHL4 and STXBP2 for FHL5. FHL can also be presented with hypopigmentation as in Griscelli syndrome type 2 (RAB27A) and Chediak-Higashi syndrome (LYST) that also involve proteins associated with vesicle trafficking (7). Our understanding of the immunopathological mechanisms responsible for MAS development has gained from the availability of several murine models of FHL. Cytotoxic-deficient mice (such as *Prf1*, *Rab27a*, or *Unc13d* or *Lyst* deficient mice) develop a MAS-like syndrome after infection with viruses like lymphocytic choriomeningitis virus (LCMV) (8) or mouse cytomegalovirus (MCMV) (9). In the absence of cytotoxic activity, an accumulation of antigen-presenting cells that continuously activate cytolytic T cells (CTLs) was reported

in *Prf1*^{-/-} mice. Hypersecretion of IL-2, TNF and IFN- γ by CTLs is linked to failed disengagement of *Prf1*^{-/-} NK cells or CTLs from their targets, causing a prolonged activating synapse (10). The hyperactivated CTLs thus secrete high levels of IFN- γ , which appears critical for the development of MAS-like symptoms as shown using blocking antibodies (8). Indeed, prolonged systemic exposure to physiologically relevant levels of IFN- γ is sufficient to cause acute cytopenias and hemophagocytosis by macrophages, the hallmark of this syndrome (11). IFN- γ acts directly on macrophages *in vivo* to alter endocytosis/phagocytosis and provoke blood cell uptake. Moreover, IFN- γ induces production of IL-6 and other cytokines by multiple cell types (10), an effect that can be blocked by JAK1/2 inhibitors (12). The crucial role of CTLs for MAS-like development has been highlighted by experiments in which depletion of CTLs but not NK cells prevents MAS-like manifestations (8). Nevertheless, NK cells appear to exert a certain control on overly activated cells as the presence of perforin competent NK cells in an otherwise perforin deficient host diminishes both overactivation of CTL and macrophages and ameliorates survival upon infection with LCMV (13).

The inflammasome (14) is an innate immune platform activated in response to danger signals and infections, leading to pro-Caspase-1 maturation. Caspase-1 is an inflammatory caspase leading to the processing and release of pro-IL-1 β and pro-IL-18. Canonical Caspase-1 activation takes place within a multiprotein complex, the inflammasome, which includes a sensor, and an adaptor, ASC. Several inflammasomes involving different stimulus/sensor pairs have been described. The NLRP1, NLRP3, NLRC4, AIM2 inflammasomes respectively sense bacterial proteolytic toxins, membrane stress, bacterial type-3 secretion system and cytosolic flagellin and both exogenous and endogenous cytosolic DNA. Two recent studies reported de novo missense mutations affecting the nucleotide-binding domain of the inflammasome component NLRC4 causing familial early-onset systemic auto-inflammation and MAS (called NLRC4-MAS) (3, 4). These mutations induce constitutive NLRC4 activation and subsequent production of IL-1 and IL-18. These studies suggest that a macrophage-intrinsic defect can drive the MAS phenotype in the absence of a primary cytotoxic defect, thus providing a new paradigm for the pathogenesis of MAS. This hypothesis is supported by other works showing that repeated injections of TLR9 ligands in T cell-deficient mice may lead to MAS-like

syndrome (15). Monocytes from NLRC4-MAS patients constitutively produce high amounts of IL-1 β and especially IL-18 during *ex vivo* culture. How the inflammasome and these cytokines drive the typical symptoms of MAS remain however unsolved. One possibility is that IL-1 β and/or IL-18 may act on other cells to induce various other cytokines such as IFN- γ (16). IL-18 induces considerable IFN- γ production by NK cells and T cells when combined with IL-12 (17). Several articles also suggested that NK cell cytotoxicity could be impaired in patients with autoinflammatory syndromes (18–20), perhaps as a result of negative regulation by the chronic inflammatory reaction.

Circulating levels of IL-18 are very high in patients with primary cytotoxic defects or secondary forms of MAS and IL-18 levels correlate with the various MAS markers (21). In the context of cytotoxic defects, these data suggest that IL-18 is produced secondary to the lack of control of virus infections and IFN- γ production by CTLs, which may promote the establishment of a vicious circle by further enhancing IFN- γ production by NK and CTLs. This hypothesis is supported by the previous observation that in a mouse model of FHL3, loss of MyD88 (the adaptor downstream of IL-1R and IL-18R among others) conferred protection from MAS upon LCMV infection, without affecting the anti-LCMV T cell response (22). Although blocking IL-18 using IL-18 binding protein (IL-18BP), a natural inhibitor of IL-18 function, is not sufficient to block MAS development in mouse models of FHL, it alleviates some of the inflammatory biological parameters (23). Moreover, in inflammatory diseases with secondary MAS, a few case reports also indicate an efficacy of anti-IL1 (24) or IL-18BP (25) treatment, thus suggesting a pathological role of IL-1 family members in this inflammatory condition. Whether several IL-1 members may synergize to induce IFN- γ production by NK and CTLs is not clear. Previous studies showed that both IL-1 α and IL-1 β could induce IFN- γ production by murine NK cells when combined with IL-12 (26). This synergistic effect was not as pronounced as in the case of IL-12 and IL-18 association, but it could contribute to the excessive IFN- γ production observed during MAS.

Altogether, genetic studies show that the development of MAS can have at least two different causes: a defect in cytotoxic function or deregulated inflammasome activation, which suggests a functional link between both events. Our working hypothesis was that inflammasome activation could be a key mechanism of MAS, driving the overt

inflammation observed during this syndrome and that it could be direct (eg. patients with NLRC4 mutations) or indirect (due to deregulated cytokine secretion following hyper-activation of CTLs in FHL patients). To test this hypothesis, we used mouse models of MAS in which inflammasome activation was prevented by genetic means. We also made the reciprocal hypothesis that chronic inflammation caused by inflammasome deregulation could decrease cytotoxic capacities of NK and CTLs, thus creating a negative vicious feedback loop leading to MAS. The latter hypothesis was tested by monitoring activity of peripheral NK cells in a cohort of patients with sJIA.

Results

Inflammasome activation is dispensable for the development of MAS in perforin-deficient mice infected with LCMV

To address the role of inflammasome activation in FHL, we crossed *prf1*^{-/-} mice with *asc*^{-/-} (also known as *pycard*^{-/-}) or *casp1/11*^{-/-} (hereinafter referred to as *casp1*^{-/-}) mice to generate *prf1*^{-/-}*asc*^{-/-} mice or *prf1*^{-/-}*casp1*^{-/-} mice. *Asc* deletion abolishes activation of most inflammasomes, while *casp1* deletion abolishes activation of all canonical inflammasomes (including inflammasomes not strictly dependent on ASC, such as NLRC4-dependent inflammasomes). Groups of *prf1*^{-/-}, *prf1*^{-/-}*asc*^{-/-}, *prf1*^{-/-}*casp1*^{-/-} and control mice (WT, *asc*^{-/-}, or *casp1*^{-/-}) were infected with LCMV (Armstrong strain). As previously reported, most *prf1*^{-/-} mice succumbed to infection within 8-20 days (Figure 1A), which correlated with pronounced weight loss (Figure 1B) and lack of virus control (Figure 1C) compared to control mice of either genotype. Similar observations were made for *prf1*^{-/-}*asc*^{-/-} and *prf1*^{-/-}*casp1*^{-/-} mice, despite a consistently delayed death and reduced weight loss for *prf1*^{-/-}*casp1*^{-/-} mice (Figure 1A, B). We monitored several biological parameters associated with MAS including blood platelet and neutrophil counts, spleen weight, as well as blood hemoglobin, triglycerids, ferritin and hepatic enzymes (ALAT and ASAT). For all these parameters, *prf1*^{-/-}, *prf1*^{-/-}*asc*^{-/-}, and *prf1*^{-/-}*casp1*^{-/-} mice grouped together, aside from control C57BL/6, *asc*^{-/-} and *casp1*^{-/-} mice (Figure 1D-J), which correlated with the overall survival. In particular, platelet and neutrophil counts and hemoglobin levels dropped after infection while triglycerides, ferritin, spleen weight and hepatic enzymes notably increased. To correlate these biological parameters with immune mechanisms, we measured the level of serum cytokines IFN- γ and IL-18. Serum levels of IFN- γ , known to be a critical factor in the development of MAS, were significantly elevated in all three *prf1*^{-/-} murine models compared to control groups, thus demonstrating that IFN- γ production in this MAS model is independent of inflammasome activation (Figure 1K). As expected, IL-18 levels were strongly reduced in mice deficient for Caspase-1 or ASC, but still consistently higher than background levels (Figure 1L). IL-1 β levels were undetectable in all mice analyzed (data not shown). The LCMV-specific CD8 T cell response was also monitored using LCMV NP-specific MHC tetramers. In all three *prf1*^{-/-} murine models, the percentage of NP-specific CD8 T cells was decreased at day

10 and especially at day 16 post infection compared to control groups, in both spleens and livers (Figure 1M). A similar trend was observed for the percentage of NK cells in both spleen and liver (Figure 1N). No significant difference was noted between *prf1*^{-/-}, *prf1*^{-/-}*asc*^{-/-}, and *prf1*^{-/-}*casp1*^{-/-} groups for either NP-specific CD8 T cells or NK cells. We then quantified the macrophage infiltration in the liver of infected animals. Inflammatory macrophages were defined as Ly6C⁺ CD64⁺ (Figure 1O), as previously reported (27). All perforin-deficient mice had a strong infiltrate of inflammatory macrophages 10 and especially 16 days after infection, irrespective of the inflammasome status, in both spleen and liver. *prf1*^{-/-}*asc*^{-/-} mice displayed a significantly increased macrophage infiltration compared to *prf1*^{-/-}, and *prf1*^{-/-}*casp1*^{-/-} mice.

Thus, based on physiological, biological and immunological parameters, we concluded that MAS induction following LCMV challenge in perforin-deficient mice is mostly independent on inflammasome components.

Inflammasome activation is dispensable for the development of MAS in perforin-deficient mice infected with MCMV

Likewise for the LCMV model, perforin-dependent cytotoxicity is also essential to control MCMV, and *prf1*^{-/-} mice develop fatal MAS upon infection (9). We decided to test the MCMV infection model as well in our study because many FHL patients develop MAS following infection by cytomegalovirus (28). Moreover, infection of C57BL/6 mice with MCMV induces a strong innate immune response culminating with the expansion of antiviral NK cells bearing the Ly49H receptor, that binds to the viral m157 MHC-like molecule on infected cells (29). Previous articles have established the essential role of dendritic cells, IL-18 and IL-12 in inducing protective IFN- γ secretion by NK cells and in promoting the expansion of the Ly49H positive subset (reviewed in (30)). This observation prompted us to test the role of inflammasome in MAS development in the MCMV model. *prf1*^{-/-}, *prf1*^{-/-}*asc*^{-/-}, *prf1*^{-/-}*casp1*^{-/-} and control mice (WT or *casp1*^{-/-}) were thus infected with MCMV (Smith strain) at the 5 x 10⁴ PFU dose. As previously described (9), most *prf1*^{-/-} mice died following infection within 4 to 8 days (Figure 2A). This correlated with pronounced weight loss (Figure 2B). Biological analyses at day 6 revealed thrombocytopenia, and neutropenia (Figure 2C,D). However there was no hepato-

splenomegaly (Figure 2E) and blood hemoglobin levels (Figure 2F) were normal in all perforin-deficient animals. Hepatic enzymes were only elevated in inflammasome-deficient animals, either Caspase-1 or ASC-deficient, irrespective of perforin expression (Figure 2G). When we compared serum cytokine levels in the different mouse groups, we noted that *prf1*^{-/-} mice had an increased level of IFN- γ at early time points compared to all other mouse groups. At this time-point, the T cell response is still negligible, suggesting that early production of IFN- γ by NK cells is dependent on inflammasome-derived cytokines in the MCMV model of MAS. However, at later time points, the level of serum IFN- γ was highly increased in all perforin-deficient groups compared to control groups, and at least four-fold higher than at early time points (Figure 2I). IL-18 levels progressively increased in *prf1*^{-/-} mice compared to all other genotypes. As expected, the deletion of *asc* or *caspase-1* prevented this increase (Figure 2H). As in the LCMV model, we also monitored the viral-specific CD8 T cell response, NK cell percentage and inflammatory macrophage infiltration. As shown in figure 2J-M, a strong MCMV-specific CD8 T cell response was detected in all mouse groups in both spleen and liver. The percentage of CD8 T cells was however decreased to the same extent in *prf1*^{-/-}, *prf1*^{-/-}*asc*^{-/-}, and *prf1*^{-/-}*casp1*^{-/-} mice compared to control mice in both organs. No major difference was observed for the percentage NK cells between the different mouse groups in either organ (Figure 2L). The percentage of inflammatory macrophages was low in the spleen of all mice groups but was much increased in all perforin-deficient mice groups compared to control mice in the liver and the absence of ASC or Caspase-1 did not influence this manifestation (Figure 2M).

Altogether, our data demonstrate that MAS induction is independent of inflammasome components in Perforin-deficient mice.

NK cells from patients with sJIA display normal degranulation and perforin expression

The high incidence of MAS in autoinflammatory diseases such as sJIA and Still's disease has led to the hypothesis of an impaired cytotoxicity in these patients. Heterozygous missense variants in perforin or other FHL genes have been identified in these patients (18, 19, 31), but a vast majority of patients do not bear any pathogenic mutations in these

genes. However, the chronic inflammation characteristics of this disease may negatively impact CTL and NK cell cytotoxicity, as demonstrated in other chronic diseases like cancer (32) or HIV infection (33). Indeed, by analogy with what is observed in FHL, reduced cytotoxicity may lead to secondary MAS. To formally test this hypothesis, we performed a phenotypical and functional analysis of NK cells in patients with Still's disease or sJIA (see table I for clinical characteristics) in comparison with healthy individuals or FHL2 patients. Of note, five of the 14 sJIA patients included in this cohort had history of MAS. Although a previous study reported low perforin expression in NK cells from a small number of sJIA patients (18), most patients in our study were comparable to healthy individuals for this parameter and not in the range of authentic FHL2 patients, regardless of the MAS status (Figure 3A). PBMC were co-cultured in the presence or absence of MHC-I deficient K562 cells and we measured their degranulation by means of CD107a (also known as Lamp1) exposure to the cell's surface. As shown in figure 3B, despite inter-individual variation, the average degranulation was similar in sJIA/Still patients and in healthy controls. Degranulation and perforin expression by NK cells were also not correlated with CRP levels at the time of sampling (data not shown). However, the percentage of NK cells within PBMC and NK cell absolute count in the blood were significantly decreased in sJIA patients compared to healthy patients (Figure S1). The percentage of CD56^{bright} NK cells and CD57⁺ NK cells were respectively increased and decreased among NK cells, indicative of a decreased maturation of peripheral NK cells. The percentage of NKG2C⁺ "adaptive" NK cells was also similar in sJIA and healthy individuals. Since immune deficiencies may also be caused by defects in immune receptors expression, we also monitored the expression of various inhibitory or activating NK cell receptors. However, no significant difference was observed for any of the receptors we studied (NKG2D, NKp30, NKp46, CD16, KIR2DL1, KIR2DL2, KIR3DL1, Figure S1).

Thus, autoinflammatory diseases such as Still's disease and sJIA are not linked with an impaired cytotoxicity pathway or a major alteration of NK cell phenotype that could explain MAS development.

Discussion

IL-18 is produced at exceptionally high levels in the serum of patients undergoing MAS (21). It was previously suggested that IL-18 and other IL-1 family members may play a role in MAS based on the evidence that i) disruption of MyD88 signaling prevents MAS development in a mouse model of FHL3; ii) IL-1 blockade with anakinra can successfully treat MAS in the context of sJIA (34); iii) IL-18BP treatment alleviate some of the symptoms observed in the course of the disease in a mouse model of FHL2 (23). However, the latter treatment may not optimally block IL-18 activity in all tissue or cellular microenvironments such as the immunological synapses (35). Besides, other inflammasome-dependent IL-1 family members may compensate IL-18 blockade to induce IFN- γ production by NK and CTLs. To circumvent this issue and further test the role of inflammasome-dependent cytokines in FHL, we blocked inflammasome activation by deleting *asc* or *caspase-1* in *prf1*^{-/-} mice infected with LCMV or MCMV. Of note, IL-18 and IL-1 β levels were not null in *prf1*^{-/-} *asc*^{-/-} or *prf1*^{-/-} *casp1*^{-/-} mice upon LCMV infection, suggesting that ASC or Caspase-1 are not completely essential for production of these cytokines. The nature of the inflammasomes activated during LCMV or MCMV infection remains unclear; AIM2 at least is activated during MCMV infection (36). Moreover, other proteases may activate IL-1 family cytokines, as previously reviewed (37). Despite this limitation, our data clearly show that the reduction of more than 80% of the total levels of IL-18 does not prevent MAS development and fatal outcome. This is likely due to an absence of a role or a certain level of redundancy for IL-18 in the induction of IFN- γ production at the peak of the inflammation, as shown by the absence of correlation between IL-18 and IFN- γ levels in infected mouse sera. In consequence, inflammasome-dependent IL-1 family cytokines are not essential drivers of MAS development in mouse models of FHL. Targeting activated T cells in this group of diseases, as is the current practice, is therefore a much better therapeutic option. This can be efficiently achieved using etoposide, that selectively ablates activated T cells (38). Moreover, a recent study showed that blocking antibodies directed against ST2, the receptor for the inflammasome-independent IL-1 member IL-33 reduced FHL symptoms in *prf1*^{-/-} mice infected with LCMV, by decreasing the number of activated T cells (39). However, in MCMV-infected mice, deletion of ST2 encoding gene *Il1r1* had the opposite outcome, IL-33 signaling

being crucial for Treg cell accumulation after MCMV infection (40). Targeting IL-33 may therefore not be appropriate in all FHL contexts.

MAS are a complication of autoinflammatory and autoimmune diseases, most often in patients with sJIA or Still's disease. Severe MAS develops in about 10% of these patients (41) and milder "occult" forms could be far more common and poorly reported (42). NK cell dysfunction was previously reported in sJIA patients (20, 43). However, a more recent evaluation showed a globally intact cytotoxic profile in a cohort of ten sJIA patients (44). Our own results confirm the latter observation, as NK cell degranulation measured at the single-cell level was overall in the normal range among sJIA patients, while the expression of perforin and different NK cell receptors was mostly unchanged compared to healthy individuals. These results rule out an impairment of cytotoxic function as a general cause of MAS in sJIA patients, even though it does not exclude the possibility that a mild impairment of this function may contribute to MAS in a few individuals with heterozygous pathogenic mutations in FHL genes, as previously reported (18, 31). Several articles have reported the characterization of gain-of-function activating mutations in NLRC4 (3, 4) or more recently in NLRP1 (45) or Pypin (46) in patients with autoinflammatory diseases. In NLRC4-MAS, constitutive activation leads to pathogenic macrophage activation, uncontrolled production of active forms of IL-1 and IL-18 and increased pyroptosis. This macrophage-intrinsic defect may drive the MAS phenotype in the absence of a primary cytotoxic defect. Accordingly, the conditions of these patients improved upon IL-1 blockade (3). This treatment was however ineffective in the case of another NLRC4 mutated patient, the condition of the latter improving only after introduction of IL-18BP as neutralizing agent of free IL-18 (25). Thus, in the context of autoinflammatory diseases with proven inflammasome mutations, targeting IL-1 family cytokines is the treatment of choice. Targeting IFN- γ using antibodies may also be effective as shown in a transgenic mouse model overexpressing human IL-6 (47). However, this therapeutic option should be taken with caution in sJIA patients undergoing viral infection as IFN- γ is also a crucial antiviral cytokine that play a protective role that depends, however, on the genetic context (48).

In conclusion, we show here that different immune mechanisms underlie MAS development in FHL and autoinflammatory diseases. In particular, we demonstrated i)

that reducing the production of IL-1 family cytokines via genetic suppression of inflammasome activation does not reduce IFN- γ levels and MAS development in perforin-deficient mice, and ii) reciprocally, that NK cell degranulation and perforin expression are preserved in sJIA patients, excluding a cytotoxic defect as a general cause of MAS in sJIA. Intriguing links between cytotoxicity and inflammasome activation remain however, as best exemplified by the case of type 2 X-linked proliferative disease (XLP2) caused by *XIAP* loss-of-function mutations. XLP2 patients are indeed highly susceptible to infection by herpesviruses such as EBV, similarly to FHL patients (49). These patients present several autoinflammatory symptoms due to a role for XIAP in negative regulation of inflammasome activation (50). More in-depth studies will be required to address the nature of inflammasomes activated during MAS and how cytotoxicity or lack thereof may influence this activation.

Material and methods

Patients

14 sJIA and 5 FHL2 patients were included in this study. Clinical and immunological characteristics of sJIA individuals are listed in Table 1. 4 of the 14 patients had MAS history and were referred to as “sJIA-MAS”. Blood samples were obtained upon informed consent. Blood samples from healthy volunteers were obtained from Etablissement Français du Sang.

Animals

This study was carried in strict accordance with the French recommendation in the Guide for the ethical evaluation of experiments using laboratory animals and the European guidelines 86/609/CEE. Procedures including animals were approved by local ethics review board under ENS2015-014 agreement. C57B6J mice were obtained at Charles River laboratories (L'Arbresle, France). *asc*^{-/-} mice were obtained at Vishva Dixit Laboratory (Genentech, South San Francisco, CA). *casp1*^{-/-} mice were obtained from Denise Monack's Laboratory (Stanford University, California). *prf1*^{-/-} mice were obtained from The Jackson laboratory (Bar Harbor, Maine). All mouse strains were maintained in our animal facility. *prf1*^{-/-} mice were crossed with *asc*^{-/-} and *casp1*^{-/-} mice to generate *prf1*^{-/-}*asc*^{-/-} and *prf1*^{-/-}*casp1*^{-/-} mice.

Virus production

LCMV-Armstrong titers were determined by plaque assay on Vero cell line and propagated on BHK cell line. MCMV-smith was propagated by infecting 3 weeks old Balb/c mice with 50 PFU of MCMV cultured until passage 2 on primary BALB/c mouse whole fetus cells. Briefly, salivary glands were collected on ice in 3% FCS DMEM, homogenized with an organ dissociator and centrifuged at 800g for 5 min at 4°C. Supernatant was collected and stored at -80°C until use. Titers were determined by plaque assay using NIH-3t3 cells.

Infections

Mice were infected intraperitoneally with 1×10^5 PFU of LCMV Armstrong or 5×10^4 PFU of MCMV Smith. Mice were weighted and scored for pain indicators daily. For LCMV, spleen and liver were harvested at d+10 and d+16 post infection (p.i.). For MCMV, organs were harvested at d+6 p.i.. Spleen and liver were separated in two parts for flow cytometry analysis and RNA extraction. For cytokine, Hg and platelets level determination, blood was collected by retro-orbital sinus rupture.

Flow cytometry

Mouse:

Blood was collected in 0.5M EDTA and red blood cells were lysed with ACK lysis buffer. Mononuclear cells were isolated from spleen and liver injected with complete RPMI 1640 5% FCS 0.4 mg/ml Collagenase IV (Serlabo Technologies, Vedène, France) and 0.1mg/ml DNase I (Roche), cut into small pieces and incubated at 37°C with 150RPM shaking for 30 min. After incubation, organs were homogenized and cells from the liver were purified using Percoll (GE Healthcare) density gradient separation. Virus specific CD3+CD8+ lymphocytes were identified using H-2D(b) LCMV NP396 396-404 FQPQNGQFI –BV421 or H-2D(b) MCMV M45 985-993 HGIRNASFI – BV421 (NIH tetramer core facility) tetramer staining for 30 min at 4°C followed by CD3-FITC (145-2C11), CD8-APC-efluor780 (53-6.7) and NK1.1-APC (PK136) staining. Inflammatory macrophages were identified as Lin- F4/80+ CD11b+CD64+LY6Chigh. Flow cytometry was carried out on a FACS Fortessa (Becton-Dickinson). Data were analysed using FlowJo (V10, Treestar)

sJIA patients:

The following antibodies were used: anti-KIR2DL2 FITC (Biolegend), anti-KIR3DL1 AF700 (Biolegend), , anti-CD16 Pacific Blue (Biolegend), anti-NKp30 PE (Biolegend), , anti-NKG2D PE-Cy7 (Biolegend), anti-NKp46 BV421 (Biolegend), anti-NKG2C AF488 (R&D systems), anti-CD19 ECD (Beckman Coulter), anti-CD4 ECD (Beckman Coulter), anti-CD14 ECD (Beckman Coulter), anti-CD56 APC (Beckman Coulter), anti-CD3 APC-

AF750 (Beckman Coulter), anti-KIR2DL1 PE-Cy7 (eBiosciences), anti-CD57 eF450 (eBiosciences), and anti-perforin PerCP-eF710 (eBiosciences).

Phenotypic analysis of NK cells and NK cell frequency

100 µL of whole blood was lysed and stained for 15 minutes for extracellular markers. Perforin was stained after Cytofix/Cytoperm (BD biosciences) permeabilization. Absolute NK cell count was obtained by multiplying the percentage of CD3-CD4-CD14-CD19-CD56+ cells among lymphocytes by the absolute lymphocyte count determined using the hematological analyzer SYSMEX (Roche).

Functional analysis of NK cells

Peripheral mononuclear cells (PBMC) were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation. Flow cytometric analysis of CD107a was evaluated after co-culture of PBMC with K562 cells (ATCC). Briefly, PBMC and target cell lines were resuspended at 2×10^6 cells/mL in RPMI 1640 containing L-glutamine (Gibco), 10% FBS (PanBiotech), 100 IU/ml penicillin (Gibco) and 100 µg/mL streptomycin (Gibco). 100µL of cells were co-cultured at a 1:1 effector to target ratio in 96-well plates. After washing, cells were resuspended in PBS and activated cell sorting analysis was performed on a Navios flow cytometer (Beckman Coulter).

Hemostatic parameters and cytokine measurements

Platelets, Hemoglobin and neutrophil levels were assessed on XN-10 analyzer (Sysmex). IFN-γ (DuoSet, RnD) , IL1β (Quantikine, RnD), IL-18 (MBL), IL-6 (DuoSet, RnD) and Ferritin (Abnova) levels in serum were measured by ELISA. Triglycerids levels were dosed using enzymatic method (abbot/architect C16). Serum ASAT and ALAT were measured by enzymatic method (Abbott Architect).

Semi-quantitative RT-PCR

Spleen and liver pieces were immediately put in Trizol on ice, homogenized using Precelys (Bertin Technologies) and kept at -80°C until analysis. Relative LCMV levels were obtained by RT-qPCR (Reagents) using the following primer pairs. LCMV; RT-Ms-

LCMV-F: CATTACCTGGACTTTGTCAGACTC, RT-Ms-LCMV-R:
GCAACTGCTGTGTTCCCGAAAC.

Statistical analysis

Data were analyzed with GraphPad Prism 6 software. Survival and HLH incidence curves were analyzed by using the Mantel-Cox test. Other analyses were performed by using one-way ANOVAs. Differences were considered to be statistically significant when $p < .05$ (* if $p < .05$, ** $< .01$, *** $< .001$, **** $< .0001$).

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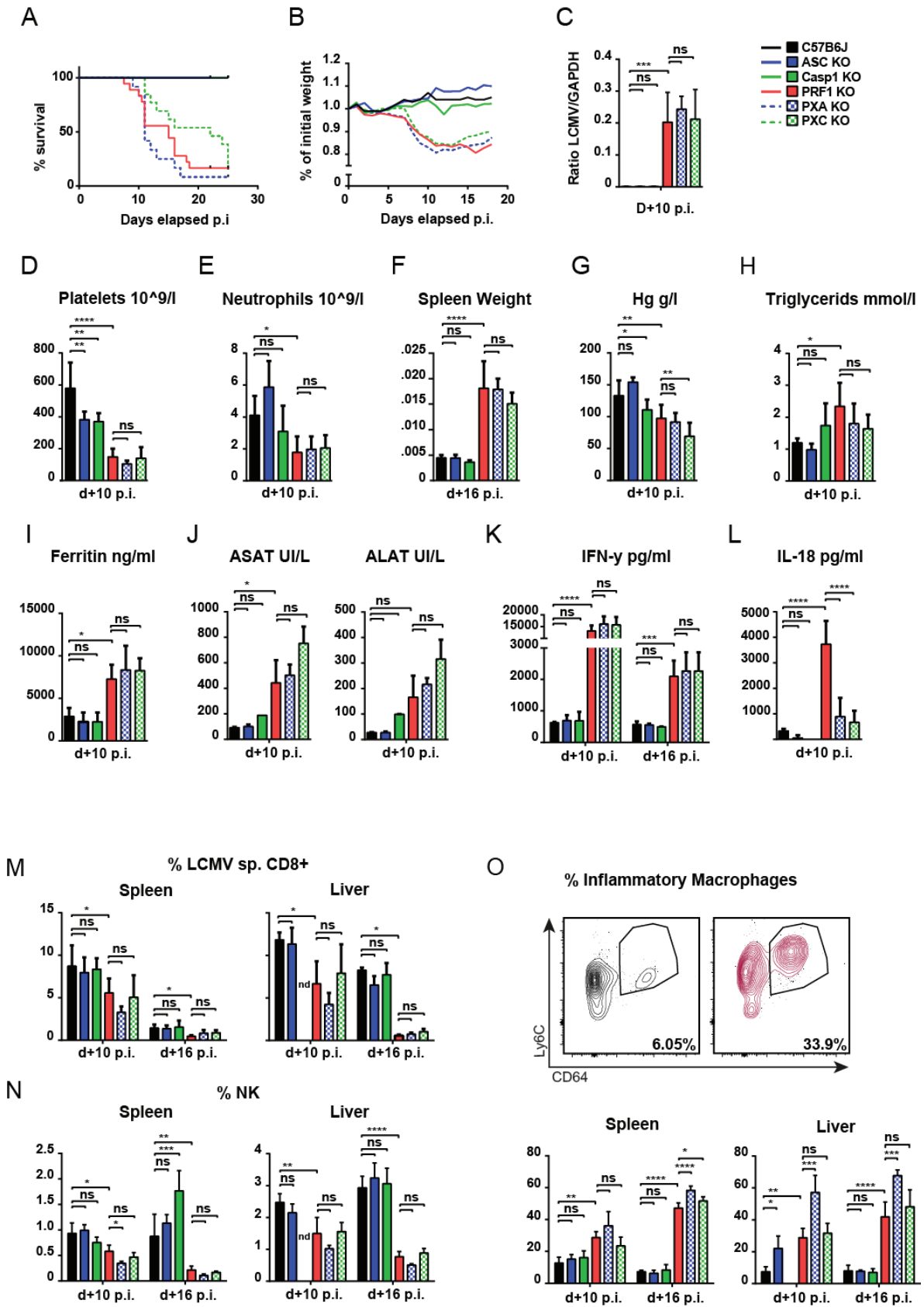


Figure 1: Inflammasome abrogation does not prevent HLH in *prf1*^{-/-} mice infected with LCMV

A) Representative longitudinal follow up of survival of C57BL/6J (black), *asc*^{-/-} (blue), *casp1*^{-/-} (green), *prf1*^{-/-} (red), *prf1*^{-/-}*asc*^{-/-} (PxA, blue dotted or checked) and *prf1*^{-/-}*casp1*^{-/-} (PxC, green dotted or checked) after infection with 1x10⁵ PFU of LCMV – Armstrong. The graph shows % of survival against days elapsed post infection (experiment n=3, >6 mice per genotype, range 6-22). **B)** Weight loss in function of days elapsed post infection. Mouse weight is expressed in % of initial weight at the day of infection **C)** Semi quantitative viral load in the spleen 10 days post infection LCMV RNA levels were compared to GAPDH and results are expressed as a ratio of LCMV early gene transcription to GAPDH. This graph shows the ratio ± SD. **D)** Platelets and **E)** Neutrophils levels on day 10 post infection. **F)** Spleen weight expressed as % of mouse weight at day +10 post infection. **G)** Blood hemoglobin, **H)** Serum triglycerides and **I)** ferritin levels on d+10 post infection. **J)** Serum ASAT and ALAT, **K)** IFN-γ **L)** and IL-18 day+10 or d+16 post infection. Data is representative of 3 experiments. 3-4 mice per genotype were analyzed. **M)** Percentage of LCMV-NP396 specific CD8T cells were determined in NK1.1⁻ CD3⁺CD8⁺ lymphocytes for spleen and liver. **N)** Percentage of NK cells was determined on total lymphocytes that are NK1.1⁺CD3⁻CD8⁻ for spleen and liver. **O)** Percentage of inflammatory macrophages were determined as Lin⁻F4/80⁺CD11b⁺ CD64⁺LY6C^{high} in the Lin⁻F4/80⁺CD11b⁺ compartment. Upper: Gate example of inflammatory macrophages. D-O data are represented as mean ± SD. *P < .05; **P < .01; ***P < .001; ****P < .0001. ALAT: alanine aminotransferase; ASAT: aspartate aminotransferase; Hg: Hemoglobin.

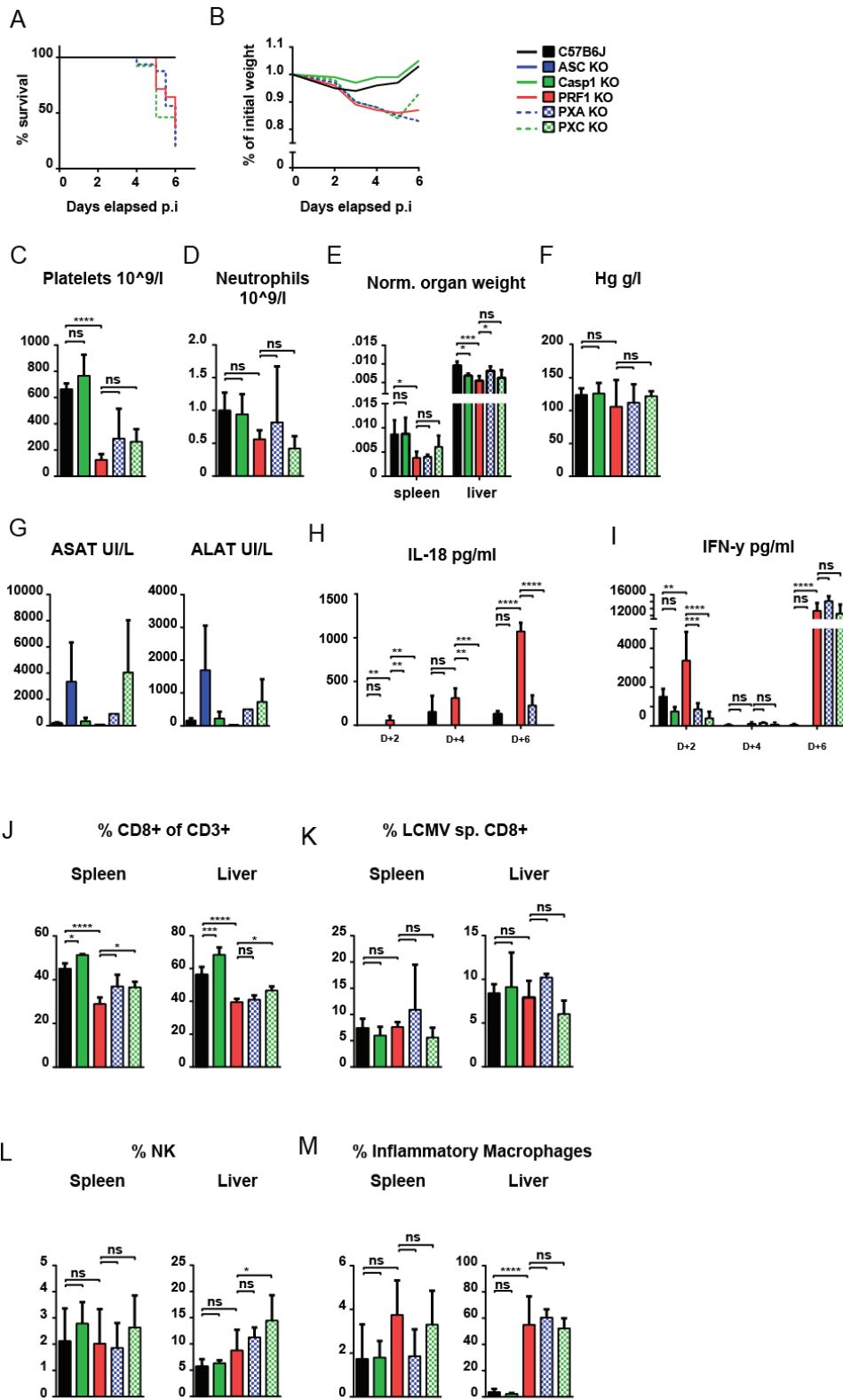


Figure 2: Inflammasome abrogation does not impact HLH development triggered by MCMV

A) Representative longitudinal follow up of survival of C57BL/6J (black), *asc*^{-/-} (blue), *casp1*^{-/-} (green), *prf1*^{-/-} (red), *prf1*^{-/-}*asc*^{-/-} (PxA, blue dotted or checked) and *prf1*^{-/-}*casp1*^{-/-} (PxC, green dotted or checked) after infection with 5x10⁴ PFU of MCMV – Smith. The graph shows % of survival against days elapsed post infection (experiment n=3, >6 mice per genotype, range 6-14). **B)** Weight loss in function of days elapsed post infection. Mice weight is expressed in % of initial weight at the day of infection **C)** Platelets and **D)** Neutrophils levels on day 6 post infection. **E)** Spleen weight expressed as % of mice weight at day +6 post infection. **F)** Blood hemoglobin, **G)** Serum ASAT and ALAT on day+6 post infection **H)** IL-18 and **I)** IFN- γ levels on d+2, 4 and 6 post infection. **J)** Percentage of CD8⁺ out of CD3⁺ cells in spleen and liver **K)** MCMV- M45985-993 specific CD8 T cells were determined as NK1.1-CD3⁺CD8⁺MCMV⁺ lymphocytes for liver and spleen. **L)** Percentage of NK cells was determined on total lymphocytes that are NK1.1⁺CD3⁻CD8⁻ for liver and spleen. **M)** Percentage of inflammatory macrophages were determined as Lin⁻F4/80⁺CD11b⁺ CD64⁺LY6C^{high} in the Lin⁻F4/80⁺CD11b⁺ compartment. Data in D-M is representative of 3 experiments. 3-4 mice per genotype were analyzed. Graphs show mean that are expressed \pm SD. *P < .05; **P < .01; ***P < .001; ****P < .0001. ALAT: alanine aminotransferase; ASAT: aspartate aminotransferase. Hg: Hemoglobin.

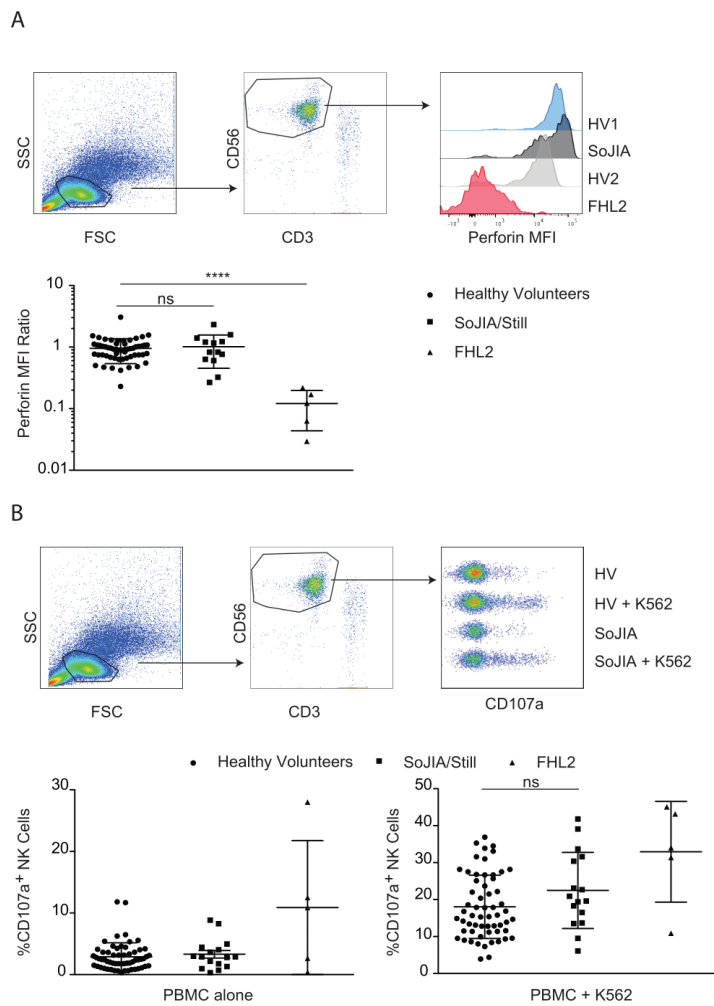


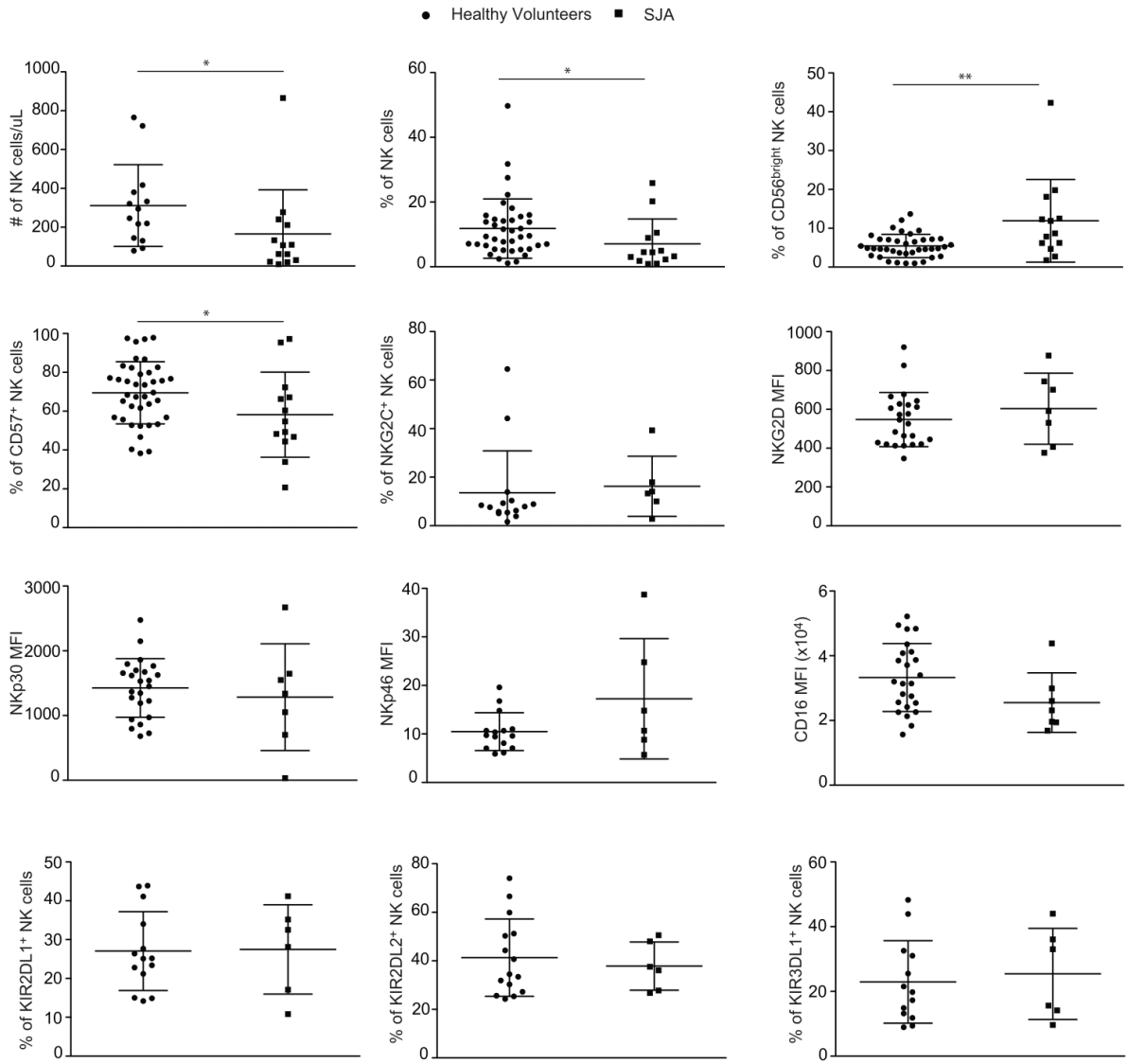
Figure 3: Patients with sJIA display no NK cell deficit of functionality.

Patient's PBMCs were stained for intracellular perforin shortly after venous puncture blood collection. NK cells were identified as CD3-CD56⁺ among lymphocytes. A) Upper: gating example of perforin levels. As different flow cytometers were used for the measurements, Perforin levels are normalized by dividing the MFI for the perforin staining by the mean MFI of healthy volunteers on each cytometer. B) Upper: gating example of CD107 α NK cells. Left: % of CD107 α in steady state PBMC after incubation. Right: after K562 target coculture. Graphs show ratio of MFI or percentage \pm SD. *P < .05; **P < .01; ***P < .001; ****P < .0001. Experiment were performed once upon patients' sample reception.

Table I: Clinical information on sJIA patients

	n	Reference Values	Patients' Values
Gender (boys)	14		7/14 (50%)
Current age years	14		11,2 (5,7 - 18)
CRP (mg/L)	14	<5	92,7 (0,6 - 424,7)
Proteins (g/L)	12	58 - 76	70 (57 - 81)
Ferritin (µg/L)	6	10 - 250	1548 (56 - 6379)
ASAT (U/L)	14	5 - 34	54 (18 - 218)
ALAT (U/L)	14	10 - 35	45 (6 - 291)
Leukocytes (G/L)	14	4 - 10	14,5 (3,6 - 38,6)
Hemoglobin (g/L)	14	120 - 160	122 (96 - 149)
Platelets (G/L)	14	150 - 400	399 (124 - 874)

Figure S1



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Discussion Section I

Il a été proposé que l'IL-18 joue un rôle important lors de la HLH car cette cytokine est produite à des niveaux extraordinairement élevés durant cette pathologie et aussi parce que l'abrogation de MyD88, un adaptateur en aval du récepteur à l'IL-18, entrave le développement de la HLH [53]. De plus, le traitement à l'IL-18BP permet la résolution partielle des dommages hépatiques lors de l'infection à MCMV chez la souris PRF1 -/- [67]. Ce traitement n'est cependant pas suffisant pour empêcher le déclenchement de la HLH. Néanmoins, l'IL-18BP a récemment été utilisée avec succès chez un patient présentant une HLH consécutive à l'activation constitutive de NLRC4 [75]. Sur ces différentes bases, nous avons fait l'hypothèse que l'IL-18 produite au cours de la HLH était un élément essentiel à la maladie par son action inductrice d'IFN- γ chez les lymphocytes NK et T. Pour tester cette proposition, nous avons vérifié l'hypothèse que l'IL-18 produite au cours de la HLH résultait de l'activation de l'inflammasome.

Dans la première partie de ce manuscrit, nous présentons donc l'effet de l'inactivation de l'inflammasome sur le déroulement de la HLH. La souris PRF1 -/- développe une HLH sévère lorsqu'infectée avec LCMV ou MCMV [43, 67]. Nous avons exploité ce modèle pour générer deux lignées de souris double KO en croisant la souris ASC -/- ou la souris Caspase1 -/- Caspase11 -/- (appelée CASP1 -/- plus avant) avec la souris PRF1 -/-. La protéine ASC, rappelons-le (*cf.* Figure 2 p.18), est une protéine échafaud permettant l'assemblage des récepteurs NLRs et de la pro-caspase-1 dans une structure multimérique appelée l'inflammasome.

Comme attendu, lors de l'infection par LCMV ou MCMV, le modèle PRF1 -/- développe l'essentiel du spectre biologique associé à la HLH : hauts niveaux d'IFN- γ , chute du niveau de plaquettes et de l'hémoglobine, cytopénie, augmentation des triglycérides, de la ferritine, de l'IL-18 ainsi que des transaminases hépatiques, ce qui est accompagné par l'infiltration importante de macrophages inflammatoires et la splénomégalie. Les souris qui sont PRF1 -/- présentent toutes une mortalité accrue en absence de contrôle viral, ce qui se met en place au jour 12 post infection (p.i.) Notons finalement la prolifération importante des lymphocytes T CD8+ spécifiques de LCMV qui sont responsables du déclenchement de la maladie.

L'abrogation de l'inflammasome n'empêche pas la mise en place de la HLH.

L'invalidation de l'inflammasome par ASC ou Caspase-1 ne permet pas d'amenuiser les niveaux sériques d'IFN- γ , la cytokine centrale de la HLH, à J+10 p.i.. La sécrétion tardive de l'IFN- γ est donc indépendante de l'activation de l'inflammasome, qui ne semble pas essentiel au déroulement du syndrome. Cette observation est appuyée par l'absence d'amélioration de la vaste majorité des paramètres biologiques lors de l'invalidation de l'inflammasome. Néanmoins, nous observons une mortalité retardée chez la souris PRF1 -/- CASP1 -/-, ce qui n'est pas retrouvé chez la souris PRF1 -/- ASC -/-. La survie transitoire des souris PRF1 -/- CASP1 -/- lors de l'infection à LCMV laisse supposer que Caspase-1 est impliquée, bien que de façon facultative, dans le déroulement tardif du syndrome alors qu'ASC ne l'est pas. La nature des inflammasomes activés lors de l'infection à MCMV ou LCMV n'est pas complètement connue mais nous savons que MCMV active AIM2 et LCMV active NLRP1 indépendamment de ASC [76, 77]. Une explication possible de la mortalité retardée chez la souris PRF1 -/- CASP1 -/- pourrait être l'induction de l'inflammasome par l'interaction alternative de NLRP1 ou encore NLRC4 directement avec Caspase-1 lors de la HLH, un phénomène qui ne nécessite pas strictement la présence d'ASC. En effet, NLRC4 et NLRP1 possèdent un domaine d'interaction CARD qui leur permet d'interagir directement avec la pro-Caspase-1 et ainsi, induire sa maturation [78]. En absence de Caspase-1, cette interaction est impossible. Si l'inflammasome et en particulier NLRP1 ou encore NLRC4 sont impliqués dans le déroulement de la HLH lors de l'infection à LCMV, il est possible que l'abrogation de Caspase-1 puisse provoquer le délai que nous observons dans la dynamique de mise en place. Ceci soulève la question des interactions alternatives entre les composantes de l'inflammasome lors de la HLH, ce que nous n'avons pas abordé au cours de ces travaux. L'impact que peut avoir l'abrogation de Caspase-1 lors du déroulement de la HLH est toutefois insuffisant pour empêcher son déclenchement. Il est également probable que ce phénomène soit inhérent au modèle d'infection. En effet, lors de l'infection par MCMV, ce délai de mortalité n'est pas observé.

L'inflammasome représente la source principale d'IL-18 lors de la HLH

Les niveaux d'IL-18 sont sévèrement impactés par l'abrogation de l'inflammasome. Nous démontrons formellement pour la première fois, que l'inflammasome est la source majeure d'IL-18 lors du déclenchement de la pHLH. Bien que cette structure ne soit pas essentielle au déroulement du syndrome, il n'en demeure pas moins qu'elle participe à la tempête cytokinique. Cette sécrétion a le potentiel d'influencer le niveau d'activation des lymphocytes NK et T, ainsi que leur production d'IFN- γ [66, 79]. La diminution des niveaux d'IL-18 tel que nous l'avons observé n'est toutefois pas suffisante pour prévenir le déclenchement du syndrome. En dépit de l'invalidation de l'inflammasome, les niveaux d'IL-18 ne sont

cependant pas nuls. Il est donc plausible que l'inflammasome ne soit pas l'unique source d'IL-18. Il est également possible que l'homéostasie sanguine soit suffisamment perturbée pour libérer l'IL-18 qui serait normalement séquestrée par l'IL-18BP. Nous n'avons pas mesuré les niveaux d'IL-18BP lors du développement de la HLH. Cependant, nous avons observé une neutropénie importante. Les neutrophiles expriment également la pro-forme de l'IL-18 ainsi que la machinerie enzymatique nécessaire à sa maturation, ils pourraient donc représenter une source potentielle de cette cytokine [80]. Finalement, il a récemment été démontré que la source principale d'IL-18 lors de l'activation constitutive de NLR4 se localisait au niveau de l'épithélium intestinal [81]. Ce tissu étant fortement irrigué par le système sanguin, il pourrait également être la source de l'IL-18 sérique lors de la pHLH.

La sécrétion d'IFN- γ par les lymphocytes NK dépend de l'inflammasome lors de la HLH

Dans un second temps, nous avons étudié le déroulement du déclenchement de la HLH dans le contexte de l'infection à MCMV. D'une part parce que MCMV est un *herpesvirus* qui est phylogénétiquement proche de CMV, souvent retrouvé dans le contexte de la HLH. D'autre part, parce que MCMV mène à l'activation des lymphocytes NK, ce qui nous permet de suivre la réponse immunitaire précoce [82, 83]. En effet, ce modèle d'infection nous permet de suivre la réponse IFN- γ précoce dont les lymphocytes NK sont principalement la source. À l'inverse de LCMV, MCMV est un virus cytopathique qui, en absence de contrôle, induit rapidement la mort par défaillance des organes lors de la première semaine d'infection. Possiblement en raison du déroulement plus rapide de cette infection, les souris PRF1 $-/-$ développent une HLH qui n'est pas accompagnée par tous les signes cliniques habituels. Notamment, l'hémoglobine était normale et les transaminases hépatiques élevées seulement pour la souris ASC $-/-$ à j+6 p.i.. En dépit de ce déroulement, la grande majorité des souris porteuses de la mutation PRF1 $-/-$ sont mortes entre les j+4-6 p.i.. À l'instar de l'infection à LCMV, les souris porteuses de la mutation PRF1 $-/-$ présentent de très hauts niveaux d'IFN- γ au pic de l'infection. Cependant, durant la phase précoce, les niveaux d'IFN- γ n'étaient pas aussi élevés pour les souris PRF1 $-/-$ invalidées pour l'inflammasome que pour leur contrepartie PRF1 $-/-$. Ceci suggère que l'inflammasome influence la sécrétion de l'IFN- γ lors des phases très précoces de l'infection. Cette situation corrobore l'hypothèse selon laquelle, l'IL-18 sécrété par l'inflammasome joue un rôle dans le développement précoce de la réponse immunitaire et peut-être également de la HLH. Comment, en influant sur la phase précoce de l'infection, l'inflammasome peut-il avoir une influence sur le déroulement tardif de la HLH tel qu'il est observé lors de l'infection à LCMV pour la souris PRF1 $-/-$ CASP1 $-/-$? L'IL-18 est une cytokine qui potentialise la production de l'IFN- γ par les lymphocytes T et NK [66]. Il est possible que l'altération de la sécrétion de l'IFN- γ par les lymphocytes NK

ait un effet sur la cinétique d'infection mais également sur la potentialisation de la réponse T, laquelle est nécessaire pour induire la pleine extension de la HLH [43, 84]. Cet effet n'est toutefois que cinétique et ne permet pas de résoudre le développement de la HLH, lequel est essentiellement gouverné par la réponse des lymphocytes T spécifiques. Dans le modèle d'infection par MCMV, nous n'avons pas observé de délai de mortalité pour les souris dont l'inflammasome est abrogé. Toutefois, il est vrai que la nature des inflammasomes activés est différente et cette fois, dépendante à la fois de Caspase-1 et de la protéine échafaud ASC.

Il a récemment été démontré qu'en absence de Caspase-1, la protéine FADD peut s'associer à ASC pour activer la caspase-8 et provoquer l'apoptose en lieu et place de la pyroptose des macrophages [85]. Dans le contexte de notre modèle, ceci mènerait à un niveau d'inflammation paradoxalement moins important en absence de Caspase-1. Il est possible que ce phénomène soit conscrit aux étapes initiales du déclenchement du syndrome, ce qui temporiserait la cinétique de mise en place de la HLH. Au moment de soumettre ce manuscrit de thèse, nous étions en train d'évaluer la cinétique de la HLH lors des jours 6 p.i., au début de la réponse T, ainsi qu'au jour 21 p.i., moment auquel les souris PRF-1 $-/-$ Casp1 $-/-$ montrent un délai dans la mortalité lors de l'infection à LCMV. Nous effectuons également une délétion des lymphocytes NK afin d'évaluer si l'inflammasome, en modulant la réponse IFN- γ des NK, a un rôle sur les niveaux d'IFN- γ retrouvés lors de la phase terminale du syndrome. Soulignons tout de même que l'expression des caspases chez l'humain est différente de chez la souris. Les caspases inflammatoires 4 et 5 remplacent en effet la caspase 11, ce qui représente une limite expérimentale de ce modèle murin [86].

De plus en plus d'indices laissent supposer que les inflammasomes ne fonctionnent pas en parallèle. Plusieurs récepteurs NLRs peuvent en effet se retrouver au sein des mêmes inflammasomes. Il semblerait donc qu'un senseur puisse en recruter un autre via son domaine NACHT. C'est le cas de NLRC4 qui recrute NLRP3 lors de l'infection à *Salmonella typhimurium* [87]. Ce type d'intégration des signaux de stress par l'inflammasome permettrait de moduler finement l'activation de cette structure en fonction de l'environnement. Est-ce que des relations entre différents NLRs existent lors du déroulement de la HLH ? Cela demeure inconnu. Il n'est toutefois pas exclu que l'effet cumulatif de plusieurs senseurs de l'inflammasome soit impliqué lors du déroulement de la HLH, un paramètre qu'il nous est également impossible de contrôler dans ce modèle. Ce type d'interaction pourrait toutefois représenter une explication supplémentaire de la survie strictement transitoire des souris PRF-1 $-/-$ Caspase-1 $-/-$. En effet, il n'est pas exclu que l'effet cumulatif du stress cellulaire et de la détection de patrons moléculaires, endogènes ou non, mène à l'activation de l'inflammasome lors de la HLH, cela en dépit de l'absence d'ASC.

Dans les deux types d'infections que nous avons montrés, les niveaux d'IL-18 sont fortement impactés par l'absence de l'inflammasome. Ceci n'est cependant pas suffisant pour entraver le déroulement de la HLH, réitérant le fait que l'inflammasome ne soit pas nécessaire pour l'enclenchement du syndrome et joue probablement un rôle secondaire. Néanmoins, il semble être impliqué de façon précoce dans le déroulement de la maladie, ce qui pourrait éventuellement représenter une cible thérapeutique prophylactique intéressante dans le contexte de prédispositions immunitaires à la HLH.

Pour récapituler, avons montré que l'inflammasome n'est pas une structure essentielle au déclenchement de la HLH. Nous avons néanmoins illustré que cette dernière était responsable des niveaux d'IL-18 sériques et qu'à cet effet, elle pouvait avoir un impact sur la réponse IFN- γ des lymphocytes NK. Toutefois, les niveaux d'IL-18 ainsi que l'altération de la réponse NK ne suffisent pas à contrer le déclenchement de la HLH. Ce phénomène est intrigant alors que la neutralisation thérapeutique de l'IL-18 par l'IL18BP permet d'abroger le déroulement de la HLH dans le contexte de l'activation constitutive de NLRC4. Plusieurs questions subsistent :

Est-il possible que les niveaux résiduels d'IL-18 que nous avons observés soient suffisants pour le déclenchement de la HLH ? Nous ne savons pas s'il existe des sources d'IL-18 alternatives et le cas échéant si la neutralisation de ces derniers aurait un impact sur la mise en place de la HLH.

Lors de l'abrogation de l'inflammasome, les niveaux d'IFN- γ demeurent étrangement élevés lors de la phase tardive du syndrome alors qu'ils ne le sont pas lors de la phase précoce du syndrome. L'IFN- γ produit par les lymphocytes NK semble donc avoir un impact limité sur l'activation des lymphocytes T.

Bien que l'activation constitutive de l'inflammasome soit suffisante pour générer une HLH, son abrogation ne permet pas d'empêcher le déroulement de la HLH dans le cadre du modèle murin de la FHL-2. Nous avons montré que la caspase-1 avait peut-être un rôle dans l'exacerbation du syndrome, par un mécanisme qui demeure inconnu. L'identité des senseurs cytosoliques et des caspases impliqués dans la constitution de l'inflammasome dans le contexte du déclenchement de la HLH demeure également inconnue. Nous devons encore nous pencher sur la cinétique de mise en place afin de mieux comprendre le déclenchement de ce syndrome.

Les patients SoJIA présentent une cytotoxicité globalement intacte

Comme nous en avons discuté en introduction, le SAM est une complication occasionnelle des maladies immunitaires. En effet, environ 10% des patients présentant une SoJIA ou encore son versant adulte, la

maladie de Still, développent un SAM sévère. En tenant compte des évolutions infra-cliniques, ce serait environ 50% des patients souffrant de SoJIA qui développeraient un SAM [36]. Plusieurs rapports font état que l'inflammation chronique associée à la SoJIA pouvait impacter négativement la cytotoxicité NK, un effet possiblement médié par la désensibilisation du récepteur de l'IL-18 en contexte pro-inflammatoire [39, 88, 89]. Alternativement, il a également été décrit que l'exposition chronique à l'IL-1 β diminue la sensibilité des lymphocytes NK à l'IL-15 [90]. Tenant également compte de la description de polymorphismes et de mutations dans les gènes de la voie de la cytotoxicité chez environ 30% de ces patients, nous avons émis l'hypothèse que le SAM dans le contexte d'un SoJIA pouvait être la conséquence d'un défaut de cytotoxicité [39]. Afin de tester cette hypothèse, nous avons procédé à une analyse de la fonction des lymphocytes NK ainsi que de leur phénotype chez une cohorte de patients souffrant de SoJIA. Parmi ces patients, au moins 5 ont souffert d'un SAM.

Comme le montrent les résultats présentés dans l'article 1 (Figure 3), les patients souffrant d'une SoJIA présentent des niveaux de perforine globalement normaux ainsi qu'une capacité de cytotoxicité intacte. Notons néanmoins, de façon anecdotique, la distribution quasi binomiale du niveau de dégranulation des lymphocytes NK en présence de cellules tumorales K562 (*cf.* Figure 7). À titre d'observation, les points représentant les patients ayant développé au moins un SAM ont été colorés. Nous remarquons que ces patients sont également ceux qui présentent le niveau constitutif de perforine le plus bas.

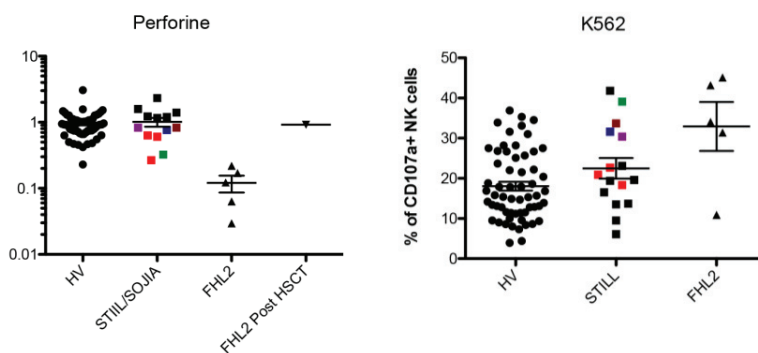


Figure 7 : Niveau de Perforine en fonction du niveau de dégranulation lors du challenge avec K562

Le niveau de perforine est évalué à l'état basal, les résultats sont exprimés en MFI normalisée par date de manipulation. Le niveau de dégranulation est évalué en pourcentage de lymphocytes NK exprimant CD107a, un marqueur lysosomal, après incubation avec des cellules tumorales. En couleur, les patients ayant souffert d'au moins un épisode de HLH. Chaque point noir représente un patient, deux points de la même couleur représentent le même patient.

Bien que le nombre de cas observés ne soit pas suffisant pour conclure et que l'amplitude de ce changement demeure dans l'intervalle d'expression des volontaires sains, il est possible que les patients présentant des niveaux de perforines moins élevés dégranulent davantage. Ceci rappelle ce qui est observé chez les lymphocytes NK dont la cytotoxicité est déficiente [20]. En effet, chez la souris PRF1 -/- ou Granzyme AB -/-, les lymphocytes NK procèdent à une synapse immunologique prolongée au cours de laquelle ils subissent une activation répétitive, ce qui a pour effet d'induire une hypersécrétion cytokinique. Nous ne savons pas si cette hypersécrétion prend également place lors de la dégranulation cytotoxique. Il n'existe, à notre connaissance, qu'un seul rapport démontrant que les niveaux de CD107a sont plus élevés sur les lymphocytes NK suite à la mise en présence de cellules tumorales K562 lors de la FHL-2 [91]. Les patients souffrant d'une SoJIA ne présentent pas une déficience évidente de cytotoxicité *ex vivo* mais il n'est pas exclu que ce mécanisme puisse contribuer au déclenchement de la HLH. En raison de l'accès limité à ces échantillons, nous n'avons pu tester cette hypothèse mais la corrélation entre le niveau d'interaction NK/cellule cible chez les patients atteint d'un SoJIA et présentant un syndrome d'activation macrophagique est une question qui demande à être clarifiée. Dans la cohorte étudiée, le niveau de dégranulation n'était cependant pas corrélé au marqueur d'inflammation CRP (non-montré). Toutefois, nous avons observé une présence moindre de NK en périphérie ainsi qu'une maturation légèrement impactée. En effet, le pourcentage de NK CD56^{bright} était élevé par rapport aux patients contrôles alors que le pourcentage de NK CD57+ était diminué. Étant donné que la déficience immunitaire peut être causée par une altération de l'expression de récepteurs immunitaires, nous avons également évalué l'expression des récepteurs les plus répandus chez les lymphocytes NK, sans trouver de différence notable entre les volontaires sains et les patients souffrant d'une SoJIA. Pour conclure, les patients souffrant d'une SoJIA ne présentent pas de défaut majeur de cytotoxicité ou encore de perturbation homéostatique majeure de leurs lymphocytes NK. Conséquemment, la cause du SAM dans le contexte du SoJIA est vraisemblablement différente d'un défaut de cytotoxicité tel que nous pouvons le mesurer *ex vivo*.

Nous avons montré que dans une cohorte de 14 individus souffrant de SoJIA, la fonction cytotoxique des lymphocytes cytotoxiques était globalement intacte. En dépit d'une possible corrélation entre le niveau de perforine et la capacité à dégranuler, nous ne pouvons affirmer avec certitude que ces faibles niveaux d'altération puisse être responsables du SAM en contexte de SoJIA et par le fait même, que le défaut de cytotoxicité puisse expliquer le SAM.

Section II : Invalidation du gène *Styk-1* spécifique des lymphocytes Natural Killer

2.1 Les lymphocytes NK

Sur base de leur phénotype et de leurs capacités fonctionnelles, les lymphocytes NK sont classés en tant que membres du groupe I des cellules lymphoïdes innées (Figure 2.1) [92]. Ils sont responsables du contrôle immunitaire précoce des cellules infectées, stressées ou tumorigènes. Suite à leur activation, ces lymphocytes produisent de grandes quantités d'IFN- γ ainsi que de la perforine et du granzyme-B, ce qui leur confère à la fois la capacité de potentialiser la réponse immunitaire adaptative ainsi que d'opérer l'élimination *in situ* des cellules cibles par cytotoxicité. La mobilisation de la fonction cytotoxique des lymphocytes NK est déclenchée par l'intégration de la balance entre les signaux activateurs et inhibiteurs qui sont délivrés par les récepteurs NK lors de l'interaction avec leurs cibles [93].

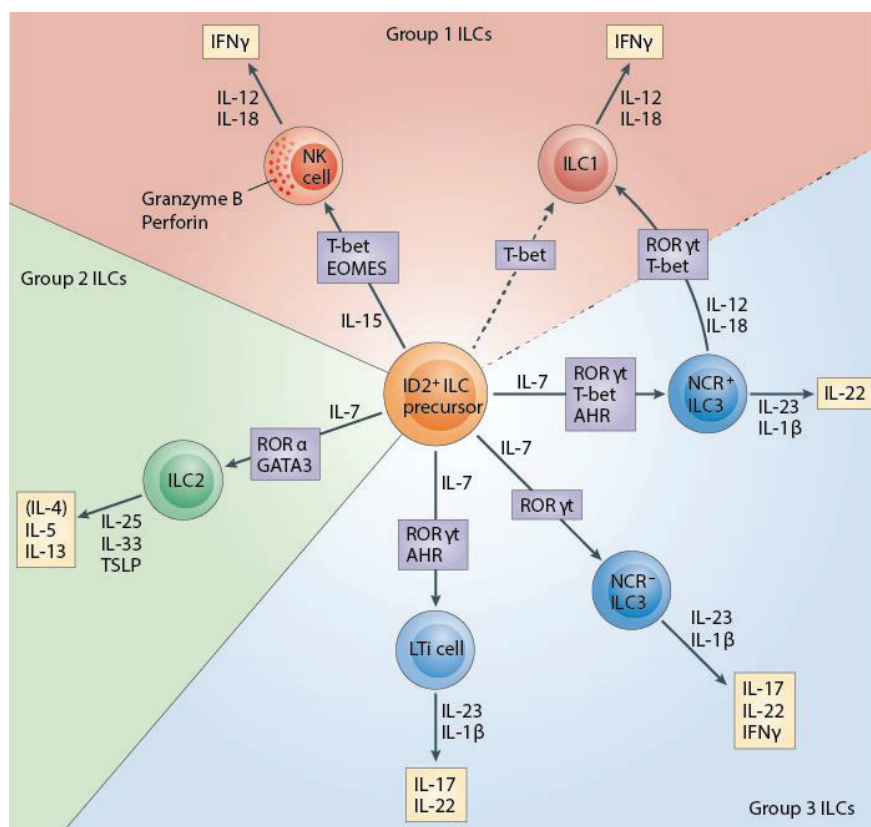


Figure 2.1 : Classification des ILCs

Les NK n'expriment pas les marqueurs CD3, CD4, CD8, CD19 et Ter119. Leur développement est sous le contrôle de l'IL-15 et est caractérisé par l'expression des facteurs de transcription T-bet et EOMES. Suite à la stimulation par l'IL-12, ils produisent et sécrètent de l'IFN- γ , ce qui est potentialisé par l'IL-18. Ces lymphocytes expriment constitutivement l'ARNm du granzyme B et de la perforine. Tiré et adapté de [92].

2.1.1 Maturation et développement des NK

Le développement précoce des lymphocytes NK murins se déroule en plusieurs stades, en grande partie au sein de la moelle osseuse. La cellule souche hématopoïétique donne d'abord lieu à un progéniteur lymphoïde commun (CLP). Ce dernier exprime alors CD127, CD117 (C-kit), Sca-1 et CD135 (Flt-3) et ne possède pas les marqueurs de lignage spécifiques (CD3, CD4, CD8, CD19, Ter119, Gr-1 et NK1.1) [94]. L'engagement du CLP dans la lignée NK est caractérisé par l'acquisition progressive de l'expression des facteurs de transcription, ID2, Ets-1, Nfil3 (E4BP4), TOX, T-bet et Eomes [95-102]. Au stade le plus précoce, les lymphocytes NK expriment CD122, la chaîne beta du récepteur à l'IL-15. Cette étape est cruciale car elle marque le premier stade à partir duquel la maturation des NK se fait sous l'influence de l'IL15. Les précurseurs NK (NKp) sont alors de phénotype CD127+CD122+NK1.1-DX5- [103]. Ils se développent ensuite en NK immature (NKi) en acquérant alors les marqueurs NK1.1, NKp46, TRAIL et en perdant l'expression de CD127 (Figure 2.2) [104]. C'est le premier stade capable de cytotoxicité. Les lymphocytes NK immatures acquièrent alors la capacité de produire de l'IFN-γ ainsi que de sortir de la moelle osseuse en exprimant le récepteur S1P5 et désensibilisant CXCR4 [105, 106]. S1P5 est le récepteur de S1P, un lipide présent à une concentration qui suit un gradient entre le sang, où il est le plus concentré et les organes lymphoïdes.

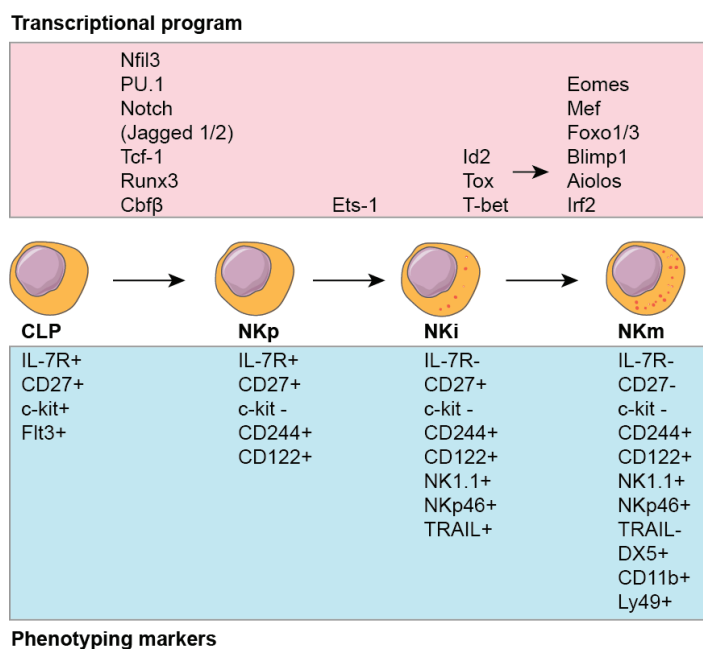


Figure 2.2 : Phénotype et programme transcriptionnel associés au développement des lymphocytes NK

Les lymphocytes NK sont issus du progéniteur lymphoïde commun (CLP), un processus qui est engagé lors de l'expression du facteur de transcription (FT) Nfil3 (E4BP4) et est marqué par l'expression de la chaîne β du récepteur de l'IL-15 (CD122). Le précurseur NK (NKp) se développe en NK immature (NKi) en exprimant le FT Ets-1 et perdant le récepteur de l'IL-7 (CD127). Ce premier stade capable de cytotoxicité est caractérisé par l'expression de surface des récepteurs NK1.1, NKp46 et TRAIL. Les NKi deviennent matures (NKm) par un processus transcriptionnel complexe qui implique l'expression des FT T-bet et Eomes, ce qui est concomitant à la perte du récepteur CD27 et au gain de l'intégrine CD11b. Inspiré de [104].

Chez la souris, l'acquisition des capacités cytotoxiques est progressive et accompagne l'expression séquentielle de CD11b, des récepteurs de type lectine C (Ly49) et de CD49b. Les lymphocytes NKi progressent ensuite du stade immature au stade mature (NKm) en gagnant l'expression de CD11b, une intégrine, tout en perdant progressivement l'expression de CD27, de la famille des récepteurs du TNF [107]. Le phénotype des NK est donc CD27^{hi}CD11b^{lo} au stade précoce, suivi d'un intermédiaire CD27^{hi}CD11b^{hi}. Ceux-ci achèvent leur différenciation au stade CD27^{lo}CD11b^{hi}. Ceci est accompagné par la diminution de l'expression du facteur de transcription Eomes et le gain de T-bet (Jiang *et al.*, Sous Presse, EJI *cf.* Annexe 1) (Figure 2.3). L'abondance relative des différents stades de maturation varie en fonction des organes, les stades immatures étant davantage présents au niveau de la moelle osseuse.

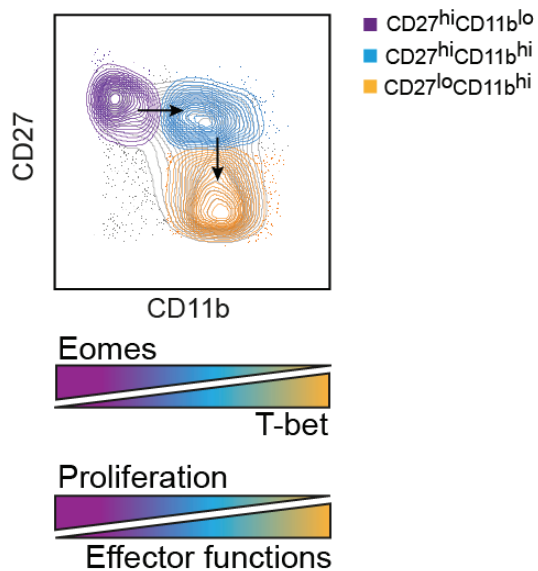


Figure 2.3 : Maturation et fonctions effectrices des lymphocytes NK

Les lymphocytes passent séquentiellement du stade immature CD27^{hi}CD11b^{lo}, au stade mature CD27^{lo}CD11b^{hi} en passant par un intermédiaire CD27^{hi}CD11b^{hi}. Ce processus de maturation est concomitant au gain d'expression du FT T-bet et la perte de EOMES. En parallèle, les lymphocytes gagnent progressivement leurs capacités effectrices mais perdent leur capacité proliférative au stade le plus mature.

2.1.2 Signalisation IL-15-AKT-mTOR

La maturation des lymphocytes NK ainsi que l'acquisition graduelle de leurs capacités effectrices sont gouvernés par la signalisation par l'IL-15. Le récepteur de l'IL-15 est composé des chaînes IL15-R α , IL-2R β (CD122) et de la chaîne commune γ c. Il transduit le signal IL-15 via Jak/Stat5, ce qui mène à la phosphorylation d'AKT ainsi que de mTOR dans le contexte du complexe mTORC1 plus en aval [108]. L'activation de mTOR au sein du complexe mTORC1 mène, quant à elle, à la phosphorylation de S6 Kinase,

ce qui peut être mesuré par le niveau de phosphorylation de son substrat, la protéine ribosomale S6 (Figure 2.4)

L'importance de cette voie pour les lymphocytes NK est illustrée par l'interruption de la maturation des lymphocytes NK au stade immature $CD27^{hi}CD11b^{lo}$ en absence de mTOR ainsi que par la dépendance à l'IL-15 pour leur survie. [109, 110]. L'intégration de ce signal est également nécessaire au plein déploiement des capacités effectrices des NK. En effet, en absence de mTOR, les lymphocytes NK ont une capacité moindre d'intégration des signaux pro-inflammatoires et ainsi que de prolifération en contexte infectieux [108, 111]. La voie de signalisation AKT-mTOR occupe donc un rôle pivot non seulement pour l'homéostasie et la maturation des NK mais également pour l'acquisition des fonctions effectrices. Cette dualité fonctionnelle est vraisemblablement entretenue par la réponse différentielle des NK à différents niveaux d'exposition à l'IL-15 [108]. En effet, la stimulation des DC par divers stimuli mène à l'augmentation des niveaux d'IL-15 trans-présenté [112, 113]. Dans le contexte d'un challenge immunitaire, les lymphocytes NK sont donc certainement exposés à des concentrations locales de l'IL-15 qui sont différentes de celles auxquelles ils sont exposés en condition homéostatique.

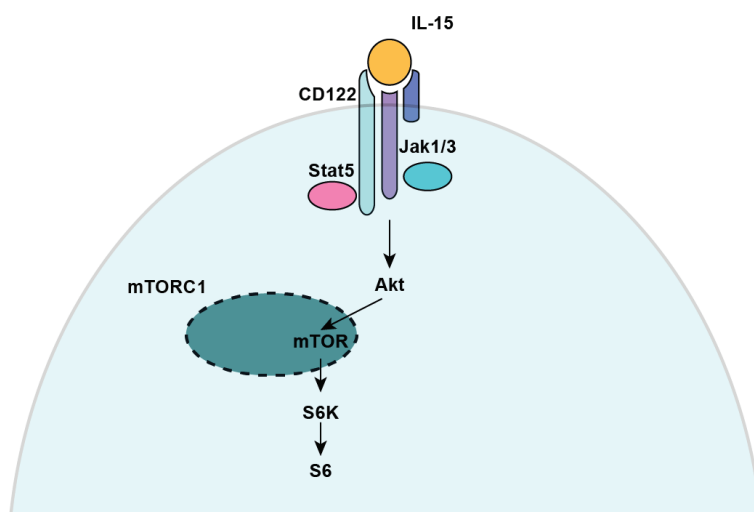


Figure 2.4 : Signalisation de la voie IL-15-AKT-mTOR-S6

2.1.3 Répertoire

La reconnaissance des cellules infectées, allogènes, tumorigènes ou stressées par les lymphocytes NK repose sur l'expression par ces derniers d'un vaste répertoire de récepteurs activateurs et inhibiteurs. La plupart des récepteurs inhibiteurs reconnaissent le Complexe Majeur d'Histocompatibilité de classe I (CMH-I), permettant ainsi la tolérance des cellules du soi et la détection du soi manquant (*missing-self*) [114, 115]. À l'inverse, les ligands des récepteurs activateurs sont des protéines virales ou encore des molécules dont l'expression est induite ou augmentée par le stress cellulaire. C'est la balance entre ces deux signaux opposés qui permet aux lymphocytes NK de distinguer très spécifiquement les cellules à éliminer, tout en limitant les dommages collatéraux [116, 117].

Récepteurs activateurs

Les récepteurs activateurs contribuent au seuil d'activation des lymphocytes NK en transduisant les signaux de stress cellulaire ou encore d'infection. La plupart de ces récepteurs sont exempts de motifs intracellulaires de signalisation. Cependant, ils possèdent une portion trans-membranaire chargée positivement par un résidu lysine ou arginine, ce qui leur permet de s'associer à des protéines adaptatrices à motif ITAM (*Immunoreceptor Tyrosine-based Activation Motif*), chargées négativement [118]. La famille de récepteurs activateurs la plus connue est certainement le groupe des récepteurs de cytotoxicité naturelle (NCR) dont NKp46, NKp30 et NKp44 font partie. Ce sont des récepteurs de type immunoglobuline. La transduction du signal activateur des NCR se fait via les protéines adaptatrices CD3 ζ seules ou en association avec FCR- γ , DAP12 ou encore DAP10 [119]. Leur engagement induit la phosphorylation des motifs ITAM par les kinases de la famille SRC, ce qui entraîne le recrutement et la phosphorylation de Syk et ZAP70. Cette signalisation converge vers la phosphorylation de PI3K ainsi que de VAV-1, un acteur important de la réorganisation de l'actine [120]. NKG2D est un autre récepteur activateur, mais de la famille des lectines de type C. Il reconnaît plusieurs antigènes associés aux cellules transformées [121]. Finalement, mentionnons la famille des récepteurs Ly49 qui comporte aussi des membres activateurs, ils font également partie de la famille des lectines de type C. Ly49H est le membre le plus étudié de ce groupe, il reconnaît la protéine m157, du virus MCMV, qui est mimétique du CMH-I [122-124].

Récepteurs inhibiteurs

Il existe trois types de récepteurs interagissant avec des molécules du CMH-I : Les récepteurs de type Killer-cell Immunoglobulin-like Receptor (KIR) chez l'humain, les récepteurs de la famille Ly49 retrouvés chez la souris et les récepteurs hétérodimériques NKG2/CD94 qui sont partagés entre l'humain et la souris [121]. Les récepteurs inhibiteurs Ly49 sont localisés au sein du complexe NK (NKC), une région génomique très dense qui code également pour Nkrp1 (CD161) ainsi que pour NKG2A (Figure 2.5) [125]

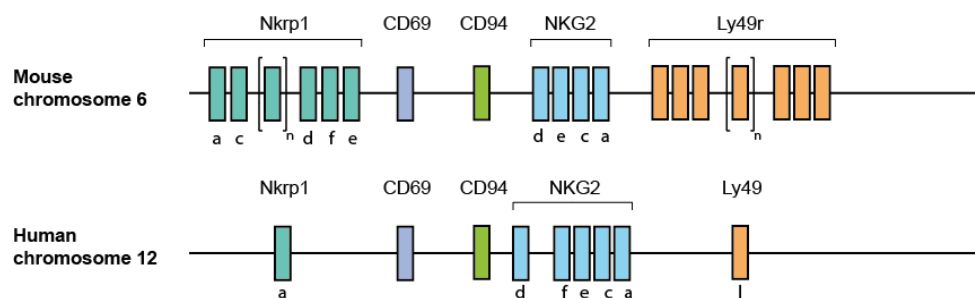


Figure 2.5 : Environnement génomique des récepteurs Ly49r au sein du complexe NK

Les récepteurs Ly49 sont localisés au sein même du complexe NK, un environnement génétique dense regroupant également CD161 et les récepteurs hétérodimériques NKG2/CD94. Le NKC est localisé au chromosome 6 chez la souris et au chromosome 12 chez l'humain.

Le caractère inhibiteur des récepteurs Ly49 repose sur la transduction cytosolique du signal par leurs motifs ITIM. Leur engagement mène au recrutement et à la phosphorylation des phosphatases SHP1 et 2 qui antagonisent l'effet des récepteurs activateurs en déphosphorylant leur cible, VAV-1 [126]. Chez la souris C57BL/6J, il existe environ une dizaine de gènes Ly49 codant pour des protéines fonctionnelles [127]. Leur transcription est binaire, stochastique et les NK peuvent en exprimer différents nombres et combinaisons [128]. Ceci a pour effet de générer un répertoire de lymphocytes NK qui expriment de nombreuses combinaisons différentes de récepteurs et qui ont, en conséquence, différentes spécificités (Figure 2.6). Ce dispositif générateur de diversité existe possiblement pour palier à l'absence de mécanisme créateur de répertoire comme il existe chez les lymphocytes T et B, qui réorganisent certaines parties de leur TCR/BCR via la recombinaison RAG. Cependant, cela reste à démontrer formellement.

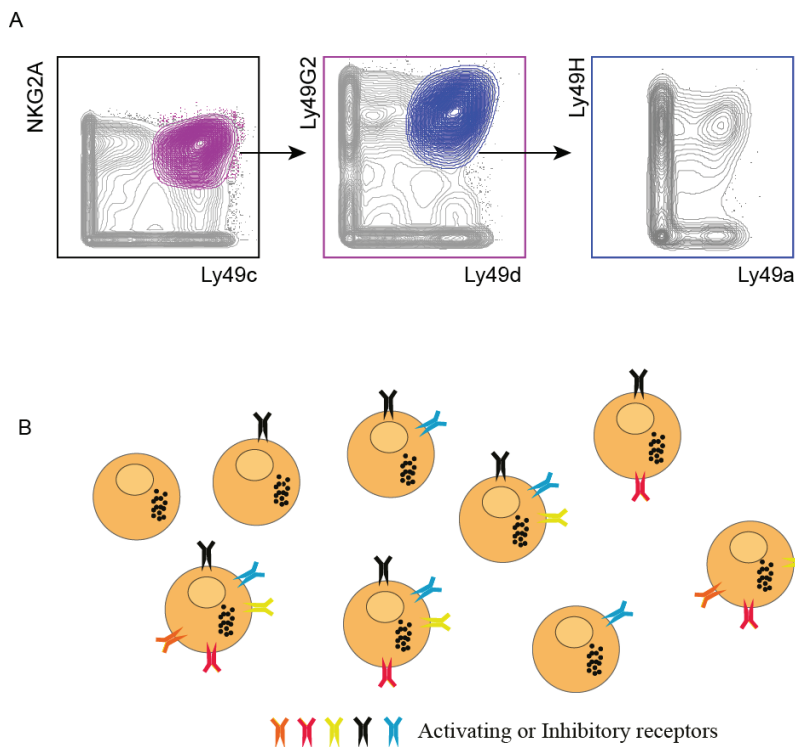


Figure 2.6 : Diversité combinatoire des récepteurs activateurs et inhibiteurs

Les récepteurs inhibiteurs ainsi que certains récepteurs activateurs ont un profil d'expression binaire de telle sorte que toutes les combinaisons sont possibles. **A)** Exemple du patron d'expression des récepteurs activateurs et inhibiteurs, données acquises en cytométrie de masse. En rose, la sélection des lymphocytes NK Ly49C+NKG2A+, reporté dans la fenêtre de la même couleur. A l'intérieur de cette sélection l'expression de deux autres récepteurs est illustrée, et ainsi de suite. **B)** Schématisation du répertoire des récepteurs inhibiteurs et activateurs chez les lymphocytes NK. **B)** est adapté de [128].

2.1.4 Éducation

Bien que cela puisse sembler contre-intuitif, la capacité des lymphocytes NK à répondre aux stimuli est dépendante de l'engagement chronique de leurs récepteurs inhibiteurs. En effet, l'exposition des récepteurs inhibiteurs à leur ligand, le CMH-I, est une condition préalable au maintien des capacités effectrices. Lorsqu'exposés à un environnement exempt du CMH-I la sensibilité des NK aux stimuli est fortement amenuisée. Ce phénomène, désigné « éducation » ou encore « licensing » est néanmoins plastique. En effet, l'exposition à de hauts niveaux cytokiniques, l'infection ou encore le transfert adoptif vers un environnement où le CMH-I est présent restaure les capacités cytotoxiques des lymphocytes NK autrement anergiques [117]. La compréhension du phénomène de *licensing* est encore incomplète. Cependant, plusieurs théories ont été proposées pour l'expliquer.

Une des hypothèses prépondérantes pour expliquer le phénomène de l'éducation présume que les NK sont intrinsèquement inertes. L'engagement des récepteurs inhibiteurs permettrait l'acquisition de compétences effectrices par un mécanisme « d'armement » ou de *licensing* qui serait dépendant de l'interaction entre ces derniers et le CMH-I (Figure 2.7A) [129]. *A contrario*, les NK pourraient également être intrinsèquement réactifs [130]. Dans le cas particulier où aucun récepteur inhibiteur n'est engagé, ce serait plutôt l'insensibilisation à la stimulation chronique via les récepteurs activateurs qui permettrait la tolérance immunitaire du soi et le « désarmement » des NK (Figure 2.7B) [131].

Alternativement, le modèle du « rhéostat » a été suggéré et semble réconcilier les modèles de l'armement et du désarmement. Ce modèle suppose que la réactivité des NK serait plutôt modulée en fonction du nombre de récepteurs inhibiteurs capable d'engager les molécules du soi. Ainsi, les lymphocytes NK d'un hôte exprimant plusieurs allèles du CMH-I seraient davantage aptes à répondre à la stimulation agoniste de leurs récepteurs activateurs que leurs contreparties se développant en absence de CMH-I ou en présence de moins d'allèles (Figure 2.7C) [132, 133]. Inversement, les lymphocytes présentant plus de récepteurs inhibiteurs potentiellement engagés sont plus réactifs.

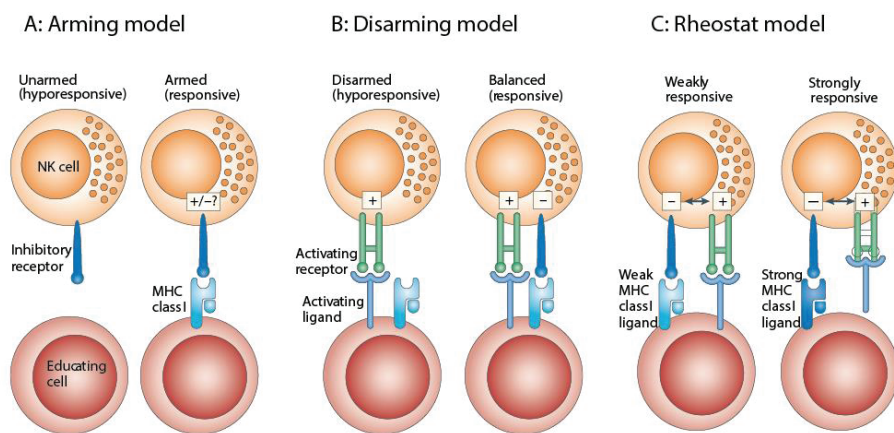


Figure 2.7 : Modèles de l'éducation des lymphocytes NK

La capacité à répondre des lymphocytes NK dépend de l'engagement chronique des récepteurs inhibiteurs, un processus désigné éducation ou *licensing*. Plusieurs théories sont proposées pour l'expliquer **A)** Armement : La mise en place des capacités effectrices des NK, autrement inertes, nécessite l'engagement via le récepteur inhibiteur. **B)** Désarmement : Les lymphocytes NK sont intrinsèquement réactifs, en situation de stimulation chronique et non opposée par les récepteurs inhibiteurs, les NK deviennent anergiques. **C)** Le rhéostat : La capacité de répondre à l'engagement par les récepteurs activateurs dépend du nombre et de la force de l'interaction entre les récepteurs inhibiteurs et leur ligand, le CMH-I. Tiré et adapté de [116]

Chez la souris C57BL/6J, seuls Ly49C et NKG2A sont engagés respectivement par H2-Kb et H2-Db [134, 135]. Bien que plusieurs autres récepteurs inhibiteurs soient exprimés, ils sont *a priori* inertes. Les lymphocytes exprimant Ly49C ou NKG2A ou une combinaison des deux présentent une activité accrue en réponse à l'engagement des récepteurs activateurs [132, 136]. Le travail présenté dans l'article en Annexe 2, montre que le niveau d'activation de la voie mTOR ainsi que la capacité de réponse à l'engagement des récepteurs activateurs est proportionnel au nombre de récepteurs inhibiteurs engagés. Nous présentons également que l'engagement chronique des récepteurs activateurs amenuise le niveau d'activation de mTOR. À la lumière de ces résultats, nous proposons que la kinase mTOR fait office de rhéostat moléculaire de la théorie éponyme. mTOR permettrait l'intégration signalétique du contexte environnemental et régirait ainsi la capacité de réponse des NK [111].

2.1.5 Activation et Synapse immunitaire

La finalité de l'activation des lymphocytes NK est le déploiement de leurs capacités effectrices qui peuvent être dichotomisées en fonction A) sécrétrice de médiateurs immunitaires et B) de cytotoxicité. La médiation de la cytotoxicité fait intervenir la mort par apoptose de la cible suite à la formation d'une synapse immunitaire qui mène au déversement polarisé du contenu des granules cytotoxiques (Figure 4). Comme nous l'avons évoqué dans la section I, la synapse immunitaire est un processus finement régulé par un ensemble de protéines, ce qui empêche tout déversement accidentel de médiateurs cytotoxiques. Son processus est séquentiel et fait intervenir les étapes que nous distinguons ainsi :

- 1) Adhésion
- 2) Capacités effectrices
- 3) Terminaison

Lors de l'adhésion les intégrines LFA-1 et CD11b se concentrent rapidement au niveau de la synapse immunitaire lors de l'interaction avec la cellule cible [137]. Notons par ailleurs que l'activation de l'intégrine LFA-1 est plus importante chez les lymphocytes éduqués, qui forment une conjugaison plus stable avec leur cible que leur contrepartie non-éduquée [138]. L'engagement des intégrines initie une cascade de signalisation précoce qui fait intervenir la phosphorylation de VAV-1 [139]. Cette étape précède la réorganisation de l'actine ainsi que l'accumulation des radeaux lipidique au niveau de la synapse immunitaire.

Suite à l'adhésion, la reconnaissance d'une cellule qui présente un ratio favorable de signaux activateurs/inhibiteurs permet la stabilisation de la synapse immunitaire. Une réorganisation profonde du réseau d'actine ainsi que l'agrégation des récepteurs sont alors opérés [137]. La réorganisation du cytosquelette permet la polarisation des granules cytotoxiques, lesquelles se déplacent vers le centre organisateur de microtubule (MTOC) qui lui-même migre vers la synapse immunitaire par un mécanisme qui demeure à clarifier mais qui est dépendant de VAV-1 [140]. Ce phénomène est suivi par la dépolymérisation d'une partie du réseau d'actine au niveau de la synapse immunitaire, permettant ainsi aux granules cytotoxiques d'accoster la membrane cellulaire [141]. C'est un processus, nous l'avons vu, qui fait intervenir la GTPase RAB27a ainsi que Munc13-4 (cf. Section I). Ensemble, ces molécules assurent l'accostage et l'amorçage de la vésicule cytotoxique, qui sera suivi par sa fusion avec la membrane cellulaire.

La mise en œuvre des capacités effectrices, qui sont dépendantes du contact étroit entre le lymphocyte NK et sa cible, repose sur l'expression de molécules cytotoxiques. Un de ces effecteurs est la perforine, une protéine formant des pores au niveau de la cellule cible lors de son incorporation à sa membrane. Elle est sécrétée par des lysosomes modifiés qu'on appelle également les granules cytotoxiques. Son ARNm est constitutivement exprimé par les lymphocytes cytotoxiques ce qui permet l'élimination prompte des cellules cibles. Alors que la perforine est constitutivement exprimée chez l'humain, la souris nécessite sa traduction, ce qui est rapidement mis en place suite à l'activation des NK [142]. Les granules cytotoxiques transportent également le granzyme B, un autre effecteur important de la cytotoxicité. Il n'est pas encore clair si le granzyme B transite par les pores formés par la perforine, mais son passage cytosolique en est dépendant. Le déversement du granzyme B au cytosol de la cellule cible déclenche une cascade apoptotique initiée par la catalyse de nombreux intervenants dont la caspase-3, BID et ICAD (Figure 2.8B). Cette mort est immunologiquement silencieuse pour l'environnement immédiat.

Finalement, la synapse immunologique est résolue par la modération du niveau d'expression des récepteurs de surface. À l'instar des lymphocytes T qui internalisent le TCR à la suite de la synapse immunitaire, les NK réduisent alors l'expression des récepteurs activateurs NKG2D, NKp46 et 2B4 [137]. Il semblerait également que le détachement de la cellule cible soit subordonné à la mort de celle-ci [20].

Suite à leur activation, les lymphocytes NK sécrètent rapidement un panel de médiateurs immunologiques qui permettent de potentialiser la réponse adaptative (cytokines) et d'attirer d'autres effecteurs sur le lieu de l'infection (chemokines). Entre autres, ils sont une source majeure d'IFN- γ de TNF- α , ce qui leur permet de jouer un rôle important dans le contrôle antiviral [93].

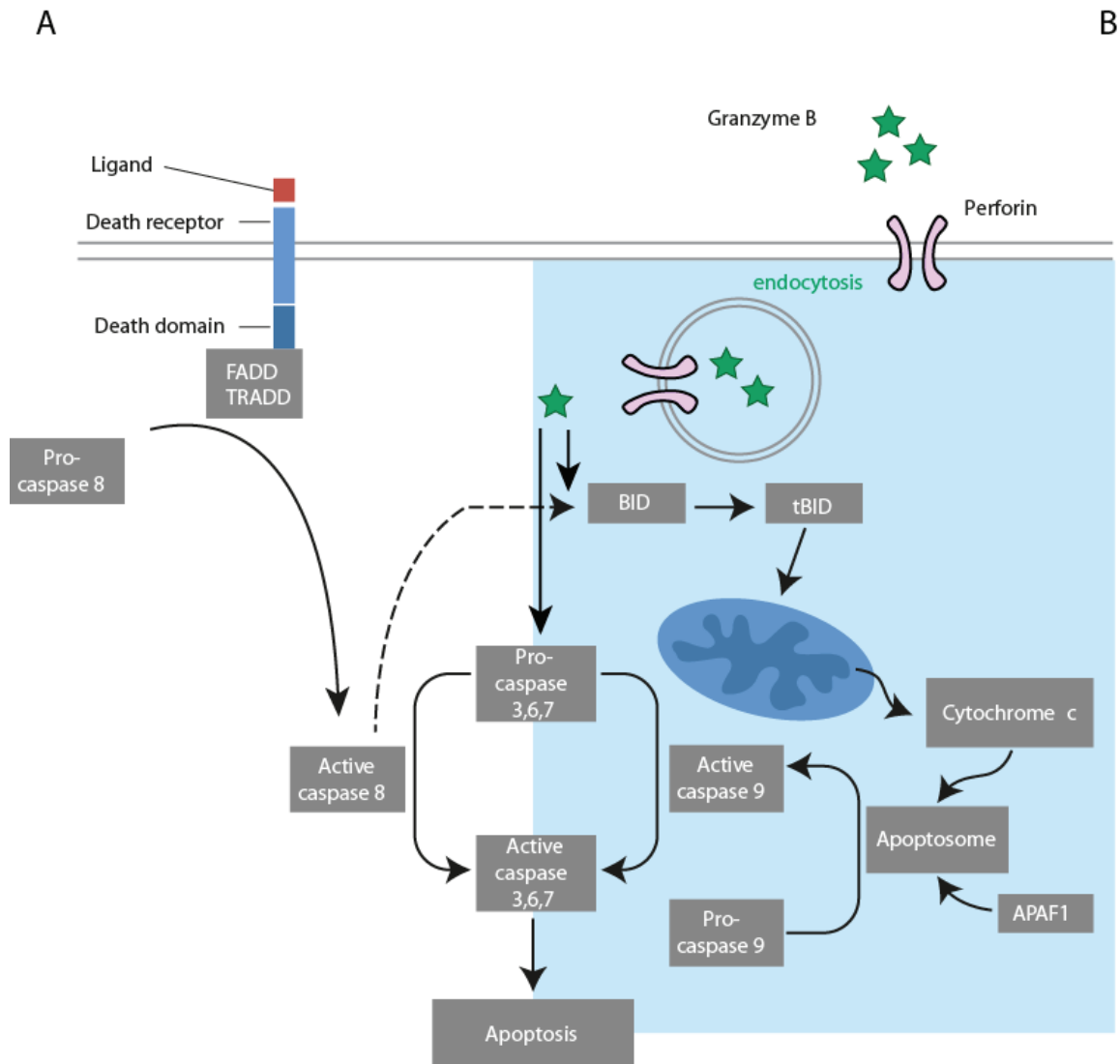


Figure 2.8 : Signalisation de l'induction de l'apoptose par les lymphocytes cytotoxiques

A) L'interaction FAS/FAS-L ou TRAIL/TRAIL-R mène au recrutement et l'activation d'adaptateurs FADD/TRADD contenant des domaines de mort. Ces derniers permettent la maturation de la pro-caspase-8 dans sa forme active. À son tour, la caspase-8 active les caspases effectrices 3, 6 et 7, ce qui mène à la mort cellulaire par apoptose. **B)** Suite à sa translocation au cytosol de la cellule cible, le granzyme B clive de nombreux substrats dont BID, qui provoque la relâche du cytochrome c au cytosol et donc la formation de l'apoptosome, une plateforme constituée de APAF-1, du cytochrome c et de la pro-caspase-9. L'activation de cette dernière mène également à l'activation des caspases effectrices 3, 6 et 7. Tiré et adapté de [143].

2.1.6 Récepteurs de mort cellulaire

La cytotoxicité des lymphocytes NK s'articule également lors d'un processus d'élimination des cellules cibles par l'engagement des récepteurs de mort cellulaire. Les NK expriment en effet FAS-L et TRAIL, qui engagent respectivement FAS et TRAIL-R [144, 145]. Suite à leur trimérisation, les récepteurs de mort cellulaire recrutent la protéine adaptatrice FADD ce qui permet la maturation de la pro-caspase 8 et marque le déclenchement d'une cascade pro-apoptotique (Figure 2.8A) [143]. La caspase-8 active les caspases 3 et 7 et clive également BID, ce qui cause la relâche au cytosol du cytochrome C. Le cytochrome C est quant à lui, un activateur de la caspase 9. Ensemble, les caspases 8 et 9 sont des activateurs des caspases effectrices 3, 6 et 7 dont l'activation mène à la mort cellulaire par apoptose. Il est intéressant de noter ici la convergence partielle entre la signalisation médiée par la stratégie de l'engagement des récepteurs de mort cellulaire et celle causée par la cytotoxicité dépendante du granzyme. *In fine*, ces deux voies font intervenir les mêmes médiateurs.

L'engagement des récepteurs de mort cellulaire joue un rôle important dans l'homéostasie du système immunitaire. Ceci est illustré par le développement de syndromes lymphoprolifératifs chez les souris déficientes en FAS-L [146]. Notons par ailleurs que la souris doublement déficiente pour la PRF-1 et pour FAS-L déclenche spontanément un syndrome réminiscent de la HLH [147].

2.1.7 Contrôle de l'homéostasie immunologique par les NK

Les conditions exactes du contrôle homéostatique de l'immunité par les NK dans le contexte des infections et des maladies auto-inflammatoires demeurent encore largement incomprises. Néanmoins, les cellules NK présentent la capacité de contrôler la réponse des cellules présentatrices d'antigènes suite à l'infection [148]. Ceux-ci ont notamment la capacité d'activer les DC via la sécrétion de TNF- α et l'IFN- γ mais également d'éliminer ces dernières par un mécanisme faisant intervenir le récepteur de mort cellulaire TRAIL [149]. De plus, ils régulent l'amplitude de la réponse par les lymphocytes T en contexte infectieux [150]. Dans le contexte de la HLH, le syndrome auto-inflammatoire présenté à la section I, la présence de NK compétents pour la perforine dans un hôte autrement déficient permet d'amenuiser la sévérité du syndrome (*cf.* Section 1.1.3 et figure 4). Ce contrôle homéostatique de l'immunité confère donc la possibilité aux NK de modérer l'intensité de la réponse immunitaire et de participer à sa résolution en limitant le bassin de cellules présentatrices d'antigènes et de lymphocytes T activés.

2.1.8 Mémoire des lymphocytes NK

Bien que traditionnellement perçues comme des cellules de l'immunité innée en raison de leur cytotoxicité intrinsèque, les NK ont des caractéristiques s'apparentant à la mémoire dont sont dotées les cellules de l'immunité adaptative. L'exemple le plus éloquent de cette capacité est représenté par l'expansion clonale que subissent les NK Ly49H+ suite à l'infection à MCMV [151]. En l'occurrence, Ly49H reconnaît la protéine virale mimétique du CMH-I, m157 exprimée par MCMV. Suite à l'infection par MCMV, un important pool de NK Ly49H+ est généré suite à leur prolifération, ce qui s'apparente à l'expansion clonale des lymphocytes T. Celle-ci est suivie d'une phase de contraction après laquelle des NK dits mémoires persistent dans le temps. Ceux-ci répondent de façon plus efficace à la restimulation par le même antigène. En parallèle, il a été démontré que l'établissement d'une réponse similaire à la mémoire peut être générée de façon non-spécifique d'un antigène, suite à la sensibilisation cytokinique préalable des NK [152].

2.1.9 Singularité des NK.

Les lymphocytes T CD8+ et les NK sont à plusieurs égards très semblables. D'abord parce que l'initiation de leur programme ontogénique est similaire [153]. Mais également parce qu'ils partagent un profil transcriptionnel ainsi que des fonctions effectrices comparables [154]. À l'instar des NK, les lymphocytes T CD8 effecteurs et mémoire expriment T-bet et Eomes (Zhang et al. Annexe 1). T-bet et Eomes contrôlent l'expression de l'IFN- γ , de PRF1 ainsi que du granzyme B. Bien que les lymphocytes T ne soient pas activés de la même façon – Via le TCR – que les NK – via la balance des signaux activateurs/inhibiteurs – ces sous-types immunitaires partagent la capacité effectrice d'exprimer le granzyme B, la perforine et l'IFN- γ . Une fois activés, les NK et CD8 partagent également l'expression d'une multitude de récepteurs (CD25, CD69, CD122, CD62L, etc.) [152]. Par ailleurs, l'expression des récepteurs Ly49 et NKp46 n'est pas unique aux NK. En effet ces récepteurs sont également exprimés par certains sous-types de lymphocytes T et ILCs [155, 156]. L'identification de la signature transcriptionnelle exclusive aux NK est donc un sujet toujours en suspens. Devant le peu d'éléments caractérisant la singularité des lymphocytes NK, notre équipe (non publié) et le consortium Immgen ont entrepris la caractérisation de la signature transcriptionnelle des NK [154]. Cet effort expérimental nous a permis d'identifier une suite de gènes spécifiques des lymphocytes NK. Parmi ceux-ci, nous retrouvons le gène *Styk1*, inconnu au sein du système immunitaire (Figure 2.9), ce que nous avons rapidement confirmé par qPCR (Figure 2.10).

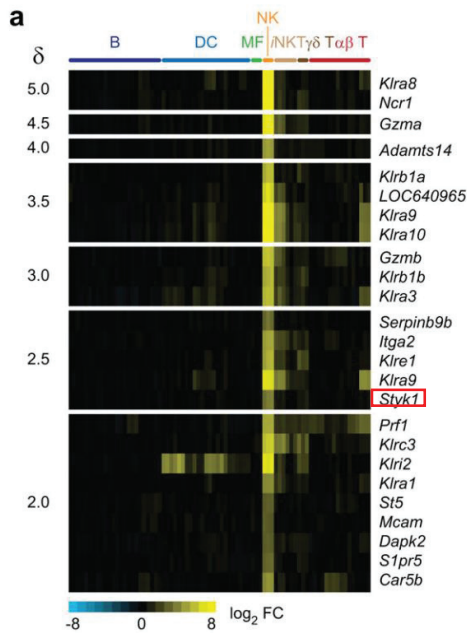


Figure 2.9 : Expression relative de transcrits caractérisant les lymphocytes NK

Tiré et adapté de [154].

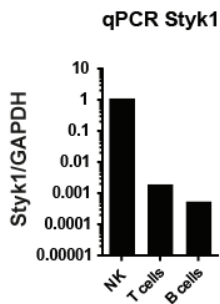


Figure 2.10 : Confirmation par RT-qPCR de l'expression spécifique de Styk1

Notre intérêt pour l'expression de Styk1 chez les NK s'est accru lorsqu'il a été formellement démontré que cette kinase était impliquée dans la voie PI3K-AKT [157]. En raison de l'importance, nous l'avons vu, de cette voie dans le maintien des fonctions cytotoxiques mais également dans l'homéostasie des lymphocytes NK, nous avons décidé d'investiguer plus avant le rôle de Styk1 chez les lymphocytes NK en générant un modèle murin dont son expression est invalidée.

2.1.10 Styk1

Très peu de choses sont connues concernant la kinase Styk1. C'est une sérine thréonine tyrosine kinase de 422 acides aminés impliquée dans la progression d'une grande variété de cancers, notamment les cancers du pancréas, colorectal, des ovaires et de la prostate [158-164]. Styk1 était d'ailleurs originalement connue sous l'appellation NOK pour Novel Oncogene Kinase [165]. Son rôle dans le système immunitaire est à ce jour entièrement inconnu.

Styk1 est évolutivement conservée. Elle est localisée sur le chromosome 6 chez la souris et 12 chez l'humain tout juste en aval du NKC (*cf.* Article 2 Figure 1A). Elle code un ensemble de 11 exons qui contiennent un domaine transmembranaire mais ne semblent pas comprendre de domaine extracellulaire [165]. En dépit de son homologie avec les récepteurs protéine tyrosine kinase (RPTK), Styk1 ne contient aucun peptide d'adressage à la membrane. Cette kinase présente un faible degré d'homologie avec les récepteurs du facteur de croissance des fibroblastes (FGFR) et du facteur de croissance dérivé des fibroblastes (PDGFR), deux récepteurs de la famille des RPTK [165].

En raison de cette homologie, son implication potentielle dans la voie PI3K-AKT a rapidement été suggérée mais ce n'est qu'en 2016 que la preuve formelle est présentée dans le contexte du carcinome hépatocellulaire [160]. *In vitro*, il semblerait que sa présence favorise la phosphorylation d'AKT à la thréonine 308 et GSK-3 β à la sérine 9, des sites phosphorylés respectivement par PI3K et pAKT [157]. L'existence de ces interactions au sein du système immunitaire demeure toutefois inconnue.

La voie AKT-mTOR, nous l'avons vu, occupe une place centrale pour le maintien homéostatique ainsi que pour le déploiement des capacités effectrices des lymphocytes NK. Nous avons donc tenté d'évaluer quelle est l'implication de Styk1 au sein des lymphocytes NK. Pour ce faire, nous avons évalué le phénotype d'un modèle murin pour lequel Styk1 a été invalidé. Cela, afin de déterminer si l'abrogation de ce gène a un impact sur le développement, la maturation ainsi que les capacités effectrices des NK.

Objectif du travail

En raison du partage de l'expression de leurs récepteurs de surface, d'une grande partie de leur programme transcriptionnel ainsi que de leurs fonctions effectrices avec d'autres sous-types de cellules immunitaires, peu de choses distinguent les lymphocytes NK. Nous avons récemment mis en évidence que la protéine sérine thréonine tyrosine kinase Styk1 fait partie de la signature transcriptionnelle de ces derniers. Toutefois, le rôle de cette kinase dans le système immunitaire demeure inconnu. Il a été rapporté que Styk1 était probablement impliquée dans l'activation d'AKT ainsi que de GSK-3 β dans le contexte du carcinome hépatocellulaire. AKT, via la phosphorylation de mTOR plus en aval joue un rôle central dans le maintien de l'homéostasie, du développement ainsi que de l'acquisition des capacités effectrices des lymphocytes NK. Toutefois, nous ne savons pas si la relation entre Styk1 et AKT existe chez les lymphocytes NK. Afin de connaître l'implication de cette kinase dans le système immunitaire, nous avons généré un modèle murin pour lequel Styk1 est invalidé. Cette seconde section traite de ce modèle. Lors des travaux présentés à l'article 2, nous avons cherché à comprendre si l'ablation génétique de cette kinase avait un impact sur le développement, la maturation ainsi que la fonction cytotoxique des lymphocytes NK.

Dans un premier temps, nous avons confirmé que l'expression de Styk1 était spécifique des lymphocytes NK. En raison de l'importance que revêt mTOR pour le développement, l'homéostasie ainsi que pour la mise en place des capacités effectrices des lymphocytes NK, nous avons d'abord évalué l'intégrité de cette voie de signalisation en absence de Styk1. Ensuite, nous nous sommes intéressés aux déterminants modulant la transcription de Styk1, notamment en appréciant si le niveau d'éducation ou encore la stimulation cytokinique avait un rôle dans l'induction de Styk1. Finalement, nous avons évalué l'état de la fonction effectrice des lymphocytes NK Styk1 $-/-$ en conditions diverses.

Work objective

Because NK cells share most of their receptors, transcriptional program and effector functions with other immune cell subtypes, few determinants specifically characterize these immune cells. Recently, our team and the Immgen consortium revealed that the serine/threonine/tyrosine kinase Styk1 was part of the NK cell's transcriptional fingerprint. However, the role of Styk1 in the immune system and more particularly in NK cells remains unknown. It was reported that Styk1 is possibly implicated in the activation of AKT and GSK-3 β in the context of hepatocellular carcinoma. AKT, via the phosphorylation of mTOR, plays a central role in the development, homeostasis and effector functions of NK cells. However, if the relation between Styk1 and AKT exists in NK cells remains to be evaluated. To follow this purpose, we generated a Styk1 $-/-$ mouse model bearing a GFP reporter for Styk1 transcription. This section covers the immune phenotyping of Styk1 invalidated mice.

First, we confirmed that Styk1 expression was specific of NK cells. Because mTOR plays a pivotal role in the development, homeostasis and effector functions of NK cells, we verified the integrity of this pathway when Styk1 is absent. Then, we evaluated under what conditions Styk1 transcription was regulated, focusing on cytokinic and activating receptor stimulation or the education levels of NK cells. Lastly, we evaluated the integrity of effector functions by stimulation NK cells in various conditions.

Résultats : Article 2

Styk1 expression is a hallmark of NK cells but is dispensable for their immune function

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Abstract

Cell-type specific transcripts are often associated with important and distinctive cellular functions. These transcripts and the corresponding proteins can also be used as cell lineage markers. To gain insight into the biology of Natural Killer (NK) cells, others and we previously identified the NK cell transcriptional signature, defined as the set of transcripts which expression is highly enriched in these cells compared to other immune subtypes. The transcript encoding the Serine/threonine/tyrosine kinase 1 (Styk1) is part of this signature. However, the role of Styk1 in the immune system is unknown. Here, we report the generation of a novel transgenic mouse model, in which Styk1 expression is invalidated and replaced by an EGFP reporter cassette. We demonstrated that Styk1 is a selective NK cell marker, not expressed by other innate lymphoid cell subsets. Styk1 expression is also maintained by IL-15 and negatively correlates with educating receptors. Analysis of phosphorylation levels of mTOR substrates S6 and Akt suggest that Styk1 may contribute to the activity of the PI3K/Akt/mTOR pathway. However, Styk1 deficient NK cells develop normally and have normal *in vitro* and *in vivo* effector functions. Thus Styk1 expression is a hallmark of NK cells but is dispensable for their immune function.

Introduction

Natural Killer (NK) cells are innate lymphocytes endowed with antitumor and antiviral functions. They have the unique ability to kill cells recognized as targets without prior stimulation. Cytotoxicity is mediated by the oriented release of specialized granules containing perforin, granzymes and other proteins at the synapse with the target cell. NK cells also secrete several cytokines including $\text{IFN}\gamma$ and $\text{TNF}\alpha$ that also play different roles in viral or tumor clearance. Recognition of target cells is mediated by an array of cell surface germ-line encoded receptors called the NK cell receptors. NK cell receptor genes are clustered in two important loci, the leukocyte receptor complex and the NK cell receptor complex [1]. These receptors transduce activating or inhibitory signals in response to the engagement by their respective ligands, and the balance between these signals governs NK cell behavior, *ie* activation or ignorance. NK cells belong to the recently defined family of innate lymphoid cells (ILCs) [2] and are closely related to ILC1s, although the latter are generally less cytotoxic than NK cells and are tissue resident, unlike NK cells that circulate in the body via the blood.

Our understanding of immune cells in general and of NK cells in particular has been largely improved by whole genome analyses of gene expression. For example, we previously identified *S1PR5* as a NK-specific transcript using microarray analyses. This led to the discovery that *S1PR5* is a chemotactic receptor for sphingosine-1 phosphate essential for NK cell egress from the bone marrow [3], a breakthrough that we recently validated in human as well. Using a similar microarray approach, we also found that *NKp46* was a specific marker of NK cells across different mammalian species [4]. Further studies subsequently showed that *NKp46* was in fact not completely specific of NK cells as it was expressed in a fraction of T cells [5],[6] and in *NCR1* positive ILC3s in the gut [7],[8]. A selective marker of NK cells is therefore still lacking. Based on a comprehensive transcriptomic analysis of hundreds of immune subsets, the Immgen consortium recently reported the identification of a transcriptional signature for NK cells [9]. This signature was composed of 25 transcripts strongly enriched in NK cells compared to all other immune populations, including several of the aforementioned transcripts (*S1pr5*, *Ncr1*). Most of these genes were already known in NK cells, many of which encoding NK cell

cells was the Serine/threonine/ tyrosine kinase 1 (Styk1) mRNA, whose role in NK cells and more generally in the immune system was unknown.

Styk1 shares homology with platelet-derived growth factor/fibroblast growth factor receptors and has been shown to drive transformation of NIH3T3 and Baf/3 cell lines by regulating cell proliferation and survival through the activation of both MAP kinase and phosphatidylinositol 3'-kinase [10]. Several recent papers confirmed that Styk1 was involved in the activation of this pathway, leading to the induction of aerobic glycolysis, an outcome that may contribute to oncogenesis in cells where Styk1 is overexpressed. Styk1 is overexpressed in many tumors including breast [11], lung [12], acute leukemia [13], prostate [14] and ovarian [15] cancers and is associated with progression of renal cell carcinoma [16]. Styk1 may induce epithelial to mesenchymal transition through the PI3K/Akt pathway [17].

s a transmembrane domain but apparently no extracellular domain. Only one protein, HSP90AA1, is reported to interact with Styk1 (BioGrid database). How Styk1 is activated under physiological conditions and the pathways this kinase regulates remains unknown. Here, we report the generation of *Styk1^{EGFP/EGFP}* reporter mouse that is also a loss-of-function of Styk1 mutant. We demonstrate that Styk1 expression is a hallmark of NK cells. In particular Styk1 is expressed neither by ILC subtypes tested nor by NKT cells. Styk1 deficient NK cells display a decreased basal level of phosphorylation of mTOR substrates S6 and Akt suggesting that Styk1 may contribute to the PI3K/Akt/mTOR pathway under steady state conditions. However, NK cells develop normally in the absence of Styk1 and Styk1 deficient NK cells have normal *in vitro* and *in vivo* effector functions.

Results

Styk1 expression marks the NK cell lineage

A previous report from the *Immgen* consortium showed that *Styk1* mRNA was part of the transcriptional signature of resting mouse NK cells. More specifically, *Styk1* was among the 25 genes more highly expressed by NK cells than any other leukocyte populations [9]. Microarray data from human leukocytes also predict NK-specific expression of *Styk1* (*Immgen* website). Preferential *Styk1* expression in NK cells correlates with the conserved location of the *Styk1* gene at the boundary of the NK-complex on mouse chromosome 6 and human chromosome 12 (Figure 1A). Analyses of genomic data from 28 mouse strains suggest the presence of a functional *Styk1* allele in all of them (*Adams DJ Mam genome 2015*). Similarly, sequence variations predicted to be pathogenic are extremely rare in human, according to the exome aggregation consortium (Exac) database that consists of more than 60 000 exome sequence data.

RT-QPCR measurements further supported NK-specific expression of mouse and human NK cells compared to T and B cells (Figure 1B and data not shown). To further explore the expression of *Styk1* in mouse leukocyte subsets, we generated a *Styk1*-reporter mouse line by inserting an EGFP cassette after the exon 2 of *Styk1*, which is followed by a gene trap, resulting in an incomplete transcript (Figure 2A). We then measured EGFP expression in various leukocyte subsets from *Styk1^{EGFP/EGFP}* mice. As shown in Figure 2B, among splenocytes, EGFP expression was only detected in NK cells. EGFP expression was gradually increased during NK cell maturation and maximal in CD27⁺ CD11b⁺ NK cells (Figure 2C). We also performed a detailed analysis of EGFP expression in ILC subsets. EGFP expression was undetectable in liver ILC1, and in gut ILC3 and NCR+ILC3s, as defined by gating in figure S1. Thus, *Styk1* expression is a hallmark of the NK cell lineage and is absent from other ILC subsets.

Normal NK cell development in *Styk1*-GFP mice

Styk1 has been suggested to regulate cell proliferation and survival by activating both MAP kinase and phosphatidylinositol 3'-kinase (PI3K). Accordingly, we found a significantly lower basal level of phosphorylation of downstream signaling molecules Akt

and S6 in *Styk1^{EGFP/EGFP}* NK cells compared to control NK cells (Figure 3A). However, no difference in the level of these phosphorylation events was observed between both types of NK cells following stimulation with anti-NK1.1 antibodies or with IL-15 (data not shown). Given the important role of mTOR in NK cell development and function, we explored the NK cell compartment in *Styk1^{EGFP/EGFP}* mice. As shown in Figure 3B, the percentage of NK cells among leukocytes in different organs was comparable in *Styk1^{EGFP/EGFP}* versus control mice. The relative proportions of mature and immature cells among NK cells was also normal in the different organs of *Styk1^{EGFP/EGFP}* mice (Figure 3C), excluding a role for *Styk1* in NK cell proliferation or maturation. The expression of various receptors from the NK or the leukocyte complex was also comparable in *Styk1^{EGFP/EGFP}* and control mice (Table I). The targeted *Styk1* null allele we generated could be converted into a floxed allele upon Flipase-driven recombination (see figure 2A). *Styk1* fl/fl mice were crossed with *Ncr1-iCre* mice to generate NK-specific *Styk1* deletion. NK cells from these mice displayed normal development and distribution (data not shown).

Styk1 mRNA expression is lower in educated NK cells and is maintained by IL-15

We took advantage of the EGFP reporter allele to study the regulation of *Styk1* mRNA in different biological contexts. In C57BL/6 mice, Ly49C and the CD94/NKG2A receptor have been shown to interact with substantial affinity with self-MHC class I molecules, and NK cell subsets expressing these receptors are educated, *ie* they are more reactive than their non-educated counterparts (Fernandez et al., 2005; Joncker et al., 2009; Kim et al., 2005). We stained *Styk1^{EGFP/EGFP}* NK cells with Ly49C and NKG2A antibodies. As shown in Figure 4A, EGFP expression was significantly lower in educated Ly49C⁺NKG2A⁺ NK cells compared to uneducated Ly49C⁻NKG2A⁻ NK cells, an effect that appears to be mainly driven by NKG2A. Next, we cultured spleen cells from *Styk1^{EGFP/EGFP}* mice in the presence or absence of different cytokines or plate-bound antibodies directed against diverse activating receptors. In the presence of IL-15, EGFP expression was maintained while all other tested conditions resulted in a decreased EGFP expression (Figure 4B). We then tested the effect of cytokines or plate-bound antibodies on EGFP expression when *Styk1^{EGFP/EGFP}* spleen cells were cultured in the presence of IL-15. As shown in Figure 4C, none of the tested stimuli altered the expression of EGFP when IL-15 was

present. Taken together, these results show that *styk1* mRNA expression is lower in educated NK cells and is maintained by IL-15

Redundant role of Styk1 in NK cell function

To test the role of Styk1 in NK cell effector functions, we stimulated *Styk1*^{EGFP/EGFP} and control NK cells with cytokine cocktails, plate-bound antibodies against activating NK cell receptors, YAC1 tumor cells or PMA / ionomycin for 4 hours and measured their degranulation by staining for surface CD107a and their capacity to secrete IFN γ using intracellular staining. As shown in Figure 5A-B, all these stimuli triggered both NK cell functions at different levels, but no difference was observed between *Styk1*^{EGFP/EGFP} and control NK cells. No difference was observed either in the ability of *Styk1*^{EGFP/EGFP} and control NK cells to kill YAC1 cells in a standard cytotoxicity assay (data not shown).

Next, we challenged *Styk1*^{EGFP/EGFP} and control mice with MCMV Smith strain using a sub lethal dose for C57BL/6 mice. All *Styk1*^{EGFP/EGFP} mice also controlled the infection. We monitored IFN γ expression at day +2. At this time point, IFN γ production is mediated by NK cells in response to an array of innate cytokines produced by myeloid cells. Despite significant inter individual variation, no significant difference between *Styk1*^{EGFP/EGFP} and control mice was noted (Figure 5C). Taken together, these results suggest that Styk1 is dispensable for mouse NK cell anti-tumoral and anti-viral functions.

Discussion

Here, we show that *Styk1* is a specific marker of the NK cell lineage. In particular *Styk1* is not expressed by other ILC subsets such as ILC1s and NCR+ILC3s. This places *Styk1* above NKp46 in terms of specificity. Indeed, following our initial observation that NKp46 was a selective marker of the NK cell lineage [4], several reports challenged our conclusions by showing that rare subsets of T cells [5],[6] and ILCs [7],[8] also expressed NKp46. *Styk1* regulatory regions could therefore be used to drive NK-specific gene expression to create transgenic mouse models for the study of NK cells. In the present manuscript, we describe an EGFP reporter mouse, *Styk1*^{EGFP/EGFP} that can be used to track mouse NK cells and could be useful for NK cell developmental studies.

Cell specific transcripts usually encode for important cellular functions. For instance, *S1pr5* is essential to promote trafficking of NK cells throughout the body [3], and is likely essential for their anti-viral and anti-tumor functions; *Ly49H* encodes an activating NK cell receptor specific to NK cells is essential to promote activation and expansion of NK cells in response to MCMV infection [18]–[20]. A previous comprehensive transcriptomic study reported the identification of a set of transcripts that were enriched in NK cells compared to many other leukocyte populations [9]. Among the genes whose expression was uniquely increased in NK cells, several of them had not reported function in NK cells. These data suggested that a rich biology related to NK cells remained undiscovered. As our own unpublished analyses had pointed to *Styk1* as being specifically expressed in NK cells, we decided to generate a *Styk1* deficient mouse model.

Styk1 expression was regulated during NK cell maturation and was maximal in mature NK cells, starting at the CD11b⁺ CD27⁺ stage. The observation that *Styk1* mRNA expression was dependent on IL-15 *in vitro* fits with the recent report that *Styk1* is down regulated in *Runx3* deficient NK cells [21]. Indeed, this study showed that *Runx3* cooperates with ETS and T-box transcription factors to drive the interleukin-15-mediated transcription program during activation of NK cells. Educating receptors negatively influence *Styk1* expression, which together with the localization of the *Styk1* gene next to the NK complex constitutes a tight link between *Styk1* and NK cell receptors. However, *Styk1* does not influence expression of NK cell receptors as *Styk1*^{EGFP/EGFP} NK cells normally expressed all the NK cell receptors tested.

A pioneer study showed in stably expressing cell lines that Styk1 could concomitantly activate both MAP kinase and PI3K pathways [10]. This initial report was followed by other observations that confirmed activation of PI3K/Akt by Styk1 in other cell lines [17],[22],[23]. Styk1 overexpression leads to an increased glycolytic capacity, and decreases oxidative phosphorylation via PI3K/Akt induction [24]. These data were of particular interest when put in the context of our own previous observations of the importance of the Akt/mTOR pathway in the control of NK cell development, bioenergetic metabolism and IL-15 [25] or NK cell receptor-mediated activation [26]. This prompted us to assess the activity of the mTOR pathway and the effector functions of Styk1 deficient NK cells. We consistently observed a decreased basal level of pS6 and pAkt in freshly isolated *Styk1^{EGFP/EGFP}* NK cells compared to control NK cells, suggesting a role for Styk1 in the basal regulation of the Akt/mTOR pathway in NK cells. However, we failed to detect any developmental, differentiation or functional defect in NK cells in the absence of Styk1. Styk1 might therefore function in some specific contexts that were not tested in our study. Van Roosmalen and colleagues previously identified Styk1 as a kinase promoting tumor cell migration in a large siRNA-based screen for kinases and other molecules involved in this cellular process [27]. However, the normal distribution of NK cells in *Styk1^{EGFP/EGFP}* mice does not support such a role in NK cells.

In conclusion, we show here that Styk1 expression is a hallmark of NK cells and that Styk1 is not essential for NK cell development, activation and antiviral functions. Further studies are required to understand the role of Styk1 in NK cells but *Styk1^{EGFP/EGFP}* mice represent a novel and interesting for developmental studies of mouse NK cells.

Material and Methods

Generation of Styk1 deficient mice

A plasmid “KO-first” designed and made by the European Conditional mouse mutagenesis program (EUCOMM) and targeting the mouse *Styk1* gene was used (Clone PRPGS00082_B_F08). This plasmid contains 5' and 3' homology arms, and a gene trap cassette that allows the expression of the LacZ reporter cassette in place of *Styk1*. It also contains FRT and LoxP sites that allow Cre-mediated conditional targeting of *Styk1* following Flp-mediated recombination. We modified this vector using E/T cloning (GeneBridges) and replaced the LacZ coding sequence with the EGFP coding sequence (obtained from the Clontech plasmid pEGFP.N1). JM8.A3 ES cells (C57BL/6N) were transfected with this construct and G418-resistant clones were obtained. We checked for correct homologous recombination by PCR followed by southern blot using different probes. Chimeric mice were obtained following microinjection of ES cells into C57BL/6 blastocysts and germline transmission was monitored by PCR using different sets of primers encompassing different parts of the targeted locus. Plasmid and PCR primer sequences are available on request.

Animals

This study was carried in strict accordance with the French recommendation in the Guide for the ethical evaluation of experiments using laboratory animals and the European guidelines 86/609/CEE. Procedures including animals were approved by local ethics review board under ENS2015-014 agreement. All mouse strains were maintained in our animal facility.

Virus production and infection

MCMV-smith was propagated by infecting 3 weeks old Balb/c mice with 50 PFU of MCMV cultured until passage 2 on primary BALB/c mouse whole fetus cells. Briefly, salivary glands were collected on ice in 3% FCS DMEM, homogenized with an organ dissociator and centrifuged at 800g for 5 min at 4°C. Supernatant was collected and stored at -80°C

until use. Titers were determined by plaque assay using NIH-3T3 cells. Mice were infected intraperitoneally with 5×10^4 PFU of MCMV.

Flow cytometry

Blood was collected in 0.5M EDTA and red blood cells were lysed with ACK lysis buffer. Mononuclear cells were isolated from spleen and liver injected with complete RPMI 1640 5% FCS 0.4 mg/ml Collagenase IV (Serlabo Technologies, Vedène, France) and 0.1mg/ml DNase I (Roche), cut into small pieces and incubated at 37°C with 150RPM shaking for 30 min. After incubation, organs were homogenized and cells from the liver were purified using Percoll (GE Healthcare) density gradient separation. Gut cells were prepared as previously described [7]. Flow cytometry was carried out on a FACS Fortessa (Becton-Dickinson). Data were analysed using FlowJo (V10, Treestar). The following antibodies from eBioscience or BD-biosciences or Biolegend were used: anti-CD19 (ebio1D3 or 6D5), anti-CD3 (17A2 or 145-2C11), anti-NK1.1 (PK136), anti NKp46 (29A1.4), anti-CD11b (M1/70), anti-CD27 (LG.7F9), anti-CD122 (5H4 or TMb1), anti-CD127 (A7R34), anti-CXCR3 (CXCR3-173), anti-CCR6 (29-2L17), anti-Ly49D (4e5), Ly49E (CM4), anti-Ly49G2 (4D11), anti-Ly49H (3D10), anti-Ly49I (YLI-90), anti-NKG2ACE (20d5), anti-NKG2D (CX5), anti-KLRG1 (2F1), anti-CD146 (Me9F1), anti-Eomes (Dan11mag), anti-IFN- γ (XMG1), anti-CD107a (1D4B), anti-GR1 (RB6-8C5 or 1A8), anti-Ly6C (HK1.4), anti-TCRgd (ebioGL3), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD11c (N418), anti-T-bet (4B10), CD1d (NIH tetramer), MHCII (m5/114.15.2), F4/80 (BM8), CD49a (Ha31/8), CD49b (DX5), CD45.2 (104) and relevant isotype controls. Antibodies against phosphorylated proteins were from BD-biosciences: pAkt S473 (M89-61), or Cell Signaling Technologies: pS6 S235/236 (5316).

Functional analysis of NK cells

Splenic lymphocytes were prepared and cultured with cytokines (rmIL-15 50ng/ml; rmIL-12 25ng/ml from Peprotech and rmIL-18 5ng/ml from PBL), or on antibody coated plates (anti-NKp46, anti-NK1.1, anti-Ly49D all at 10 μ g/ml on Immulon 2HB plates) and Golgi-stop (1 μ l in 1.5ml; BD-Biosciences) in the presence of anti-CD107a for 4h. Surface and

intracellular stainings were then performed and IFN- γ production as well as CD107a exposure was measured by flow cytometry.

Semi-quantitative RT-PCR

We used High capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, USA) to generate cDNA for RT-PCR. PCR was carried out with a SybrGreen-based kit (FastStart Universal SYBR Green Master, Roche, Basel, Switzerland) or SensiFast SYBR No-ROX kit (Bioline) on a StepOne plus instrument (Applied Biosystems, Carlsbad, USA) or a LightCycler 480 system (Roche). Primers were designed using the Roche software. The following primers were used for QPCR: RT-MM-Styk1-F1;CTGGCTTCTGCCCCTAGAT, RT-MM-Styk1-R1:AAAGGCAGAGCCCTGGAC

Measurement of serum IFN γ

IFN γ level was measured in the serum using a commercial Elisa kit (DuoSet, RnD)

Statistical analysis

Data were analyzed with GraphPad Prism 6 software, using standard T-tests. Differences were considered to be statistically significant when $p < .05$ (* if $p < .05$, ** $< .01$, *** $< .001$, **** $< .0001$).

Acknowledgements

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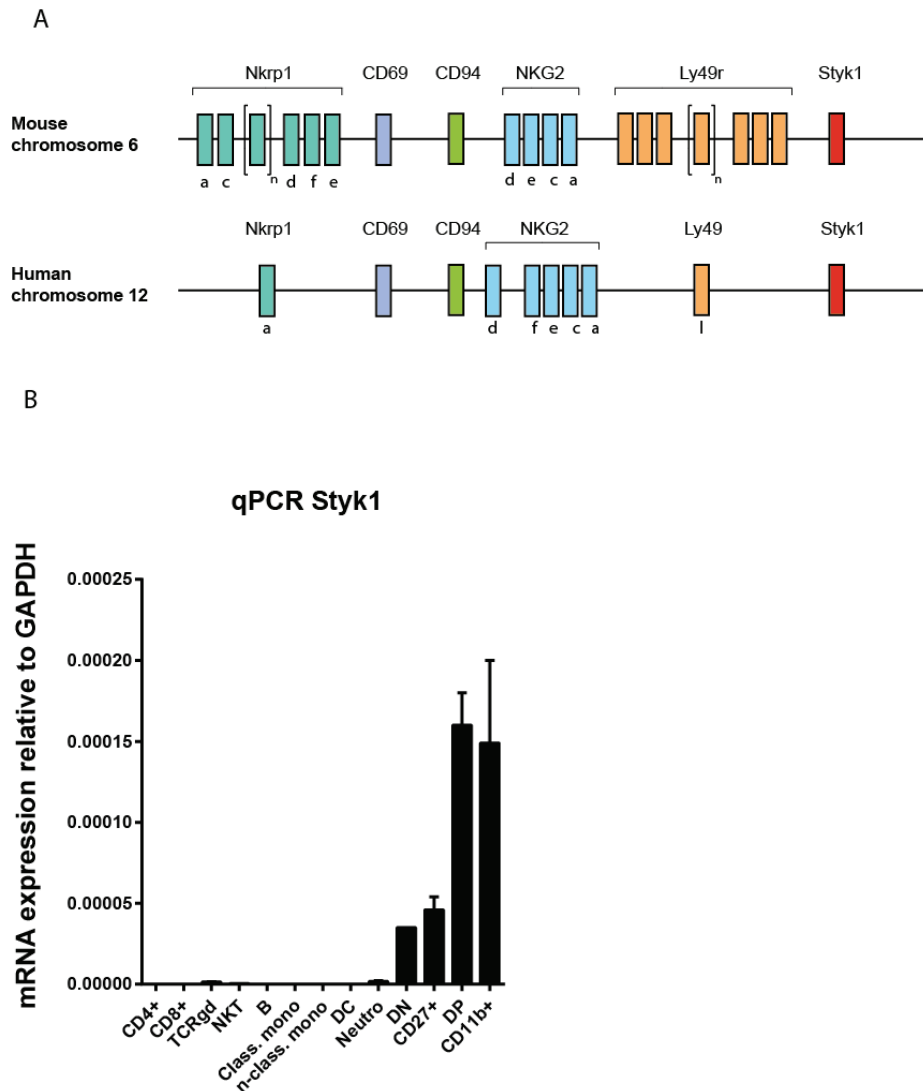
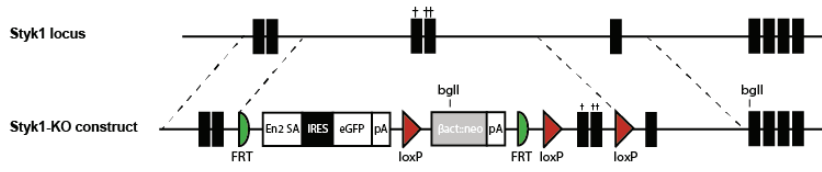


Figure 1: Styk1 mRNA is specifically expressed in mouse NK cells

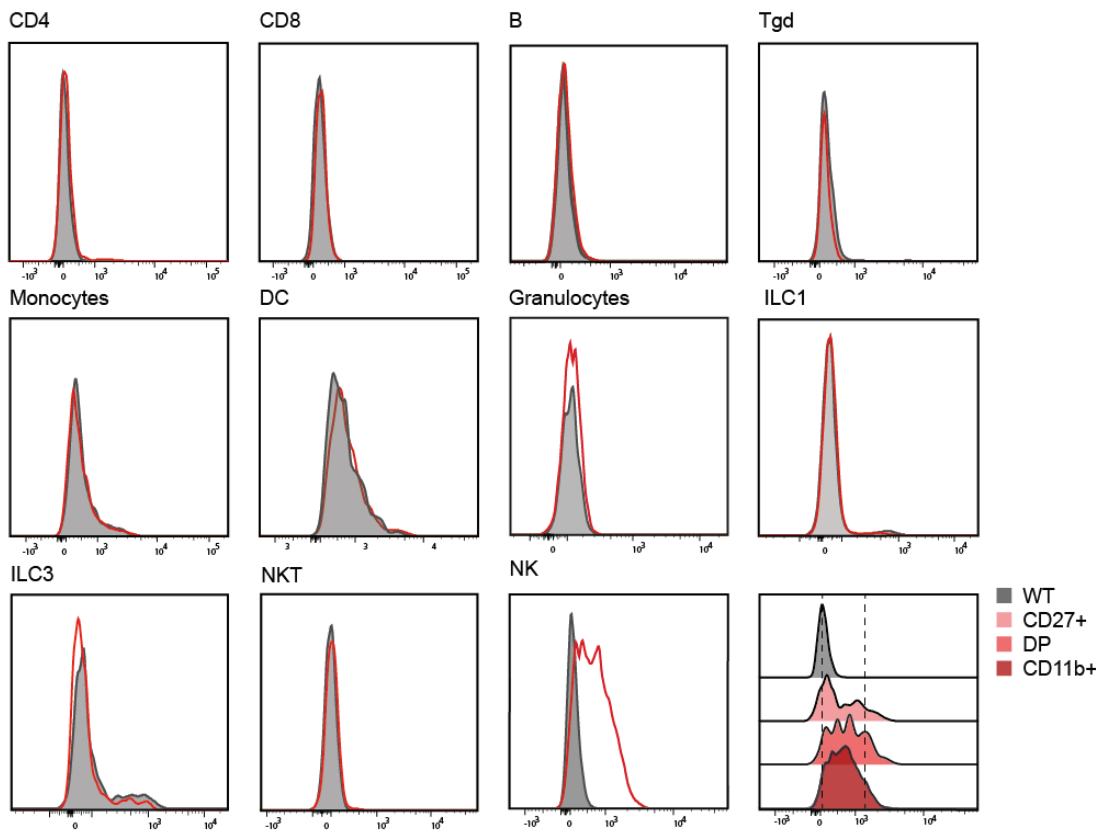
(A) Scheme of the NK complex in mouse and human showing localization of the Styk1 gene adjacent to the complex in both species. (B-C) RT-QPCR analysis of Styk1 expression in FACS sorted subsets., T cells as CD3⁺ CD19⁻ NK1.1⁻ (mouse), TCRgd as TCRγδ⁺ CD3⁺ NK1.1⁻ TCRβ⁻, NKT cells as CD1d⁺ NK1.1⁻ TCRγδ⁻ TCR β⁻, B cells were defined as CD19⁺ CD3⁻ NK1.1⁻, Classical monocyte as Ly6C^{hi} CD11b⁺ MHCII^{int or -} Lin⁻, n-classical monocyte as Ly6C^{low} CD11b⁺ MHCII^{int or -} Lin⁻, DC as MHCII⁺ F4/80⁺ Lin⁻, NK cells as NK1.1⁺ CD19⁻ CD3⁻. DN: CD27⁻ CD11b⁺, CD27⁺ are CD11b⁻, DP: CD27⁺ CD11b⁺, CD11b⁺ are CD27⁻. Data show representative results from three independent experiments.

A



Chromosome 6: 131,299,142-131,353,597
 †ENSMUSE00000318000
 †ENSMUSE00000317991
 Bact::neo : neomycine resistance cassette.
 En2 SA: Splicing site
 FRT: Flippase Recognition Target
 pA: poly adenylation site

B



C

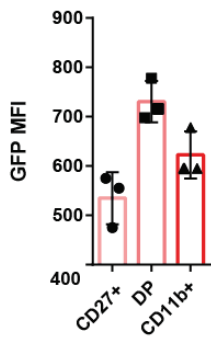


Figure 2: *Styk1* expression marks the NK cell lineage

(A) Scheme of the *Styk1* locus and of the targeting strategy. (B) Flow cytometry analysis of EGFP expression in various immune subsets from *Styk1^{EGFP/EGFP}* mice. B cells were defined as CD19+CD3-NK1.1-, CD4 T cells as CD3+CD4+TCRb+ NK1.1-, CD8 T cells as CD3+CD8+ TCRb+ NK1.1-, gd T cells as CD3+TCRgd+, NK cells as CD3-NK1.1+CD19-CD3-, monocytes as CD11b+ Ly6C+, granulocytes as CD11b+ Ly6G+, dendritic cells as MHCII+ CD11c+, ILC1 as liver Lin-NK1.1+CD49a+ CD49b-, ILC3 as gut CD3-CD127+CD49a+NKp46+CCR6+/- . (C) Flow cytometry analysis of EGFP expression in gated NK cells of different maturation status as defined by CD27 and CD11b expression. B and C show representative results of 3 independent experiments or more.

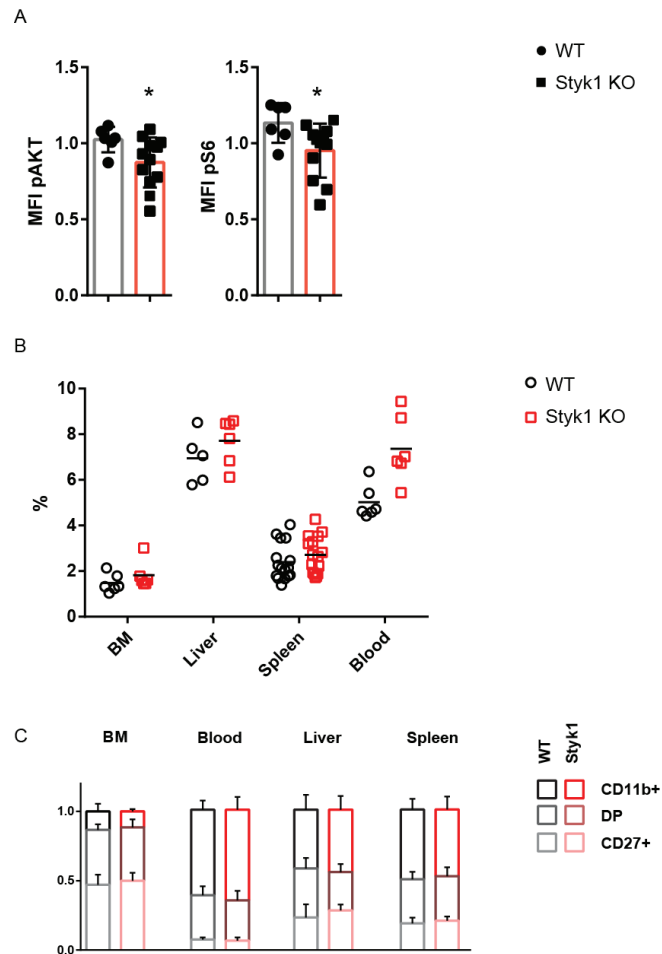


Figure 3: *Styk1*^{EGFP/EGFP} NK cells have a low basal mTOR activity but develop normally

(A) Flow cytometry analysis of pS6 and pAkt in gated NK cells from freshly isolated spleen cells. Data show normalized fluorescence intensity of the staining. Each dot corresponds to an individual mouse. **p <0.01 (Student T-test). (B) Percentage of NK cells in different organs of *Styk1*^{EGFP/EGFP} and control mice. N=6 or more mice for each organ. (C) NK cell maturation as defined by the flow cytometry analysis of CD27/CD11b expression in *Styk1*^{EGFP/EGFP} and control mice. N=6 or more mice. CD27+ is CD11b-, DP is CD27+CD11b+, CD27- is CD11b-.

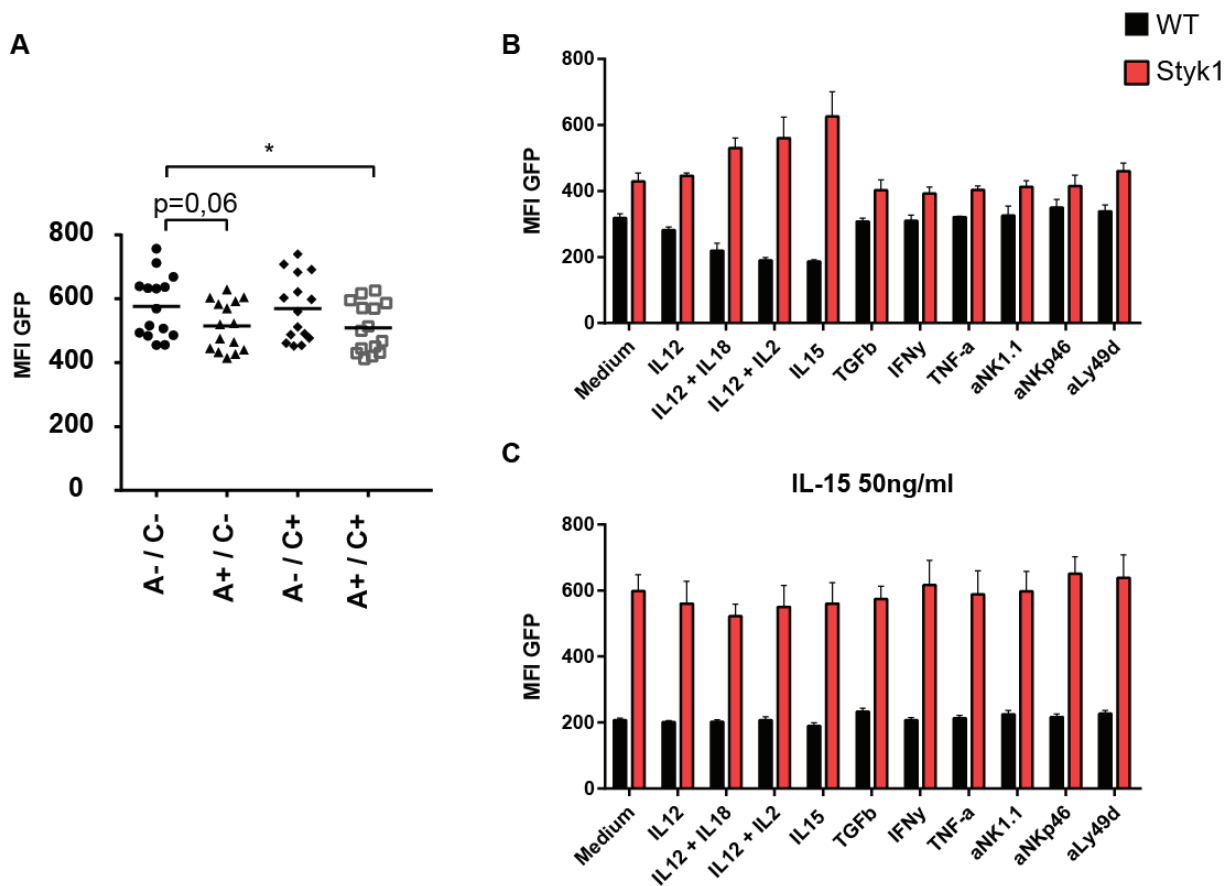


Figure 4: Styk1 expression is maintained by IL-15 in NK cells and is lower in educated NK cells

(A) EGFP expression in educated vs uneducated NK cells defined as NK cells co-expressing NKG2A (A) and Ly49C (C) and NK cells negative for both receptors, respectively. (B-C) Spleen cells from *Styk1^{EGFP/EGFP}* and control mice were cultured in the indicated conditions for 24h. EGFP expression was then measured in gated NK cells by flow cytometry. Histograms show mean fluorescence intensity of EGFP fluorescence. Data are pooled from the analysis of N=3 mice in each group.

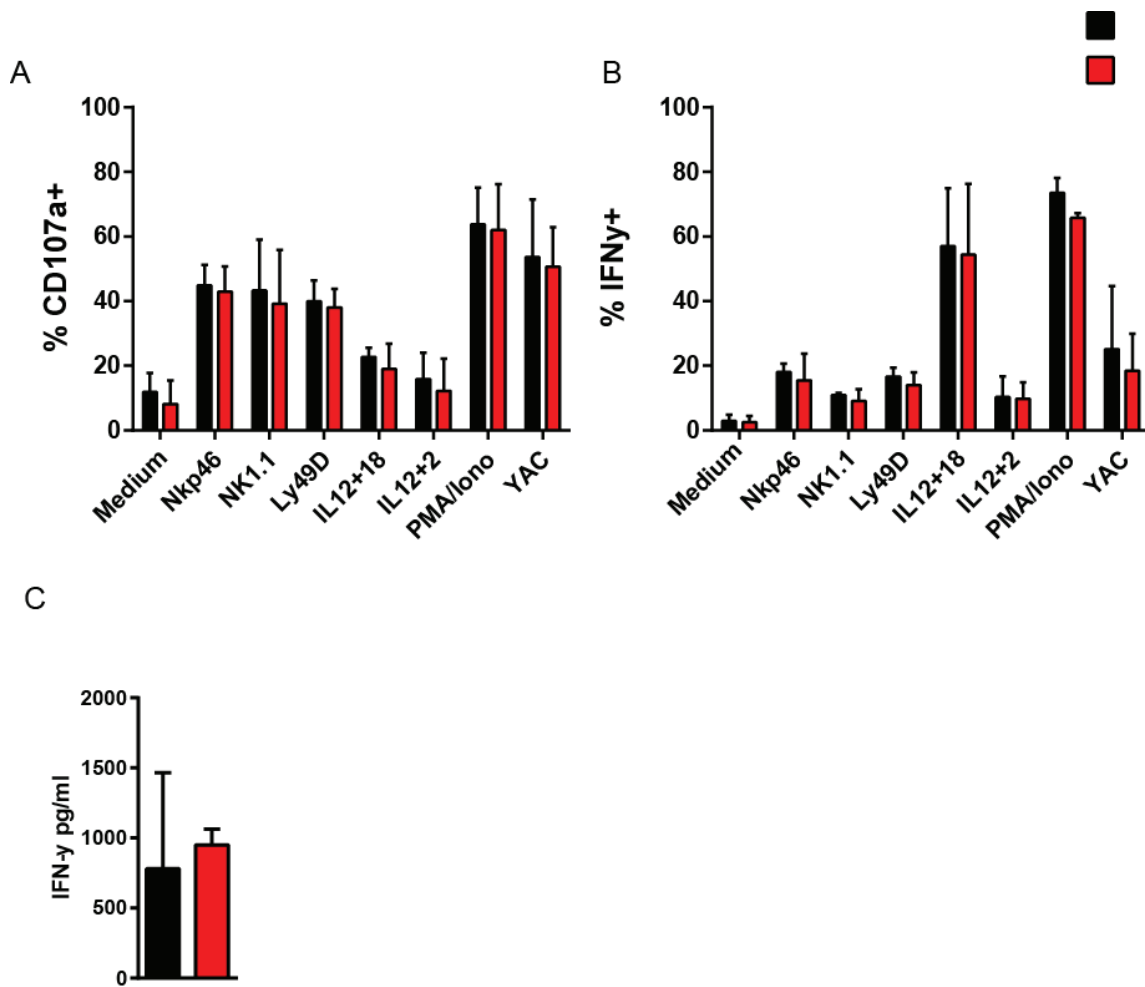


Figure 5: Normal *in vitro* and *in vivo* function of Styk1 deficient NK cells.

(A-B) Spleen cells from *Styk1^{EGFP/EGFP}* and control mice were stimulated in the indicated conditions for 4 hours. NK cell degranulation was measured by staining for surface CD107 and IFN γ secretion was measured by intracellular staining. Results are pooled from 8 mice and are representative from 2 independent experiments. (C) IFN γ levels measured by Elisa in the serum of the indicated mice 2 days post infection with MCMV.

Table I: Percentage of NK cells expressing the indicated NK cell markers in the spleen of *Styk1^{EGFP/EGFP}* and control mice.

	CD127	CXCR3	CD146	KLRG1	Ly49D	Ly49H	NKG2	Ly49E	Ly49I	Ly49G
<i>Styk1^{wt/wt}</i>	5.7 ±	54.0±	60.7±	29.2±	43.4±	49.4±	53.5±	8.3±1	48.5±	41.1±
<i>Styk1^{-/-}</i>	3.7±1	42.3±	64.9±	34.7±	48.3±	52.1±	49.2±	8.3±1	47.6±	42.3±

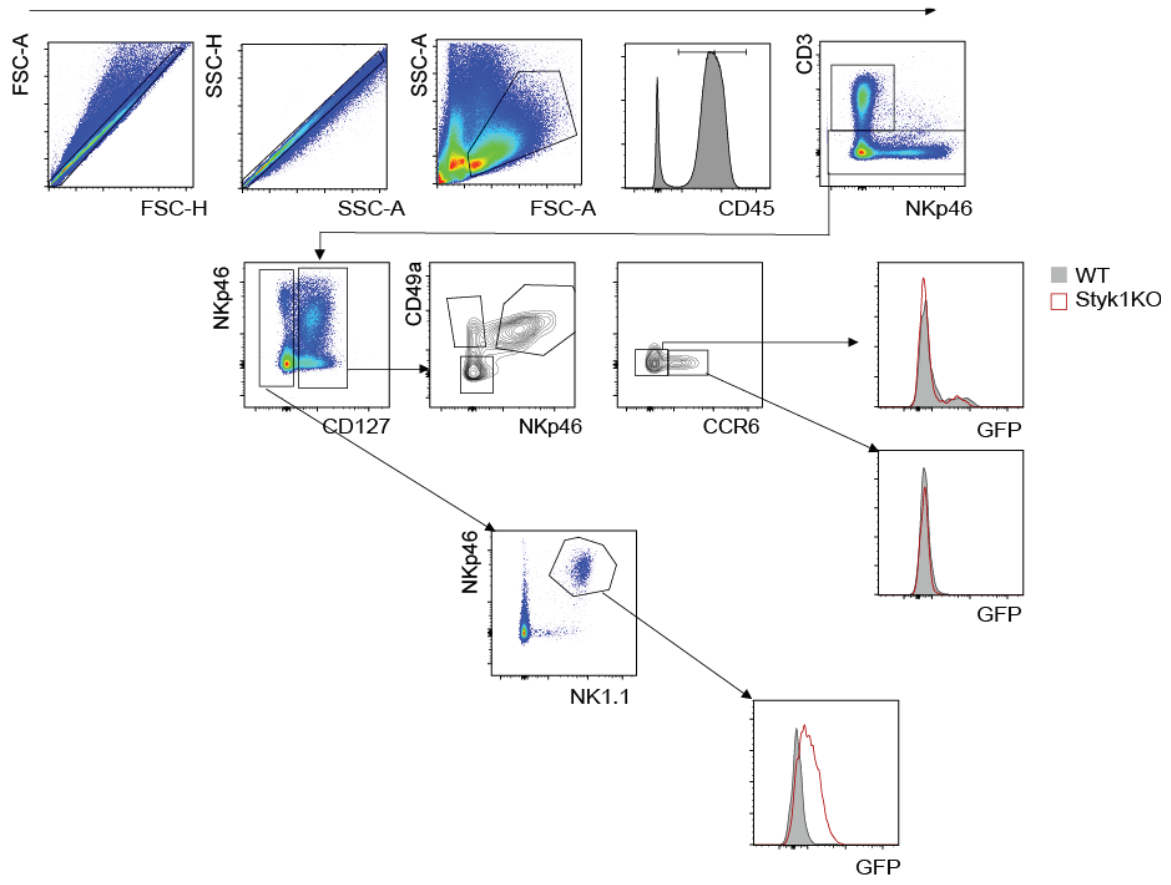


Figure S1: ILC3 gating analysis

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Discussion Section II :

Styk1 est une sérine/thréonine/tyrosine kinase dont la fonction demeure inconnue. Il a été décrit que son expression était augmentée dans une grande variété de cancers, mais son rôle dans le système immunitaire est incompris. Nous avons orienté notre intérêt vers cette kinase lorsque nous avons constaté, simultanément au consortium Immgen, que Styk1 faisait partie de l'empreinte transcriptionnelle des lymphocytes NK [154]. Subséquemment, la preuve formelle de son implication dans la voie PI3K-AKT a été faite dans le contexte du carcinome hépatocellulaire [157]. Or, comme nous l'avons abordé en introduction (*cf.* section 2.1.2), cette voie de signalisation occupe un rôle central dans 1) l'homéostasie des lymphocytes NK, 2) leur maturation et 3) l'intégrité de leur fonction effectrice. Dans l'article 2, nous présentons l'effet de l'inactivation du gène Styk1 sur le développement et la maturation des lymphocytes NK ainsi que leur distribution et l'intégrité de leurs fonctions effectrices.

L'expression de Styk1 est spécifique des lymphocytes NK

Dans un premier temps, nous avons confirmé par RT-qPCR ainsi que par l'expression d'un gène rapporteur, que l'expression de Styk1 est conscrée aux lymphocytes NK parmi les cellules immunitaires. Par ailleurs, Styk1 est exprimé au stade le plus immature CD27^{hi}CD11b^{low} démontrant une expression précoce lors du développement des lymphocytes NK. Cette expression atteint un maximum au stade intermédiaire CD27^{hi}CD11b^{hi} avant de diminuer au stade mature CD27^{low}CD11b^{low}. De façon anecdotique, ce patron d'expression suit le profil d'intégration du signal IL-15 par mTOR.

Styk1 est impliquée dans la signalisation basale de la voie AKT-mTOR

La signalisation de l'IL-15 est transduite par la kinase mTOR, un événement qui peut être mesuré par le niveau de phosphorylation de ses substrats dont la protéine ribosomale S6 – substrat du complexe mTORC1 – et la protéine AKT – substrat du complexe mTORC2 [108]. Nous avons observé que l'inactivation de Styk1, mène à la diminution des niveaux de phosphorylation d'AKT ainsi que de S6 à l'état basal. Toutefois, ce phénomène est annulé par la stimulation des lymphocytes NK avec l'IL-15. Il est donc plausible que la kinase Styk1 ait un rôle davantage orienté vers le maintien de l'homéostasie des lymphocytes NK que lors de leur stimulation. Son implication dans l'homéostasie, si elle est directe, serait en amont de mTOR. Malgré l'impact de la délétion de Styk1 sur la signalisation basale de la voie AKT-

mTOR, nous n'avons pas remarqué de différence dans la distribution ou encore la maturation des lymphocytes NK. Il est vrai que nous n'avons pas étudié plusieurs niches, notamment la peau, la décidua utérine ainsi que les surfaces mucosales. Il n'est donc pas exclu que l'inactivation de Styk1 ait un impact sur la maturation et la distribution des lymphocytes NK au sein des autres compartiments immunologiques. Néanmoins, le niveau de maturation des lymphocytes NK en fonction des organes était compatible avec ce qui était attendu. De façon globale, il semblerait donc que l'inactivation de Styk1 ait un impact nul sur la maturation et la distribution des lymphocytes NK. En absence de défaut de maturation, il est également peu probable que Styk1 puisse avoir un impact négatif sur les facteurs de transcriptions régissant le développement des lymphocytes NK tel que ID2 et TOX.

La transcription de Styk1 est impactée par l'éducation

Nous avons récemment démontré que mTOR constitue un rhéostat moléculaire de l'éducation des lymphocytes NK (cf. Annexe 2). En effet, au sein du complexe mTORC1, qui contrôle un ensemble de paramètres métaboliques nécessaires à l'homéostasie et la prolifération des NK, mTOR est plus actif chez les lymphocytes NK éduqués que chez leur contrepartie non-éduquée. L'éducation des lymphocytes NK est définie par l'acquisition de capacités effectrices grâce à l'engagement chronique de leurs récepteurs inhibiteurs. En conséquence, les lymphocytes NK exprimant NKG2A ou Ly49C, les seuls récepteurs inhibiteurs engagés chez la souris C57BL/6J, sont plus réactifs que leur contrepartie NKG2A-/Ly49C-. Afin de vérifier si l'expression de Styk1 est sous le contrôle de l'éducation des lymphocytes NK, nous avons analysé son niveau de transcription en fonction du niveau d'éducation. Les lymphocytes NK éduqués montrent paradoxalement un niveau de Styk1 moins élevé que leur contrepartie non éduquées. Ce résultat semble *à priori* contradictoire avec les niveaux de pS6 observés à l'état basal. Comment est-ce que l'absence de Styk1 impacte négativement les niveaux de pS6 à l'état basal alors que l'éducation diminue également sa transcription ? Une hypothèse pourrait être que Styk1 est surtout impliqué dans le maintien homéostatique des lymphocytes NK non-éduqués, ce que nous n'avons pas encore évalué. Styk1 et les récepteurs inhibiteurs sont localisés au sein du même complexe génétique, le NKC. Il est aussi plausible que l'expression de certains récepteurs inhibiteurs ait un impact d'exclusion ou d'inclusion génique sur Styk1. En effet, l'expression stochastique des récepteurs Ly49 est sous le contrôle d'interrupteurs nucléiques distaux [166]. Ces derniers gouvernent l'accessibilité des promoteurs des gènes Ly49. Il est tout à fait envisageable que l'induction de la transcription d'un des gènes du complexe

NK ait un impact favorable ou défavorable sur l'expression d'un autre, possiblement en révélant ou encore en dissimulant une région régulatrice de sa transcription.

L'expression de Styk1 dépend de l'exposition à l'IL-15

Nous avons vu que la transcription de Styk1 est principalement dépendante de l'IL-15. Cette observation semble cohérente avec la diminution de l'expression de Styk1 qui est rapportée chez la souris Runx3 -/- [167]. En effet, Runx3 est un facteur de transcription médiant l'expression de nombreux gènes sous l'influence de la signalisation par l'IL-15. En revanche, le maintien du pool d'ARNm est intimement lié à la survie cellulaire. En effet, la dégradation générale des ARNm codants est un évènement précoce de l'induction de l'apoptose [168]. Or, la survie des lymphocytes NK est intimement dépendante de l'IL-15 [169]. Ceci pourrait représenter un facteur confondant de ce résultat. Nous avons donc stimulé les lymphocytes NK avec d'autres cytokines, en présence et en absence d'IL-15. Cependant ni la stimulation par d'autres cytokines, ni l'activation des lymphocytes NK par la mobilisation des récepteurs activateurs ne semble impacter la transcription de Styk1. Cette kinase ne semble donc pas impliquée dans l'activation des lymphocytes NK. Cette observation est appuyée par une capacité de dégranulation essentiellement intacte en absence de Styk1. De plus, la capacité d'élimination des cellules YAC-1 n'est pas impactée par l'absence de Styk1.

Finalement, nous avons évalué le contrôle antiviral des souris Styk1 -/-. À l'instar des souris C57BL/6J, les souris Styk1 -/- survivent à l'infection par 3×10^4 PFU de MCMV. Par ailleurs, les niveaux d'IFN- γ , essentiellement issus des lymphocytes NK à j+2 p.i., sont comparables. Toutefois, nous observons une diminution presque totale du niveau de transcription de Styk1 suite à l'infection (Figure 2.8A). Ceci laisse supposer que Styk1 est régulée négativement lors du contrôle viral. Cette diminution était toutefois indépendante de l'expression du récepteur Ly49H, spécifique de l'épitope m157 de MCMV. La diminution globale de la transcription de Styk1 lors de l'infection à MCMV est donc un évènement qui semble être indépendant de la mise en place des fonctions effectrices. Pour confirmer ce résultat, nous avons injecté les souris Styk1 -/- avec du Poly:IC, un agoniste de TLR3 (Figure 2.8B). Ce composé favorise la présentation de l'IL-15 par les DC et de ce fait, l'activation des NK [170]. Très rapidement après le challenge, les NK montrent une diminution modérée de la transcription de Styk1, suggérant que cette kinase, même si elle n'est pas nécessaire à la mise en place des fonctions effectrices, est négativement régulée en conditions proinflammatoires.

Ainsi, il est possible que cette kinase soit impliquée lors de la prolifération des lymphocytes NK en conditions inflammatoires et qu'elle ait un rôle de régulation négative. Dans quelles conditions exactes cette régulation intervient ? Cette question demeure. Néanmoins, des expériences de chimérisme en condition d'infection seront informatives prochainement.

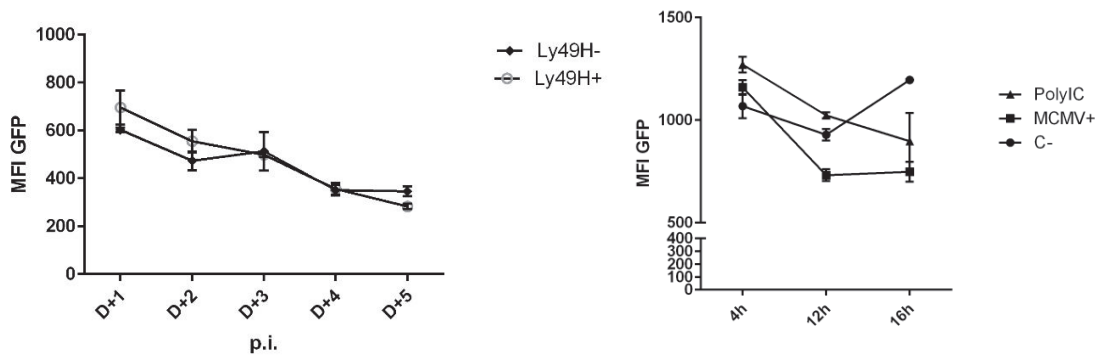


Figure 2.8 : Expression de Styk1 en contexte proinflammatoire

A) Niveau d'expression de l'ARNm de Styk1 chez les lymphocytes NK suite à l'infection par MCMV B) niveau d'expression de l'ARNm de Styk1 suite au challenge immunitaire par MCMV ou poly:IC

Pour conclure, la kinase Styk1 ne semble jouer aucun rôle dans le développement, la maturation des lymphocytes NK ainsi que l'acquisition de leurs capacités effectrices. Toutefois, sa transcription semble être sous l'influence de l'IL-15 et être négativement régulée en situations pro-inflammatoires par un mécanisme que nous ne connaissons pas. Alors qu'il est possible que cette kinase joue un rôle dans la prolifération des lymphocytes NK en conditions de challenge immunitaire, la démonstration expérimentale doit en être faite.

T-bet and Eomes in mouse and human NK cells and ILC1s

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Keywords: NK cell differentiation, T-box transcription factors

Abstract

T-bet and Eomes are T-box transcription factors that drive the differentiation and function of cytotoxic lymphocytes such as Natural Killer (NK) cells. Their DNA-binding domains are highly similar, suggesting redundant transcriptional activity. Yet, these transcription factors have different patterns of expression and the phenotype of loss-of-function mouse models suggest that they play distinct roles in the development of NK cell and other innate lymphoid cells (ILCs). Recent technological advances such as reporter mice and conditional knockouts have helped define the regulation and function of these factors at steady state and during pathological conditions such as cancer or infections. Here, we review these recent developments, focusing on NK cells as prototypical cytotoxic lymphocytes, and also discussing parallels between NK and T cells. We also examine the role of T-bet and Eomes in human NK cells and ILC1s. Taking into account divergent findings on mouse and human ILC1s, we propose here that NK cells are defined by co-expression of T-bet and Eomes while ILC1s express only one of these factors, either T-bet or Eomes, depending on the tissue or the species.

Introduction:

The T-box family of transcription factor (TFs) has been named after the discovery and cloning of the founding member Brachyury (short-tail in Greek) in a mouse line showing an altered tail development. T-box proteins harbor a similar sequence-specific DNA-binding motif, or T-box domain, which spans 180-200 amino acid residues. The T-box family of TFs is subdivided in five families classified on the basis of overall homology. Most T-box TFs have important roles during embryogenesis and development and are evolutionarily conserved. T-box proteins function as transcriptional repressors or activators and some T-box TFs have both activation and repression domains that can function in different cellular or promoter contexts¹.

Loss-of-function mutations in the genes encoding for T-box TFs have been identified in many human syndromes associated with developmental defects¹. T-bet and Eomesodermin (Eomes), respectively encoded by *Tbx21* and *Eomes* are the only T-box proteins expressed in the immune system. Their T-box DNA binding domains present a strong degree of identity, suggesting that they bind to the same sequences (**Figure 1**). By contrast, the sequence identity of other portions is quite divergent, suggesting that their interacting partners are distinct. Putative partners of T-bet and Eomes identified by high-throughput techniques support this hypothesis, as indicated in the reference database Biogrid².

T-bet and Eomes, discovery and role in T cells

In the immune system, T-bet and Eomes have been first studied in T cells, where their expression is inducible upon activation and differentiation. T-bet is the Th1-lineage-specifying transcription factor. It plays an important role in regulating IFN- γ production³. T-bet not only promotes Th1 cell differentiation but also prevents the development of other T helper cell lineages. Indeed, in the absence of T-bet, T helper cells adopt the functional features of Th2 or Th17 cells because they fail to repress their genetic programs. IL-12 induces T-bet in a STAT4-dependent manner⁴. IFN- γ sustains T-bet expression, which results in a positive feedback loop during Th1 cell differentiation (see review ⁵).

Eomes has been first studied in cytotoxic T cells (CTLs) where it was suggested to cooperate with T-bet to induce the expression of CD122, the beta chain of the IL-15

receptor and to repress Th17-type cytokines⁶. Eomes is expressed at lower levels in CD4 T cells when compared to CD8 T cells and its role in CD4 T cell functions remains ill-defined. Enforced expression of Eomes rescues IFN- γ production by T-bet deficient T cells. Moreover, Eomes overexpression in CD4 T cells promotes IFN- γ production⁷⁻⁹ and cytotoxicity¹⁰. Like T-bet, Eomes can also inhibit IL-5 production in memory Th2 cells¹¹ and repression of Eomes is necessary for Th17 cell induction¹². Therefore, most of the described roles for Eomes in CD4 T cells appear to be redundant with T-bet. Although it is generally assumed that T-bet and Eomes play comparable roles in T and NK cells, previous studies clearly showed context-dependent function of these TFs in epigenetic regulation¹³. The following sections discuss more specifically the role of T-bet and Eomes in NK cells and Innate Lymphoid Cells (ILCs).

NK cell development and maturation

NK cells are innate lymphocytes that contribute to the early defense against intracellular pathogens and to the immunosurveillance of tumors. They have been recently reclassified as members of group 1 ILCs and presented as the innate counterpart of cytotoxic CD8 T cells¹⁴. They are indeed endowed with perforin-dependent cytotoxic properties that can be enhanced upon activation by IL-15¹⁵. NK cells produce large amounts of IFN- γ rapidly after pathogen infection, as well as other cytokines and chemokines that have important roles during the early steps of the immune reaction¹⁶. This property is shared with other innate lymphocytes such as NKT cells, ILC1, and adaptive lymphocytes such as memory CD8 T cells that behave like innate lymphocytes during the first phases of infections¹⁷. The triggering of NK cell effector functions is controlled by inhibitory and activating NK cell receptors expressed at the cell surface. Most inhibitory receptors bind to MHC class I molecules. Hence, MHC-I deficient cells are recognized and killed by NK cells. Three families of NK cell receptors recognize MHC class I: the primate KIR, the murine Ly49 receptors, and the CD94-NKG2 receptors shared by both rodents and primates. All three families encode both inhibitory and activating family members¹⁸.

NK cells develop in the bone marrow (BM) from NK cell precursors deriving from common innate lymphoid precursors¹⁹. In mouse, acquisition of the NK1.1 epitope marks their commitment to the NK cell lineage. Next, they undergo a sequential maturation program

that includes three discrete steps marked by surface levels of CD27 and CD11b. CD11b⁻ CD27⁺ NK cells express high levels of NKG2A and low levels of Ly49 receptors. They are mostly represented in BM and lymph nodes (LN). Upon acquisition of CD11b, NK cells massively proliferate in the BM²⁰. CD11b⁺ CD27⁺ and CD11b⁺ CD27⁻ correspond to mature NK cells mainly found at the periphery, displaying the full repertoire of Ly49 receptors, and having the highest cytotoxic potential^{21,22}. At the CD11b⁺ CD27⁺ stage, NK cells acquire high expression of S1P5 that induces their exit from the BM to the periphery²³. S1P5 binds sphingosine-1 phosphate (S1P) that is carried by albumin and different lipoproteins present at high concentration in the blood and in the lymph. In parallel, they also acquire the expression of CX3CR1²⁴ which ligand, fractalkine, is expressed on endothelial cells, and progressively lose the expression of CXCR3 and CXCR4²⁵ the former being important for the entry into inflamed LN, while the latter retains NK cells in the BM. This coordinated switch in chemotactic receptors impacts homing, mature NK cells becoming mostly blood circulating while immature NK cells are rather found within LN, BM or non-lymphoid tissues such as the liver. Terminal maturation is also marked by the acquisition of other receptors such as KLRG1²⁶ and CD146²⁷.

Regulation and role of T-bet and Eomes during NK cell development

Early NK cell development

Common ILC precursors lack T-bet and Eomes but express Id2, as shown using Id2 reporter mice²⁸. Upon commitment to the NK cell lineage, the beta chain of the IL-2/15 receptor (CD122) is induced. This event occurs before the induction of NK cell receptors, and defines the NK cell precursor (NKp)²⁹ stage. Runx3 regulates Eomes expression in CD8 T cells and is also expressed in NKp as demonstrated using a Runx3-reporter mouse model³⁰. Other factors such as NFIL3^{31,32} Ets-1³³, and TOX³⁴ may help inducing T-bet and Eomes expression as both TFs are expressed when cells progress to the immature CD11b⁻ CD27⁺ stage. However, T-bet expression remains low until NK cells are released from the BM to the periphery. Interestingly, T-bet expression is actively repressed in the BM and mature NK cells recirculating to this compartment rapidly decrease their T-bet expression³⁵. This repression may be important to promote NK cell turnover as T-bet deficient NK cells display a high proliferation rate³⁶.

Regulation of CD122 and NK cell proliferation

At the NKp stage, T-bet and Eomes are weakly expressed in the BM, suggesting that CD122 induction is not firmly dependent on T-bet and Eomes during commitment to the NK cell lineage, as it is the case of CD8 T cells⁶. Consistently, CD122^{hi} NKp and a few NK cells are present in mice deficient for both Eomes and T-bet⁴⁵. However, T-bet and Eomes are essential for correct regulation of CD122 in mature NK cells. CD122 expression is higher in T-bet deficient NK cells, along with Eomes³⁷. As CD122 expression is a rate-limiting factor for the use of IL-15, this suggests that Eomes is particularly important to promote NK cell proliferation via the induction of CD122, which would explain why there are so few NK cells in the absence of Eomes. These data may also suggest that T-bet represses CD122 expression in NK cells. However, CD122 is also expressed in NKT cells or ILC1s that express T-bet but not Eomes. To reconcile these seemingly contradictory observations, we propose a refined model of CD122 expression control in which both Eomes and T-bet would be capable to induce CD122, the former being more potent than the latter to transactivate CD122 expression, both TFs competing for the same binding sites. As T-bet represses Eomes expression, this model would explain why T-bet deficiency leads to high expression of both Eomes and CD122³⁵ and high NK cell proliferation³⁶ (**Figure 2**).

Eomes promotes NK cell development and maturation

The role of Eomes in NK cell development has been recently addressed, when Eomes floxed mice became available. In Vav1-Cre x Eomes^{fl/fl} mice, NK cell development is severely impaired, as evaluated by the absence of NK1.1 or NKp46 harboring NK cells³⁸. In the absence of Eomes, residual NK cells were originally considered as immature, but probably include many ILC1s. Since the conventional NK cell lineage is classically defined by Eomes expression, the discrimination between conventional but immature NK cells and ILC1s remains problematic in Eomes deficient mice. Despite this fact, most NK1.1⁺ cells in Eomes deficient mice express low amounts of CD11b and other maturation markers, suggesting that Eomes is required not only for NK cell development but also for NK cell maturation. Besides, transgenic expression of Eomes in ILC1s is sufficient to

induce some attributes of conventional NK cells such as CD49b expression³⁹. In the absence of Eomes expression (ILC1s or Eomes deficient NK cells), Ly49 receptors are weakly expressed, implying an important role of Eomes in inducing the expression of these receptors or in opening the chromatin conformation at the Ly49 locus (leukocyte receptor complex). During MCMV infection, Eomes deficient Ly49H⁺ NK cells can expand to some extent, suggesting that inflammatory conditions may partially compensate for the lack of Eomes in NK cell expansion³⁸. This proliferation may be promoted by IL-12⁴⁰ as this cytokine is a potent inducer of T-bet. In turn, T-bet may promote CD25 (IL-2R α) expression on NK cells⁴¹. CD25 expression allows NK cells to use CD4 T cell-derived IL-2⁴² to sustain their proliferation upon infection, a process limited by regulatory T cells⁴³ which express high levels of CD25.

T-bet promotes terminal maturation by cooperating with Zeb2

T-bet is required for terminal NK cell maturation; in the absence of T-bet, NK cells express less CD11b and the sialoglycoprotein CD43 and more the stem cell factor receptor c-Kit³⁶. They also fail to down regulate CD27⁴⁴ and to up regulate KLRG1 expression⁴⁵. As discussed above, T-bet is essential to limit NK cell proliferation, likely through the control of CD122 expression. T-bet also promotes viability through unknown mechanisms. Moreover, T-bet controls NK cell trafficking; in the absence of T-bet, NK cells accumulate in the BM and within LN. This may be caused by a decreased expression of S1P⁵⁴⁶, which is involved in NK cell exit from the BM and LN²³. Increased expression of chemokine receptors CXCR3 and CCR2 in the absence of T-bet may also contribute to this phenomenon³⁷. Reduced numbers of NK cells in the spleen and lung of T-bet deficient animals may account for the reduced regional control of B16 melanoma⁴⁷ or *Toxoplasma Gondii*⁴⁸ upon challenge.

In an effort to identify other TFs involved in NK cell maturation, we recently showed that the Zinc finger containing protein Zeb2 was also essential for NK cell maturation³⁷. Zeb1 and Zeb2 are TFs that can induce epithelial-to-mesenchymal transition (EMT), an important process for embryonic development and for tumor metastasis⁴⁹. Zeb1 has been previously shown to be involved in NK cell development⁵⁰ whereas Zeb2 expression is strongly induced during NK cell maturation. The phenotype of T-bet and Zeb2 deficient

NK cells was very similar, suggesting a cooperation between these factors in NK cell maturation. T-bet was found to induce Zeb2, and Zeb2 was important to sustain T-bet transcriptional activity, in a classical feed-forward loop (**Figure 3**). Overexpression of Zeb2 in T-bet deficient mice partially restored NK cell maturation, suggesting that some of the roles attributed to T-bet may be indirect via Zeb2³⁷. Blimp1 may also be part of the T-bet network, as a previous study placed this TF downstream of T-bet⁵¹. T-bet deficiency leads to attenuated IL-15-dependent Blimp-1 expression, hence controlling the peripheral maturation and proliferation of NK cells⁵¹. Blimp1⁵² and Zeb2^{53,54} are also essential players in the control of CD8 T cell differentiation, which highlights the similarity in the transcriptional mechanisms controlling NK and CD8 T cells. In effector CD8 T cells, in the absence of Zeb2, T-bet binding is altered on many promoters, suggesting that T-bet and Zeb2 act together and co-regulate the expression of several target genes. In few cases, Zeb2 promotes T-bet binding which results in promoter transactivation, while in most cases Zeb2 prevents T-bet binding, thereby repressing the expression of the target gene^{53,54}.

Control of T-bet to Eomes balance

In the periphery, Eomes expression progressively decreases during NK cell differentiation, while T-bet expression steadily increases. This is reminiscent of effector CD8 T cell differentiation⁵⁵ and indicates that high T-bet to Eomes ratios may favor differentiation over proliferation. Whether T-bet and Eomes levels are controlled by extrinsic signals in NK cells at steady state is unknown. Many cytokines and other signals regulate their expression upon activation. For example, IL-12 is a well-described inducer of T-bet in NK cells³⁶, while Eomes expression is induced by strong IL-2 signaling in T cells⁵⁶. IL-4 and type I IFN have also been shown to induce Eomes expression in innate memory T cells^{57,58}, even though they have limited effect on Eomes expression in NK cells (TW, unpublished data).

The immunosuppressive cytokine TGF- β negatively regulates both T-bet and Eomes in NK cells, with a more pronounced effect on T-bet⁵⁹. This effect could be mimicked by rapamycin, a specific inhibitor of the mTORC1 complex, which highlights the role of mTOR complex in T-bet and Eomes regulation. In CTLs, IL-12 induces a strong mTOR

activation, which is required to induce T-bet, while mTOR inhibition decreases T-bet and leads to high Eomes expression⁶⁰. At late stages of MCMV infection, inhibition of mTOR or activation of AMPK increased autophagic activity in NK cells and enhanced memory NK cell numbers through an Atg3-dependent mechanism⁶¹. Similarly, deficiency in Rictor, a key component of the mTORC2 complex leads to enhanced memory T cell formation upon infection and higher Eomes expression, suggesting that mTORC2 induces T-bet and represses Eomes⁶². mTORC2 may act via Foxo1 and Foxo3 which are known regulators of T-bet in T cells⁶³ and NK cells⁶⁴. The precise interplay between cytokines, mTOR and T-box factors in NK cells remains however to be fully elucidated. Subtle variations in T-bet/Eomes ratios may also implicate microRNAs that have a strong impact on NK cell maturation⁶⁵.

Genome wide analyses of T-bet and Eomes binding

Our understanding of the role of T-bet and Eomes in NK cell transcription and development is mainly based on the comparison of gene expression between control and T-bet deficient NK cells on one hand³⁷ and between Eomes negative ILC1s and conventional Eomes positive NK cells on the other hand^{35,66}. Chromatin immunoprecipitation (ChIP) and sequencing (ChIPseq) experiments have been performed for T-bet in mouse^{67,53,68} and human⁶⁹⁻⁷¹ T cells and Eomes for human embryonic stem cells⁷² but only one study reported the results of a T-bet ChIPseq experiment in NK cells⁷³, with limited analysis of the results. The context-dependent binding characteristics of these TFs makes uncertain to assume that T-bet and Eomes operate the same way in T and NK cells. Moreover, T-bet and Eomes likely influence each other's binding to DNA, and a clear picture of their respective transcriptional activities should take into account the level of expression of each of these TFs during NK cell differentiation or activation. Genome wide analyses in different NK cell subsets will be important to identify the mechanisms of T-bet and Eomes action in NK cells, and in particular if they compete for the same motifs during development, maturation and activation.

T-bet and Eomes in NK cell effector functions

Cytokine secretion

In T cells, IFN γ , the hallmark of Th1 cytokines, is directly induced by T-bet as shown by ChIP followed by microarray or luciferase reporter assay^{3,13}. Cooperating physically with both the histone H3K27-demethylase and the histone H3K4-methyltransferase⁷⁴, T-bet promotes IFN γ transcription through its interaction with RbBp5 and JMJD3^{75,76}. T-bet facilitates CTCF binding and chromatin looping respectively at +1kb and +66kb loci⁷⁷ (**Figure 3**). Yet, in NK cells, IFN γ expression is surprisingly normal in the absence of T-bet⁷⁸, except for a somewhat reduced ability to produce this cytokine in response to IL-12 and IL-18³⁶. Reciprocally, Eomes deficient NK cells expressed IFN γ at near-normal levels upon stimulation with IL-12 and IL-18³⁸. Although T-bet and Eomes might have important roles in driving IFN γ expression in specific contexts, the current data suggest that other TFs may be sufficient to promote IFN γ expression in NK cells in response to cytokines and to remodel the IFN γ locus during their differentiation. The recent identification of Eomes negative ILC1s (see below) has prompted their functional comparison with Eomes positive NK cells. The finding that, in addition to IFN γ , ILC1s can produce larger amounts of TNF, IL-2 and GM-CSF³⁵ than NK cells implied that Eomes may be important to further specify the NK cell lineage and restrict the range of cytokines they can produce.

Cytotoxicity

T-bet is dispensable for NK cell cytotoxic activity *in vivo*³⁶. By contrast, Eomes seems important to acquire full cytolytic machinery. Indeed, Eomes may regulate the expression of several NK cell receptors such as the Ly49 receptors in mouse³⁸ and also activates transcription of *Gzmb* and *Prf1*^{6,79,80}. As previously shown for memory CD8 T cells, Eomes could facilitate histone acetylation at the *GZMB* and *PRF1* locus in NK cells⁸¹. Accordingly, Eomes negative ILC1s express lower levels of granzymes and perforin compared to conventional NK cells³⁵. This observation has led to the proposal that NK cells constitute the cytotoxic arm of the ILC family while all other subsets are rather “helper” cells¹⁴. Yet, mice in which Eomes is deleted in NK cells seem to normally eliminate adoptively transferred MHC-I deficient cells³⁸, suggesting that ILC1s may retain *in vivo* cytotoxic activity.

Migration / trafficking

As previously discussed, S1P5 is the dominant S1P receptor in NK cells and promotes egress from LN and BM²³. In CD4 and CD8 T cells, T-bet acts directly on S1P5 expression by binding a conserved element 3' of S1P5 gene⁴⁶ and also an upstream region⁵³. Whether T-bet behaves the same way in NK cells needs to be further illustrated. The promoter region of another chemokine receptor CXCR3, which is involved in NK cell entry to inflamed LNs⁸², was recognized as a direct binding site for T-bet in NK cells^{13,83}.

T-bet and Eomes in the development of ILC1s

The existence of a peculiar NK cell population in the mouse liver has been recognized for many years. These cells were originally defined on the basis of their high expression of Trail, their low expression of CD49b (the target of the DX5 antibody) and their abundance in newborn mice⁸⁴. Although they were initially described as precursors of classical Trail-DX5⁺ NK cells^{38,84}, they are now defined as a separate ILC1 lineage, dependent on T-bet but not Eomes, the latter not being expressed in mouse ILC1s^{35,66}. Hepatic ILC1s are tissue-resident cells and do not depend on NFIL3 for their development. Given the plasticity of ILC populations, hepatic ILC1s might differentiate into NK cells under inflammatory conditions, even though this would require a strong induction of Eomes, which expression is opposed by T-bet³⁵. Multiple other ILC1s populations have been described in the gut, in the salivary gland, in the uterine tissue or in fat pads that resemble hepatic ILC1s, with some tissue-specific characteristics⁸⁵. By analogy with hepatic ILC1s, these cells may differentiate *in situ* and reside within tissues. Tissue-resident ILC1s all express T-bet at high levels and are presumably dependent on this factor for their development. As discussed above, transgenic expression of Eomes in ILC1s converts them at least in part into conventional NK cells³⁹. Reciprocally, transgenic expression of T-bet promotes the development of ILC1s in organs where they normally do not develop such as the BM, at the expense of NK cells³⁵. This highlights the role of the T-bet to Eomes ratio in the early commitment of progenitors to the ILC1 versus NK cell lineages. Tissue-specific factors may favor one TF over the other to promote either lineage

development. While the global absence of ILCs may not lead to overt immune deficiencies⁸⁶, recent studies have highlighted a possible role of ILC1s in the defense against pathogens and in anti-tumor responses^{87,88}.

T-bet and Eomes in human NK cells and ILC1s

Human ILC1s have been first identified in mucosal tissues as T-bet⁺ Eomes⁻ ILCs capable of producing Th1 cytokines but devoid of cytotoxic properties⁸⁹. However, a series of articles described that the human liver contains a large ILC population that resembles in many aspects the mouse hepatic ILC1 population⁹⁰⁻⁹³ but express high levels of Eomes and not T-bet. These cells also express high levels of CXCR6 and CD69, express less KIR receptors and appear to be less cytotoxic than conventional NK cells. Interestingly a recent study showed that NK cells with a similar CXCR6⁺ Eomes^{hi} Tbet^{lo} phenotype were present in several human fetal organs including the BM, the lung and the spleen⁹⁴. The authors further showed that Eomes^{hi} Tbet^{lo} NK cells appeared before Eomes^{lo} Tbet^{hi} NK cells during *in vitro* cultures of hematopoietic stem cells. Moreover, when analyzing NK cell maturation stages on the basis of CD34/CD117/CD94/CD16 expression as previously described⁹⁵, immature NK cells were found to express high Eomes expression with levels decreasing along with maturation. Altogether, the authors proposed that CXCR6⁺ EOMES^{hi} TBET^{lo} NK cells correspond to immature NK cells, and that, like in the mouse, NK cell maturation is coupled with a decrease in Eomes and an increase in T-bet expression⁹⁴. In this model, ILC1s would be precursors of mature NK cells, and this differentiation would be restricted by tissue-specific factors in organs like the liver. Such a differentiation would also change homing and trafficking properties, since NK cells are blood-circulating cells while ILC1s are tissue resident. To reconcile divergent findings, we propose that co-expression of high levels of T-bet and Eomes defines mature NK cells, that are mainly found within the blood circulation and the spleen, while ILC1s are defined by single expression of T-bet or Eomes, depending on the tissue (**Figure 4**). Further work will be required to precise the relationships between NK cells and ILC1s in all tissues.

Another question regards the relationship between hepatic NK/ILC1s and memory/adaptive NK cells⁹⁶, that are known to home to the liver in mouse^{97,98}. A

population of CD49a⁺ EOMES⁻ TBET⁺ NK cells with clonal-like properties has been identified in the human liver and might correspond to liver-homing adaptive NK cells⁹⁹.

T-bet and Eomes regulation and role during infections

T-bet and acute infections

Interest in the role of T-bet during infection took place when it was initially described that this TF regulates Th1 polarization and IFN- γ production by both T cells and NK cells³. In turn, IFN- γ up regulates T-bet in a positive feedback loop. One could argue that the concomitant skewing of Th1 commitment and dampened T cell effector functions observed in *Tbx21*^{-/-} mice participate to the differential susceptibility to several pathogens that was observed in numerous studies. Indeed, T-bet is required for the control of *Mycobacterium tuberculosis*¹⁰⁰, *Salmonella typhimurium*¹⁰¹, Vaccinia virus¹⁰² and *Toxoplasma gondii*⁴⁸. During the acute phase of infection with *Toxoplasma gondii*, T-bet is strongly induced in both T cells and mature NK cells⁴⁸. Whereas MCMV infection also leads to T-bet induction in NK cells¹⁰³, *Tbx21*^{-/-} mice show no alteration of viral control. MCMV resolution being NK dependent, this implies that NK cells harbor sufficient residual anti-viral activity in the absence of T-bet. The increased susceptibility to other pathogens observed in *Tbx21*^{-/-} mice may not be only due to impaired cytotoxic lymphocyte responses. Indeed, T-bet was also shown to be induced in B-cells and dendritic cells after IFN- γ stimulation¹⁰⁴ promoting respectively Ig class switch and Th1 priming.

Eomes and acute infections

Little knowledge regarding the role of Eomes was acquired in the context of infections, since Eomes floxed mice were only recently obtained¹⁰⁵ and because Eomes deficient mice are not viable¹⁰⁶. Reiner and colleagues showed that in the absence of Eomes expression in hematopoietic cells, Ly49H positive NK cells are still capable to expand to some extent upon infection with MCMV³⁸. These Ly49H positive cells also up regulate CD11b and KLRG1 expression, albeit at lower levels than control NK cells. These data suggest that in the context of acute infections, other TFs may partially compensate for the loss of T-box factors in NK cells, likewise to T cells⁵⁵. However, further studies are required to address this point in more details.

Chronic infections

NK cell effector functions have been shown to be impaired during several chronic infections, leading to the concept of NK cell “exhaustion”, as recently reviewed¹⁰⁷. The regulation and role of T-box TFs have not been addressed in this context. By contrast, an abundant literature is available on the transcriptional landscape associated with T cell dysfunction during chronic infections (reviewed in ^{108–111}) and is discussed here considering the similarities between CD8 T cells and NK cells. T-bet is down-regulated in virus-specific CD8 T cells¹¹² during chronic infection, resulting in severe exhaustion, a state of T cell dysfunction. Using the mouse LCMV chronic infection model, it has been demonstrated that low expression of T-bet leads to a high expression of the inhibitory receptor PD-1, since T-bet directly suppresses PD-1 transcription. By contrast, the expression of Eomes is elevated in exhausted CD8 T cells¹¹³. Two subsets of exhausted CD8 T cells can thus be identified in chronically infected mice based on the expression of T-bet and Eomes: Tbet^{high} Eomes^{low} T cells that give rise to Tbet^{low} Eomes^{high} T cells. The T-bet^{high} subset is also characterized by an intermediate expression of PD-1 whereas the Eomes^{high} subgroup displays a higher expression of this immune checkpoint. In human, during HIV chronic infection, the balance between T-bet and Eomes also regulates the CD8 T cell exhaustion process and the majority of HIV specific CD8 T cells display a TBET^{dim} EOMES^{high} expression profile¹¹⁴. As shown in the LCMV model, high EOMES expression is associated with poorly functional T cell phenotype. Interestingly, this elevated expression is not driven by stimulation with HIV antigens but rather by the level of inflammation^{115,116}. T-bet deficiency is also observed in virus-specific CD8 T cells from patients with chronic HBV or HCV infection¹¹⁷ compared to those with acute infection. This indicates that T-bet is required and essential for a successful and appropriate T cell response and to avoid the evolution into chronicity. T-bet has been shown to repress many type-I-IFN stimulated genes in T cells⁶⁸ and persistent production of type-I IFN has been implicated in promoting T cell exhaustion, as recently reviewed¹¹⁸. It is therefore tempting to speculate that high TBET expression is important to counter the negative effects of type-I IFN on T cell function. Further studies will be needed to test if this also applies to exhausted NK cells in the context of chronic infection. A recent paper

described that exhausted NK cells in HBV individuals express lower levels of T-BET mRNA than NK cells from healthy donors¹¹⁹, which is a first hint that similar exhaustion mechanisms may be at play in NK and T cells during chronic infections.

T-bet and Eomes regulation and role in anti-tumor responses

Tumor immune-surveillance is severely impaired in *Tbx21*^{-/-} mice, as firstly shown in the transgenic adenocarcinoma mouse prostate model¹²⁰. Although the absence of T-bet did not impact the response against the primary tumor, T-bet was required to suppress metastasis development. This indicates that T-bet regulates the immune response against late steps of cancer development. These results were confirmed in a metastatic melanoma model⁴⁷. Adoptive transfer of T-bet sufficient NK cells in *Tbx21*^{-/-} animals restored the anti-tumor response and conferred protection against melanoma. Moreover, as discussed above, T-bet and Zeb2 co-regulate multiple target genes and Zeb2 in NK cells has been shown to be essential for the control of metastatic melanoma³⁷. Animals lacking T-bet and Eomes in T cells show several alterations in anti-tumor responses that indicate a critical role for these TFs in promoting CD8 T cells migration to the tumor tissue¹²¹. Upon adoptive transfer in tumor-bearing mice, NK cells progressively decrease the expression of both T-bet and Eomes. This correlates with a rapid loss of their effector functions. However, forced expression of Eomes, but not T-bet, in adoptively transferred NK cells led to a reduction in tumor burden and enhanced survival compared with mice treated with control NK cells¹²². The mechanisms used by tumor cells to inhibit T-bet and Eomes expressions are discussed below.

Role of TGF- β

It was suggested that TGF- β produced in the tumor microenvironment can diminish CD16 mediated IFN- γ production in a T-bet dependent manner. Indeed, TGF- β can repress T-bet expression through phosphorylation of SMAD3 that binds T-bet promoter¹²³ (**Figure 5**). Moreover, constitutive TGF- β signaling in transgenic mice blocks NK cell maturation at early stages, suggesting that both T-bet and Eomes are influenced by TGF- β signaling⁵⁹. Furthermore, a very recent study also revealed that tumors may escape the

NK cell immune-surveillance by a TGF- β dependent conversion of NK cells (CD49a⁻CD49b⁺Eomes⁺) into ILC-1 (CD49a⁺CD49b⁺Eomes^{int}) that fail to control tumor progression¹²⁴. SMAD4 could oppose this pathway in tumoral contexts as this TF was recently shown to restrain non-canonical TGF- β signaling mediated by the cytokine receptor TGF- β R1 in NK cells¹²⁵. Zeb2, which is also essential for proper NK cell maturation might also contribute to this network. Indeed, Zeb2, also known as SIP1 for Smad Interacting protein 1, contains a Smad-interacting domain that can potentially interact with all Smad members¹²⁶. Under homeostatic conditions, TGF- β is dispensable for the differentiation of peripheral NK cell populations as revealed by NK-specific deletion of *Tgfb2* in mouse NK cells⁵⁹. There is however one exception to this rule regarding salivary gland ILC1s, which express Trail, CD49a and others as a result of TGF- β mediated suppression of Eomes expression¹²⁷.

Other pathways

Micro-RNA29b, a negative regulator of both T-bet and Eomes was found to be up regulated in NK cells from leukemic mice and patients, which could contribute to this phenomenon¹²⁸. The GSK3 kinase may also control T-bet expression in NK cells. Indeed, pharmacological inhibition of GS3K induces an increase in T-bet, Zeb2 and Blimp1 expression in NK cells and greatly enhances their effector functions and anti-tumor potential¹²⁹. A similar effect was observed in CD8 T cells upon combined blockade of CTLA-4, PD-1 and Lag-3¹³⁰. This highlights the important role of T-bet in coordinating different anti-tumor functions of cytotoxic lymphocytes and suggests that therapies aimed at restoring T-bet and/or Eomes expression in NK or T cells should prevent exhaustion of these cells.

Conclusions and Outstanding questions

After more than 15 years of research on T-bet and Eomes, it becomes clear that these related TFs have non-redundant activities despite their similarities, and that the balance between these two TFs determines the capacity of NK cells to proliferate, differentiate and the range of their effector functions. A wide array of cytokines and upstream TFs regulate their expression during NK cell development and upon inflammatory conditions.

In turn, T-bet and Eomes cooperate to promote NK cell maturation and homeostasis. Deregulation of either factor is associated with impaired immune responses during cancer or chronic infections. Despite recent advances, much remains to be learned on the spatio-temporal action of these TFs that are often co-expressed. Future studies will need to identify their respective target promoters and enhancers upon individual or combined expression. Proteomics analysis of interacting partners may also help to understand how they regulate transcription. Post-transcriptional regulation of these factors also occurs and should be added to the picture to fully understand their mode of action. These modifications could affect the subcellular localization of T-bet and Eomes, which is known to be regulated upon T cell activation¹³¹. New tools and reagents will be needed to address these questions. Similar efforts should also be directed toward understanding other pairs of related TFs, which are also co-expressed in cytotoxic lymphocytes such as Foxo1 and Foxo3, Id2 and Id3, Runx1 and Runx3 or Zeb1 and Zeb2.

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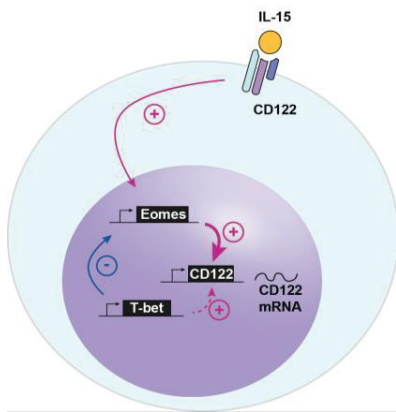


Figure 2: Regulation of CD122 / IL2R β by T-bet and Eomes

In this model, both T-BET and EOMES would be able to bind to *Ii2rb* promoter and promote transcription, but EOMES would be more potent than T-BET. As both TFs would compete for the same site, the relative amount of each TF would determine the level of CD122 expression. Moreover, CD122 expression would positively feedback on its own expression by inducing Eomes expression, perhaps through STAT5.

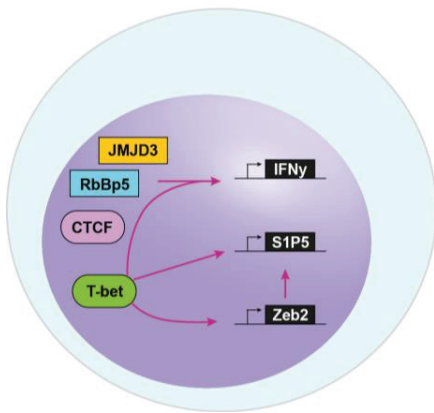


Figure 3: Regulation of IFN γ and S1P5 expression by T-bet

IFN γ and S1P5 are both regulated by T-bet. In T cells, it has been shown that IFN γ expression is turned on by promoting chromatin remodeling involving JMJD3, RbBp5 and CTCF co-factors. S1P5 expression is induced through a positive feedback loop involving Zeb2: T-bet induces Zeb2 and Zeb2 binding on S1P5 promoter enhances T-bet binding and favors transcription.

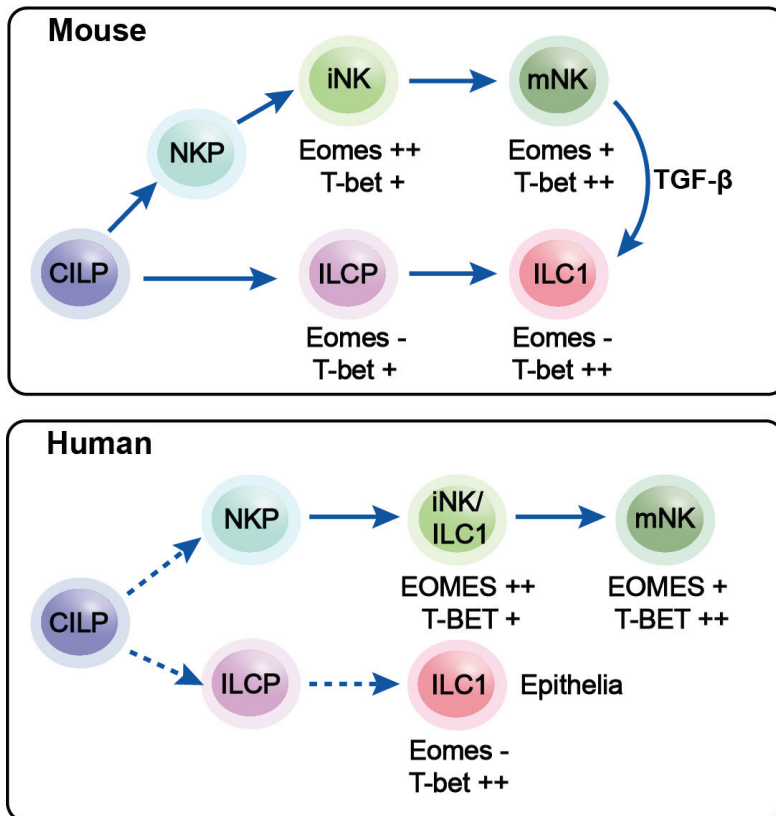


Figure 4: NK cells and ILC1 developmental pathways in mouse and human, with relation to T-bet and Eomes.

In mouse, NK cells and ILC1 are independent lineages. NK cells are defined by Eomes expression, which is induced at the immature stage. Upon maturation, T-bet expression is enhanced and represses Eomes. Moreover, in tumor microenvironments or in the salivary glands, TGF- β may promote the conversion of NK cells into ILC1 by suppressing Eomes expression. In human, immature NK cells defined as Eomes^{hi} T-bet^{lo} express many of the attributes of “classical” epithelial Eomes⁻Tbet^{hi} ILC1s. Both subsets may therefore be considered as ILC1s, from a functional point of view. In this model, mature NK cells would be defined by co-expression of high levels of both T-bet and Eomes, while ILC1s would express only one of them, either T-bet or Eomes.

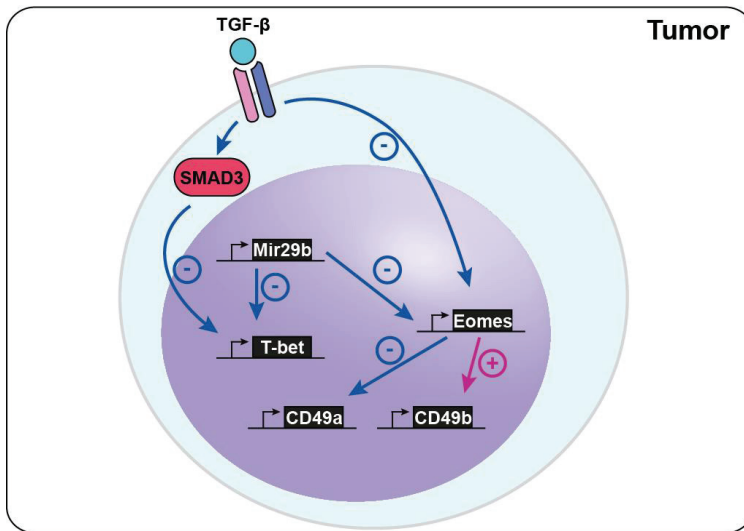


Figure 5: Regulation of T-bet and Eomes in the tumor microenvironment

Within the tumor microenvironment, several mechanisms may cooperate to suppress T-bet and Eomes expression by NK cells. Firstly, Mir29b is induced and targets the expression of both TFs. Secondly, TGF- β inhibits T-bet and Eomes expression via Smad3 binding. Eomes expression is important to repress CD49a and induce CD49b, which are often used as markers of ILC1s and NK cells, respectively.

High mTOR activity is a hallmark of reactive natural killer cells and amplifies early signaling through activating receptors

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Abstract NK cell education is the process through which chronic engagement of inhibitory NK cell receptors by self MHC-I molecules preserves cellular responsiveness. The molecular mechanisms responsible for NK cell education remain unclear. Here, we show that mouse NK cell education is associated with a higher basal activity of the mTOR/Akt pathway, commensurate to the number of educating receptors. This higher activity was dependent on the SHP-1 phosphatase and essential for the improved responsiveness of reactive NK cells. Upon stimulation, the mTOR/Akt pathway amplified signaling through activating NK cell receptors by enhancing calcium flux and LFA-1 integrin activation. Pharmacological inhibition of mTOR resulted in a proportional decrease in NK cell reactivity. Reciprocally, acute cytokine stimulation restored reactivity of hyporesponsive NK cells through mTOR activation. These results demonstrate that mTOR acts as a molecular rheostat of NK cell reactivity controlled by educating receptors and uncover how cytokine stimulation overcomes NK cell education.

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Introduction

Natural killer (NK) cells are group 1 innate lymphoid cells characterized by their ability to kill target cells and to secrete cytokines such as IFN- γ (Spits et al., 2013). Thereby, they take part in the early response against infected and neoplastic cells. Target cell recognition and NK cell activation are controlled by the balance between positive and negative signals arising from the engagement of an array of NK activating receptors (NKAr) and NK inhibitory receptors (NKIr). While normal cells express an excess of NKIr ligands, stressed cells, such as tumor and infected cells, may lose

eLife digest The cells of the immune system patrol the body to detect and destroy harmful microbes and diseased cells. Natural killer cells are immune cells with a natural capacity to kill infected or cancerous cells, as their name suggests. Importantly, they do so while sparing the surrounding healthy cells.

As natural killer cells mature they go through an “education” process to learn to distinguish between normal and abnormal cells. During education, the natural killer cells interact continuously with nearby healthy cells. However, it remains unknown how these interactions change the natural killer cells, or how these changes control their killing activity.

Marçais et al. now show that a protein called mTOR is essential to the education of natural killer cells. Comparing natural killer cells that had or had not completed the education process revealed that mTOR is more active in the educated cells. Moreover, inhibiting the activity of mTOR caused educated natural killer cells to lose their ability to identify diseased cells, while stimulating mTOR activity in uneducated natural killer cells mimicked the education process, allowing them to recognize and eliminate diseased host cells.

Certain nutrients are known to control the activity of mTOR, which suggests these nutrients could also affect how natural killer cells develop. In addition, manipulating the activity of mTOR could be used to control the response of natural killer cells to diseased host cells, and so could form part of treatments for cancer and infectious diseases. However, given that mTOR plays numerous roles within different body cells, any potential therapies that are developed would need to be able to manipulate mTOR specifically in natural killer cells.

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expression of NKir ligands (‘missing-self’) or acquire expression of NKar ligands (‘modified-self’), thus tilting the balance towards activation. NKirs, which mostly recognize classical or non-classical MHC-I molecules, are stochastically expressed, resulting in a variegated expression pattern. Depending on the species, three types of NKir interact with MHC-I: Killer Immunoglobulin-like Receptors (KIR) in primates, Ly49 receptors of the lectin-like family in rodents and the heterodimer formed by CD94 and NKG2A in these species (Vivier et al., 2008).

In addition, considerable functional heterogeneity is observed in the NK cell population. Such cell intrinsic differences led to the proposition that NK cell reactivity and consequently their ability to discriminate self from non-self is the result of an education process (Anfossi et al., 2006; Fernandez et al., 2005; Kim et al., 2005). There was however considerable debate over the molecular process leading to education. Two theories were crafted to account for these observations: the first one proposing that a priming (or arming) signal was required to confer reactivity to otherwise hyporesponsive cells, the second positing that responsiveness is a default state that is lost upon unopposed chronic stimulation of NKar (disarming) (Höglund and Brodin, 2010). The data accumulated so far are in favor of the latter model, suggesting that intrinsic reactivity is lost upon chronic engagement of NKar unless this is opposed by concomitant engagement of NKir. Indeed, there is no evidence so far that priming signals are a prerequisite for acquisition of responsiveness. In contrast, disarming is the simplest explanation to account for the tolerance to self of NK cells raised in a mosaic or chimeric environment (Johansson et al., 1997; Wu and Raulet, 1997). Moreover, the loss of reactivity consequent to exposure of NK cells to activating ligands functionally demonstrates the possibility to disarm reactive NK cells (Oppenheim et al., 2005; Tripathy et al., 2008).

At the molecular level, physical interaction between NKirs and their ligands is required to maintain responsiveness as (1) only NK cells expressing NKir engaged by MHC-I at the surface of surrounding cells are reactive and as (2) NK cells expressing NKirs but developing in MHC-I deficient humans or animals are functionally impaired (Fernandez et al., 2005; Kim et al., 2005; Zimmer et al., 1998). In addition, the inhibitory signaling module acting downstream of NKirs is required to maintain reactivity. Indeed, mutation of the immunoreceptor tyrosine-based motifs (ITIM) of inhibitory Ly49 molecules or deficiency in the phosphatase SHP-1, recruited to NKirs upon ligation, decreases responsiveness (Kim et al., 2005; Viant et al., 2014). Inhibition of the activating signal by NKir thus serves two-distinct but related purposes: it counters inappropriate NK cell

activation and it prevents the desensitization induced by chronic stimulation thereby preserving NK cell reactivity. In inbred C57BL/6 mice, Ly49C (specific for H2-K^b), Ly49I (specific for H2-K^b) and the CD94/NKG2A receptor (specific for a D^p peptide presented by Qa-1) have been shown to interact with substantial affinity with self-MHC class I molecules, while other receptors show no or marginal affinity (Hanke *et al.*, 1999; Michaëlsson *et al.*, 2000; Vance *et al.*, 1998). Consequently, NK cell populations expressing these receptors are educated in C57BL/6 mice, that is, they are more reactive than their non-educated counterparts (Fernandez *et al.*, 2005; Joncker *et al.*, 2009; Kim *et al.*, 2005). Education is a dynamic process tuned by the number of engaged NK cells and the strength of each interaction in a rheostat-like manner (Brodin *et al.*, 2009a; Johansson *et al.*, 2005; Joncker *et al.*, 2009). It is also reversible in as little as one or two days as shown in different experimental set-ups (Ebihara *et al.*, 2013; Elliott *et al.*, 2010; Joncker *et al.*, 2010). This suggests the existence of a potent cellular process integrating activating and inhibitory educating signals of variable strength (i.e. the strength of the NK cell or NK cell-ligand interaction and number of different interactions over time) and controlling the display of effector functions in response to NK cell stimulation.

Previous studies have shown that reactive NK cells are characterized by stronger calcium flux and LFA-1 integrin activation upon NK cell stimulation (Guia *et al.*, 2011; Thomas *et al.*, 2013). However, the nature of the molecular process conditioning NK cell reactivity and negatively affected by chronic engagement of NK cell is unknown. To address this question, we systematically compared phosphorylation levels of key molecules involved in immunoreceptor tyrosine-based activating motif (ITAM) signaling in reactive vs. hyporesponsive NK cells at steady-state and following NK cell stimulation. We discovered that NK cell reactivity is associated with a higher basal activity of the mammalian target of rapamycin (mTOR) pathway. Our genetic and pharmacological approaches collectively demonstrate a prominent role of mTOR signaling in controlling steady-state NK cell responsiveness.

Results

Reactive NK cells display higher activity of the Akt/mTOR pathway at steady-state and following acute NK cell engagement

Seeking to identify molecular pathways involved in NK cell education, we systematically screened the basal levels of 20 phosphorylations on 16 proteins involved in ITAM signaling between reactive and hyporesponsive NK cells by flow cytometry (complete list in Table 1). This flow-cytometry based approach allowed us to combine the advantages of single-cell analysis and comparison of equivalent cell subset thanks to electronic gating. In C57BL/6 mice, the main educating NK cells are NKG2A and Ly49C, defining four subsets of which the double-negative display the lowest, the double-positive the highest and the single positives an intermediate responsiveness (Joncker *et al.*, 2009). We also analyzed *B2m*^{-/-} NK cells that are uniformly unreactive. Most of these phosphorylations are developmentally regulated (Figure 1—figure supplement 1), thus, to exclude any developmental bias, we compared similar developmental stages defined by CD11b and CD27 (Figure 1—figure supplement 2). Strikingly, all analyzed phosphorylations in the Akt/mTOR pathway correlated positively with the level of NK cell reactivity (Figure 1A). This was true when comparing C57BL/6 and *B2m*^{-/-} NK cells as well as reactive and unreactive populations in C57BL/6 mice, regardless of the maturation stage. In C57BL/6 populations, absence of either NKG2A or Ly49C had a measurable negative effect, the absence of both leading to further decrease in the phosphorylation level. We also noted a significant correlation between education status and the level of pNFκB S529 and S468 as well as pLck Y505 and pItk Y180 (Figure 1A). However, as the most consistent differences lied in the Akt/mTOR pathway, we decided to focus our analysis on this pathway.

The phosphatase SHP-1 is required to maintain an optimal NK cell reactivity (Viant *et al.*, 2014). To test its involvement in the maintenance of the basal activity of the Akt/mTOR pathway, we measured the phosphorylation levels of the ribosomal S6 protein and Akt in NK cells deficient in *Ptpn6*, the gene encoding SHP-1. As a control, we also measured the level of phosphorylation of STAT5 in these cells. The basal activity of the Akt/mTOR pathway was specifically decreased in NK cells from *Ncr1*^{Cre/+} *Ptpn6*^{lox/lox} mice compared to control NK cells while pSTAT5 levels were unchanged (Figure 1B). Thus, basal activation of the Akt/mTOR pathway is correlated with NK cell reactivity and controlled by SHP-1-dependent signaling downstream of NK cells.

Table 1. List of the antibodies used in this study and the phosphoepitopes they recognize.

Phosphoepitope	Clone (Supplier)
pCD3 ζ (Y142)	K25-407.69 (BD)
pLck (Y505)	4/LCK-Y505 (BD)
pSyk (Y342)	I120-722 (BD)
pSLP76 (Y128)	J141-668.36.58 (BD)
pltk (Y180)	N35-86 (BD)
pPLC γ 2 (Y759)	K86-689.37 (BD)
pWIP (S478)	K32-824 (BD)
p-p38 (T180/Y182)	36/p38 (pT180/pY182) (BD)
pERK1/2 (T203/Y205)	20A (BD)
p-c-Cbl (Y698)	47/c-Cbl (BD)
pJNK (T183/Y185)	N9-66 (BD)
pNFkB p65 (S468)	#3039 (CST)
pNFkB p65 (S529)	K10-895.12.50 (CST)
pNFkB p65 (S536)	93H1 (CST)
pAkt (T308)	C31E5E (CST)
pAkt (S473)	M89-61 (BD)
pS6 (S235/236)	D57.2.2E (CST)
p4EBP1 (T36/45)	236B4 (CST)
p-mTOR (S2448)	D9C2 (CST)
p-mTOR (S2481)	#2974 (CST)

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We next compared mTOR-related signaling events arising from NKar stimulation in reactive versus hyporesponsive NK cells. To this end, we stimulated splenocytes from C57BL/6 (around 85% of NK cells are reactive in these mice) and $B2m^{-/-}$ mice by crosslinking NK1.1 and we measured phosphorylation events over time. Phosphorylation of Akt on T308 and S473 and phosphorylation of the ribosomal protein S6 were induced at higher levels in reactive NK cells compared to hyporesponsive NK cells (Figure 1C,D). By contrast, other signaling events not linked to the mTOR pathway were induced at similar levels (Figure 1C,D and Figure 1—figure supplement 3).

In summary, high activity of the Akt/mTOR pathway is a hallmark of reactive NK cells both at steady-state and following stimulation through NKars. Importantly, considering that education is not a discrete but rather a continuous process, absence of one or two of the educating NKir in C57BL/6 resulted in a commensurate loss in mTOR activity.

Chronic NK cell stimulation results in decreased phosphorylation of the Akt/mTOR pathway which parallels the loss of reactivity

Education is rapidly reverted by unopposed chronic stimulation. Indeed, transfer of reactive NK cells into a host devoid of MHC-I leads to their rapid loss of reactivity and to their tolerance to MHC-I negative cells (Joncker et al., 2010). We thus sought to test whether chronic NKar stimulation decreased the activity of the Akt/mTOR pathway in parallel with the decrease of reactivity. To this purpose, we transferred reactive C57BL/6 NK cells into control C57BL/6 or $B2m^{-/-}$ mice and measured basal Akt/mTOR phosphorylation levels and their reactivity 3 days after transfer. To quantify the intensity of NKar signaling, we took advantage of a transcriptional reporter of the TCR signaling (Moran et al., 2011). This reporter consists of a GFP under the control of the promoter sequence of *Nur77*, an orphan nuclear receptor strongly induced in response to TCR stimulation. The signaling pathways triggered by TCR or NKar engagement mobilizing the same signaling adaptors, we reasoned that the *Nur77^{GFP}* construct might also report NKar triggering. Indeed, in vitro stimulation with an NK1.1 agonist antibody or YAC-1 cells, a lymphoblastic cell line detected as foreign by C57BL/6 NK cells, resulted in an increase in the GFP fluorescence (Figure 2—figure supplement 1).

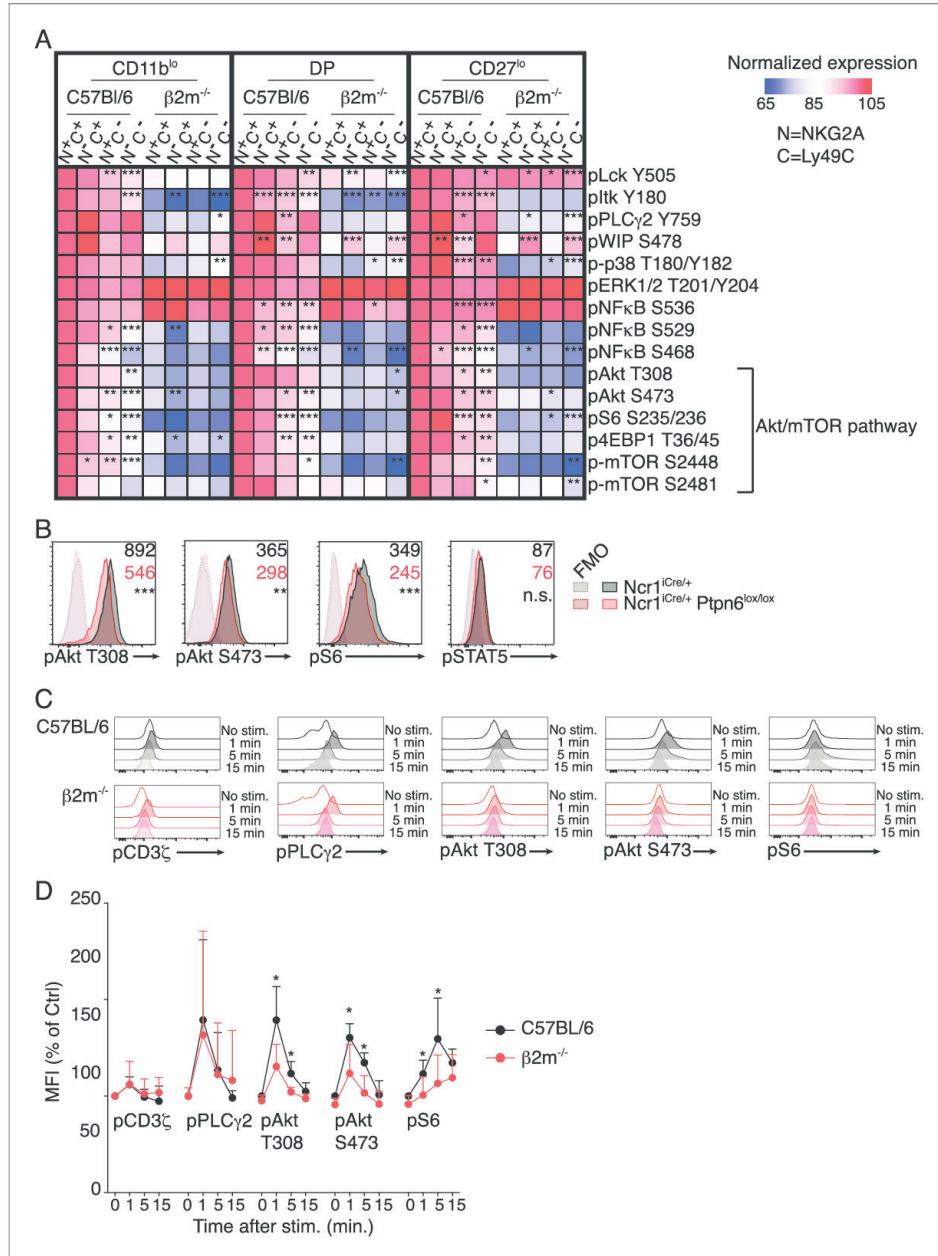


Figure 1. Basal activity of the mTOR pathway is proportional to the level of NK cell reactivity, and dependent on SHP1. (A) Heatmap representing the phosphorylation level of the phosphoepitopes indicated on the right in the different subsets of splenic resting NK cells indicated on top and gated as defined in *Figure 1—figure supplement 2*. Mean Fluorescence Intensity was recorded for each phosphoepitope in each subset. Normalized expression was calculated using the N⁺C⁺ subset of C57BL/6 mice as reference, as described in the Materials and Methods. The mean values are shown (n = 6 mice of each genotype in three independent experiments, adjusted p-values were calculated as described in the Materials and methods *Figure 1 continued on next page*

Figure 1 continued

and compare the N^+C^+ subset to the indicated subset, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (B) Histograms representing the phosphorylation level of the indicated proteins in splenic resting NK cells from $Ncr1^{Cre/+}$ or $Ncr1^{Cre/+} Ptpn6^{lox/lox}$ mice (representative of 5 mice of each genotype in three independent experiments, t-test, ** $p < 0.01$; *** $p < 0.001$, n.s. non significant). The MFI are indicated, in black for the $Ncr1^{Cre/+}$ NK cells and in red for the $Ncr1^{Cre/+} Ptpn6^{lox/lox}$ NK cells. (C–D) Phosphorylation level of the indicated phospho-epitope in splenic NK cells from C57BL/6 or $B2m^{-/-}$ mice following NK1.1 stimulation for the indicated time. (C) Histogram overlays from one representative experiment. (D) MFI of the indicated phospho-epitope (mean +SD) of 5 mice of each genotype in five independent experiments (t-test, * $p < 0.05$).

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The following figure supplements are available for figure 1:

Figure supplement 1. Bar graph showing the phosphorylation level of the indicated phosphoepitopes in the different subsets of splenic resting C57BL/6 NK cells defined by their expression of CD27 and CD11b.

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Figure supplement 2. Flow cytometry density plots presenting the analysis strategy to compare educated versus uneducated NK cells in C57BL/6 mice and the phenotypically equivalent subsets in $B2m^{-/-}$ mice.

DOI: <https://doi.org/10.7554/eLife.26423.005>

Figure supplement 3. Phosphorylation level of phospho-epitopes defined in **Table 1** was measured by flow-cytometry in splenic NK cells from C57BL/6 or $B2m^{-/-}$ mice following NK1.1 stimulation for the indicated time.

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Moreover, this increase was commensurate with reactivity so that higher GFP levels were reached in reactive $NKG2A^+Ly49C^+$ NK cells, thus validating the expression of GFP as a reporter of NKAr stimulation. Transfer of $Nur77^{GFP}$ cells into $B2m^{-/-}$ mice resulted in a transient increase in the GFP level in the reactive subsets one day after transfer indicative of ongoing NKAr signaling (**Figure 2A**). Interestingly, this was followed, 3 days after transfer, by a significant decrease in steady-state GFP level indicative of a loss of the cell capacity to signal following NKAr stimulation. As previously reported, NK cells transferred into $B2m^{-/-}$ mice lost their reactivity while reactivity was maintained upon transfer into C57BL/6 host (**Figure 2B**, anti-NK1.1 stimulation and **Figure 2—figure supplement 2**, anti-NKp46 or YAC1 stimulation). Importantly, this was paralleled by a decrease in the phosphorylation of S6 and Akt S473 and a loss of the gradient observed between the different subsets expressing Ly49C and NKG2A (**Figure 2C**).

Collectively, these results demonstrate that the basal activity of the Akt/mTOR pathway is negatively affected by persistent and unopposed NKAr stimulation. This suggests that engagement of Ly49C and NKG2A in C57BL/6 mice preserves Akt/mTOR basal activity resulting in higher basal phosphorylation in the NK cell population expressing these NKAr.

mTOR is essential for NK cell reactivity

To test if high mTOR activity was required for NK cell reactivity, we stimulated NK cells from $Ncr1^{Cre/+} Mtor^{lox/lox}$ or control mice with plate-bound anti-NK1.1 antibody or YAC-1 cells and measured NK cell degranulation relative to the expression of the major educating receptors Ly49C and NKG2A. Control NK cells responded significantly better than mTOR-deficient NK cells, irrespective of the subset analyzed (**Figure 3A**). Moreover, within control NK cells, reactive $Ly49C^+NKG2A^+$ degranulated more than the other subsets, while mTOR deficiency resulted in equally hyporesponsive subsets.

These results suggested a major role of mTOR in NK cell reactivity. However, mTOR deficiency leads to a severe NK cell developmental block that may confound the interpretation of the results (**Marçais et al., 2014**). To address this issue we took advantage of Torin2, a highly selective ATP-competitive mTOR inhibitor targeting both mTORC1 and mTORC2 (**Liu et al., 2011**). We stimulated mature NK cells from C57BL/6 and $B2m^{-/-}$ mice with plate-bound anti-NK1.1 in the presence or absence of the inhibitor. Torin2 significantly decreased the capacity of C57BL/6 NK cells to produce IFN- γ and to degranulate upon stimulation, regardless of the subset analyzed (**Figure 3B**). Moreover, treatment of C57BL/6 NK cells with Torin2 abrogated the differences between highly reactive ($Ly49C^+NKG2A^+$) and hyporesponsive ($Ly49C^+NKG2A^-$) cells. Treatment of hyporesponsive $B2m^{-/-}$ NK cells led to a further decrease in their capacity to degranulate while their production of IFN- γ was unaffected. Similar results were obtained upon NKp46 stimulation (**Figure 3—figure supplement 1**). Torin2 treated C57BL/6 NK cells thus functionally behaved like $B2m^{-/-}$ hyporesponsive NK

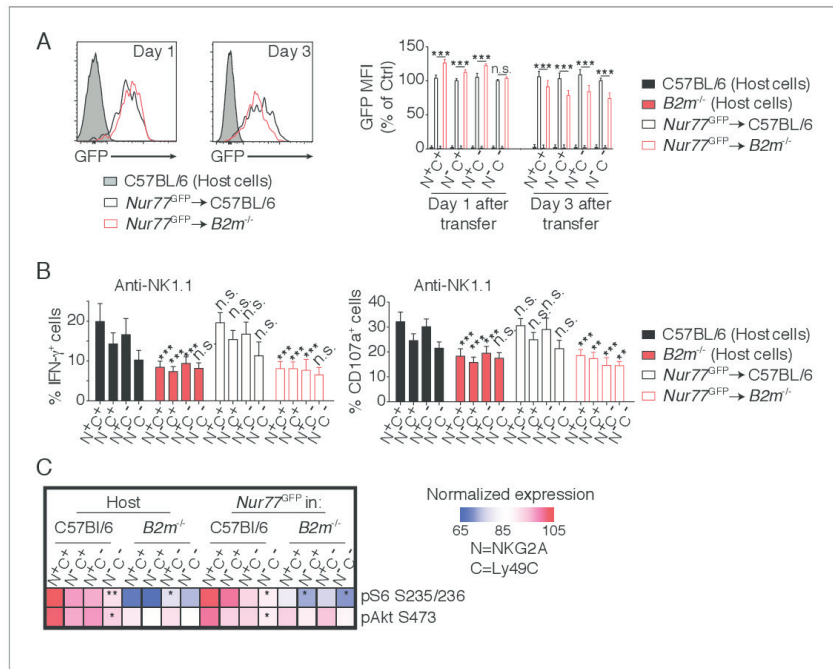


Figure 2. Reversion of education is accompanied by loss of the basal activity of the mTOR pathway. (A) *Left:* Representative histograms showing the GFP fluorescence levels of Nur77^{GFP} NK cells transferred into C57BL/6 or B2m^{-/-} mice and harvested 1 or 3 days after transfer. Non-transgenic host cells are shown. *Right:* Bar graph showing the GFP levels of the indicated splenic NK cell subsets normalized to the N:C population of Nur77^{GFP} NK cells transferred into C57BL/6 control mice (mean +SD, n = 6 mice of each genotype per time point in two independent experiments, two-way ANOVA; ***p<0.001, n.s. non significant). (B) Percentage (mean +SD) of IFN- γ ⁺ or CD107a⁺ cells among splenic host or transferred NK cells of the indicated subset following a 4 hr stimulation with coated anti-NK1.1. The experiment was done 3 days after transfer (n = 6 mice of each genotype in two independent experiments, two-way ANOVA comparing each subset to its counterpart in C57BL/6 mice, **p<0.01, ***p<0.001, n.s. non significant). (C) Heatmap representing the phosphorylation level of the phosphopeptides indicated on the right in the different subsets of splenic resting NK cells indicated on top. Mean Fluorescence Intensity was recorded for each phosphopeptide in each subset. Normalized expression was calculated using the N⁺C⁺ subset of C57BL/6 host NK cells as reference. The mean values are shown (n = 6 mice of each genotype in two independent experiments, t-tests comparing the N⁺C⁺ subset to the indicated subset, *p<0.05, **p<0.01).

DOI: <https://doi.org/10.7554/eLife.26423.008>

The following figure supplements are available for figure 2:

Figure supplement 1. *Left:* Representative histograms showing the GFP fluorescence levels of Nur77^{GFP} NK cells before or after a 4 hr stimulation with anti-NK1.1 or YAC-1 cells.

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Figure supplement 2. Percentage (mean +SD) of IFN- γ ⁺ or CD107a⁺ cells among splenic host or transferred NK cells of the indicated subset following 4 hr stimulation with (A) coated anti-NKp46 or (B) YAC-1 cells.

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cells. Similarly, Torin2 inhibited C57BL/6 NK cells from triggering YAC-1 lysis at a similar level seen in hyporesponsive B2m^{-/-} NK cells (Figure 3C). Torin2 treatment had no effect on the lytic capacity of B2m^{-/-} NK cells.

Education conditions the phenomenon of missing-self recognition. A classical readout to highlight this property is to measure the rate of rejection of MHC-I negative target cells in vivo. To test whether basal activity of the Akt/mTOR pathway was involved in this process, we transferred a mix of C57BL/6 and NK-sensitive MHC-I negative (B2m^{-/-}) target cells into C57BL/6 mice, previously

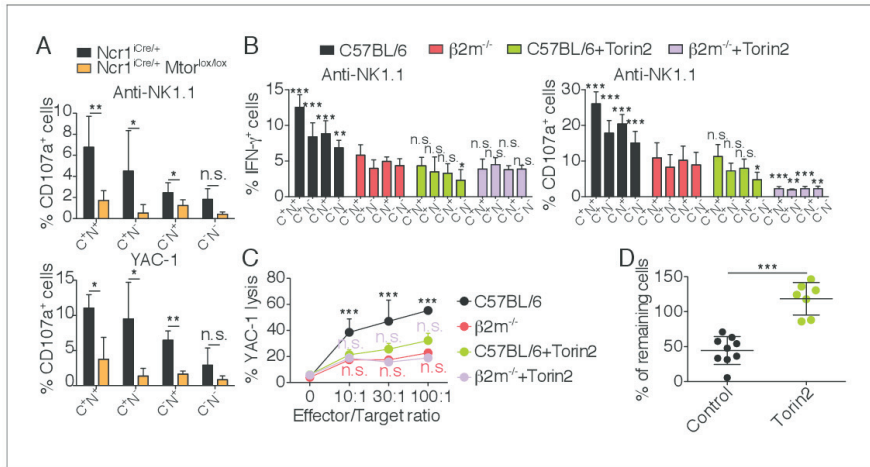


Figure 3. mTOR is essential for NK cell reactivity. (A) Percentage (mean +SD) of CD107a⁺ cells among splenic CD11b^{lo} NK cells of the indicated subset from *Ncr1*^{Cre/+} or *Ncr1*^{Cre/+} *Mtor*^{lox/lox} mice following 4 hr stimulation with coated anti-NK1.1 or YAC-1 cells (n = 5 mice of each genotype in three independent experiments, t-tests comparing each subset in both genotype, *p<0.05, **p<0.01, n.s. non significant). (B) Percentage (mean +SD) of IFN- γ ⁺ or CD107a⁺ cells among splenic NK cells of the indicated subset from C57BL/6 or *B2m*^{-/-} mice following 4 hr stimulation with coated anti-NK1.1 in the presence or absence of 250 nM Torin2 (n = 9–10 mice of each genotype in five independent experiments, two-way ANOVA comparing each subset to its counterpart in *B2m*^{-/-} mice, *p<0.05, **p<0.01, ***p<0.001, n.s. non significant). (C) Percentage (mean +SD) of dead YAC-1 cells after a 4 hr co-culture with purified NK cells of the indicated genotype at the indicated Effector/Target ratio in the presence or absence of 250 nM Torin2 (n = 9 C57BL/6 and 7 *B2m*^{-/-} mice in four independent experiments, two-way ANOVA comparing each E/T ratio of C57BL/6 + Torin2 to C57BL/6, *B2m*^{-/-} or *B2m*^{-/-} + Torin2 as indicated by the color, ***p<0.001, n.s. non significant). (D) Percentage of remaining *B2m*^{-/-} target cells following in vivo cytotoxicity experiment as described in the Materials and methods. Each dot represents a single mouse, bars indicate mean and SD (n = 9 control treated mice and 7 Torin2 treated mice in two independent experiments, t-test, ***p<0.001).

DOI: <https://doi.org/10.7554/eLife.26423.011>

The following figure supplement is available for figure 3:

Figure supplement 1. Percentage (mean +SD) of IFN- γ ⁺ or CD107a⁺ cells among splenic NK cells of the indicated subset from C57BL/6 or *B2m*^{-/-} mice following 4 hr stimulation with coated anti-NKp46 in the presence or absence of 250 nM Torin2 (n = 9–10 mice of each genotype in five independent experiments, 2-way ANOVA comparing each subset to its counterpart in *B2m*^{-/-} mice, *p<0.05, **p<0.01, ***p<0.001, n.s. non significant).

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treated or not with Torin2. While injection into control mice led to the disappearance of 50% of the target cells, this rejection was abrogated in Torin2 treated animals, underlining the importance of mTOR activity in NK cell recognition of missing-self under steady-state conditions (Figure 3D).

Altogether, these results demonstrate that mTOR is required for NK cell reactivity.

mTOR is a rheostat of NK cell reactivity through NKAr

The 'rheostat' model of education proposes that the strength of the MHC-I input translates into a quantitative modification of NK cell responsiveness (Brodin et al., 2009b). Indeed, several studies reported that the higher the number of self-MHC-I receptors expressed by NK cells interacting with their ligands, the stronger their responsiveness (Brodin et al., 2009a; Johansson et al., 2005; Joncker et al., 2009). As shown in Figure 1, the level of mTOR activity was tightly correlated with the number of educating NKs in NK cells, suggesting that mTOR could serve as the molecular rheostat translating the MHC-I input into quantitative tuning of the responsiveness. To directly test this point, we analyzed how the ex vivo modulation of mTOR activity by pharmacologic mTOR inhibitors changed NK cell responsiveness. We took advantage of four different inhibitors of graded mTOR inhibitory potential: the macrolide Rapamycin that primarily inhibits mTORC1 and three ATP-competitive inhibitors targeting both mTORC1 and mTORC2 to a varying extent: AZD2014, KU-0063794 (KU) and Torin2 (García-Martínez et al., 2009; Guichard et al., 2015; Liu et al., 2011;

Sabatini et al., 1994; Yang et al., 2013). The use of different concentrations of those compounds allowed us to modulate mTOR activity in NK cells over a dynamic range of 10-fold for mTORC1 or 2-fold for mTORC2 as measured by phosphorylation of S6 and Akt S473 respectively (Figure 4A). Of note, we confirmed that Rapamycin acted specifically on mTORC1 while AZD, KU and Torin2 inhibited both complexes. Importantly, at these concentrations no significant changes in STAT5 phosphorylation or specific toxicity over a 24 hr incubation period were noted (Figure 4—figure supplement 1A and B). We then correlated the S6 and Akt phosphorylation levels to the IFN- γ production and degranulation induced by NK1.1 crosslinking. S6 phosphorylation was positively correlated with the effector functions in all conditions tested (Figure 4B). Similar correlations were found between Akt phosphorylation and effector function upon AZD, KU or Torin2 treatment (Figure 4C). However, this correlation was lost upon Rapamycin treatment, suggesting that mTORC2 activity alone is not sufficient to sustain effector functions (Figure 4B,C). In addition, effector functions were not correlated to STAT5 phosphorylation levels (Figure 4—figure supplement 1B,C). Similar results were obtained upon stimulation of NK cells from *Ncr1^{iCre}* and *Ncr1^{iCre} Mtor^{lox/lox}* mice and measure of the phosphorylation levels of the S6 and Akt proteins in parallel thus genetically confirming the results (Figure 4—figure supplement 1D).

Overall, these results demonstrate that mTOR acts as a molecular rheostat of NK cell responsiveness. Together with results in Figures 1 and 2, they demonstrate that NK cell education relies on the modulation of mTOR activity that in turn controls NK cell responsiveness through NKars.

mTOR is essential for calcium response and integrin activation in NK cells following NKar engagement

Next, we asked whether mTOR activity could regulate signaling via NKar. Previous studies established that reactive NK cells display higher calcium flux (Guia et al., 2011) and higher integrin activation than hyporesponsive NK cells (Thomas et al., 2013). Hence we sought to test the impact of mTOR activity on these cardinal events in lymphocyte activation. We first measured the calcium flux in real time by flow cytometry following NK1.1 stimulation using fluorescent calcium probes and we quantified the intensity of the fluorescence peak. When we challenged *Ncr1^{iCre/+}* (control) and *Ncr1^{iCre/-} Mtor^{lox/lox}* NK cells, NK1.1 cross-linking resulted in a detectable calcium flux in NK cells of both genotypes (Figure 5A). However, the peak was lowered (15–20%) in the absence of mTOR. We next applied the same protocol to control C57BL/6 NK cells in the presence or absence of Torin2 to acutely inhibit mTOR. As shown in Figure 5B, mTOR inhibition resulted in a decreased calcium flux characterized by a 20%-decrease in the peak intensity, thus phenocopying the impact of mTOR deficiency.

Next, we assessed the effect of mTOR deficiency on LFA-1 integrin activation following NKar triggering of inside-out signaling. For this purpose, we incubated NK cells from *Ncr1^{iCre/+}* and *Ncr1^{iCre/+} Mtor^{lox/lox}* mice with beads coated with ICAM-1, the ligand of LFA-1, in the presence or absence of NK1.1 cross-linking. At different times, we measured by flow-cytometry the percentage of beads-associated NK cells as an indicator of LFA-1 activation in NK cells. As shown in Figure 5C, NK1.1 cross-linking failed to induce LFA-1 activation in mTOR-deficient NK cells contrary to control NK cells. In parallel, we also tested the effect of acute mTOR inhibition on LFA-1 activation in mature educated NK cells. As shown in Figure 5D, addition of Torin2 resulted in significant inhibition of LFA-1 activation induced by NK1.1 stimulation.

Thus, using genetic and pharmacological tools, we showed that the mTOR pathway lies upstream of two signaling events, calcium flux and LFA-1 integrin activation, which are elevated in reactive NK cells.

Metabolic parameters of reactive and hyporesponsive NK cells

mTOR is a well-known regulator of the cell metabolism. We thus asked whether the higher activity of mTOR measured in reactive NK cells resulted in detectable changes in metabolic activity. We first measured cell size and granularity using the FSC and SSC flow-cytometry parameters. Reactive NK cells from C57BL/6 control mice presented a slight but significant increase of both morphological indicators when compared to hyporesponsive NK cells of *B2m^{-/-}* mice (Figure 6A). Similarly, their mitochondrial content as well as glucose and fatty-acid uptake capacities estimated by measure of the uptake of the glucose fluorescent analog 2-NBDG or the fatty-acid fluorescent analog Bodipy

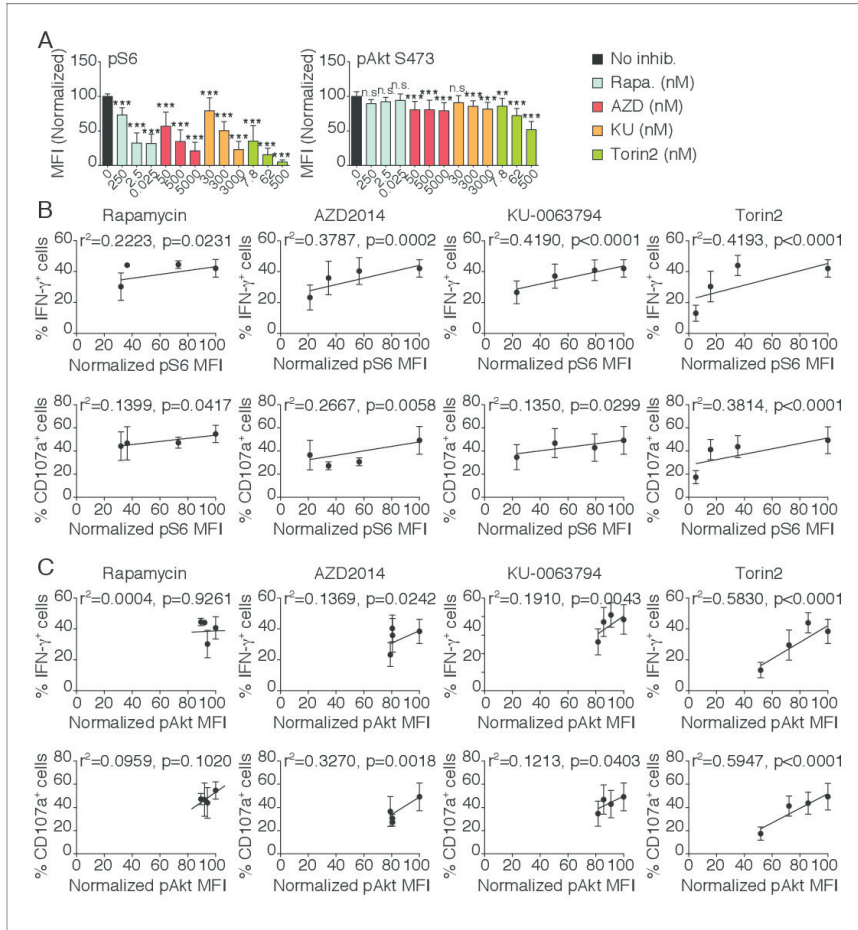


Figure 4. mTOR is a rheostat of NK cell reactivity through NKAr. (A) Bar graph showing the phosphorylation level of S6 (left) and Akt S473 (right) in splenic NK cells following 1 hr treatment with 100 ng/ml IL-15 in the presence or absence of the indicated mTOR inhibitors at the indicated concentration (mean of the MFI normalized to the No inhibitor condition \pm SD, n = 9 mice in three independent experiments, one-way ANOVA comparing the No inhibitor condition with the indicated condition, **p < 0.01; ***p < 0.001, n.s. non significant). (B–C) Linear regression plots showing the correlation between (B) pS6 or (C) pAkt S473 as indicated and the percentage of IFN- γ ⁺ or CD107a⁺ NK cells following 4 hr stimulation with coated anti-NK1.1 in the presence of 100 ng/ml IL-15 and mTOR inhibitors (mean \pm SD, n = 9 mice in three independent experiments, the r^2 and p-value calculated by linear regression are indicated).

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The following figure supplement is available for figure 4:

Figure supplement 1. Bar graphs showing (A) the phosphorylation level of STAT5 in splenic NK cells following 1 hr treatment with 100 ng/ml IL-15 or (B) the percentage of live NK cells following a 24 hr culture in the presence or absence of the indicated mTOR inhibitors at the indicated concentration (A) mean of the MFI normalized to the No inhibitor condition or (B) percentage of live cells \pm SD, n = 9 mice in three independent experiments for pSTAT5 and 4 mice in two independent experiments for Viability, one-way ANOVA comparing the No inhibitor condition with the indicated condition, n.s. non significant).

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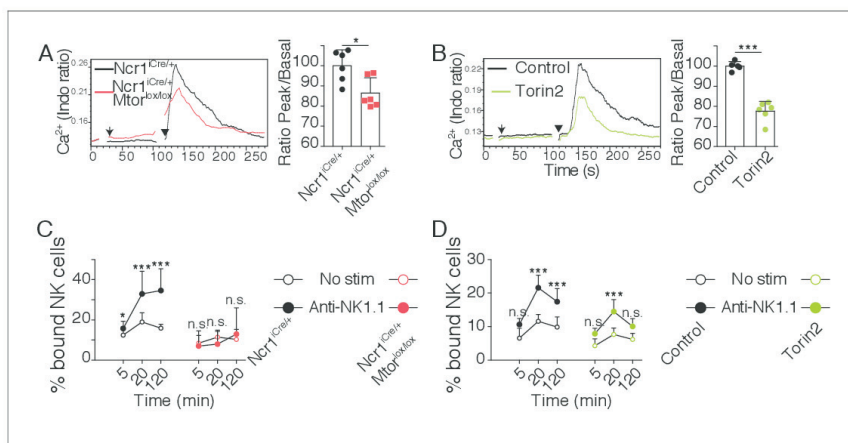


Figure 5. mTOR is essential for calcium response and integrin activation following NK cell engagement. (A) Left: Representative histogram overlay showing the Ca^{2+} flux intensity in splenic CD11b^{lo} NK cells from $Ncr1^{\text{Cre/+}}$ or $Ncr1^{\text{Cre/+}} Mtor^{\text{lox/lox}}$ mice. NK cells were activated following incubation with biotinylated anti-NK1.1 (Arrow) followed by cross-linking with streptavidin (Arrowhead). Right: Bar graph showing the Ratio Peak/basal normalized to the ratio of control NK cells (mean \pm SD, t-test $*p < 0.05$). (B) Left: Representative histogram overlay showing the Ca^{2+} flux intensity in splenic NK cells from C57BL/6 mice in the presence or absence of 500 nM Torin2. Right: Bar graph showing the Ratio Peak/basal normalized to the ratio of control NK cells (mean \pm SD, t-test $***p < 0.001$). (C) Percentage of splenic $\text{CD11b}^{\text{low}}$ NK cells from $Ncr1^{\text{Cre/+}}$ or $Ncr1^{\text{Cre/+}} Mtor^{\text{lox/lox}}$ mice bound to beads coated with ICAM-1 after the indicated incubation time with or without NK1.1 stimulation (mean \pm SD, $n = 6$ mice of each genotype in four independent experiments, two-way ANOVA comparing the conditions with or without NK1.1 stimulation, n.s. non significant, $**p < 0.01$, $***p < 0.001$). (D) Percentage of splenic C57BL/6 NK cells bound to beads coated with ICAM-1 after the indicated incubation time with or without NK1.1 stimulation, in the presence or absence of 250 nM Torin2 (mean \pm SD, $n = 6$ mice in four independent experiments, two-way ANOVA comparing the conditions with or without NK1.1 stimulation, n.s. non significant, $***p < 0.001$).

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FL-C16 were significantly higher (Figure 6B). In contrast, mitochondrial ROS production, lipid droplet content or lipid peroxidation were comparable in both cell types (data not shown). Differences were also detectable for FSC and SSC values as well as fatty-acid uptake when comparing reactive and hyporesponsive NK cell subsets present in the most mature CD27^{low} population of C57BL/6 mice (Figure 6C).

In summary, the higher activity of the Akt/mTOR pathway observed in reactive cells increased their metabolic activity compared to hyporesponsive NK cells, which may also contribute to their enhanced responsiveness.

Cytokine stimulation overcomes NK cell education by inducing high mTOR activity that restores NK cell signaling

Several studies have demonstrated that hyporesponsive NK cells can be rendered reactive (Ebihara et al., 2013; Elliott et al., 2010; Joncker et al., 2010; Sun and Lanier, 2008). The underlying molecular mechanism has however remained elusive. We reasoned that if the mTOR pathway was really a key determinant of NK cell reactivity, acute activation of this pathway should immediately restore reactivity of hyporesponsive cells. To test this hypothesis, we stimulated NK cells from C57BL/6 or $B2m^{-/-}$ mice with plate-bound antibodies stimulating NK1.1 or NKp46 and we simultaneously added IL-2, a cytokine known to potentially activate mTOR (Marçais et al., 2014). To test the requirement for the mTOR pathway in this process, cells were also treated or not with Torin2. IL-2 resulted in an increase of the cell capacity to produce $\text{IFN-}\gamma$ and to degranulate as measured by CD107a exposure (Figure 7A). This acute treatment was sufficient for hyporesponsive cells to acquire a level of reactivity equal or even higher than that of reactive NK cells from C57BL/6, regardless of the stimulating antibody. mTOR activity was required for this effect since the increase in reactivity was suppressed by mTOR inhibition (Figure 7A). Similar results were obtained when using IL-

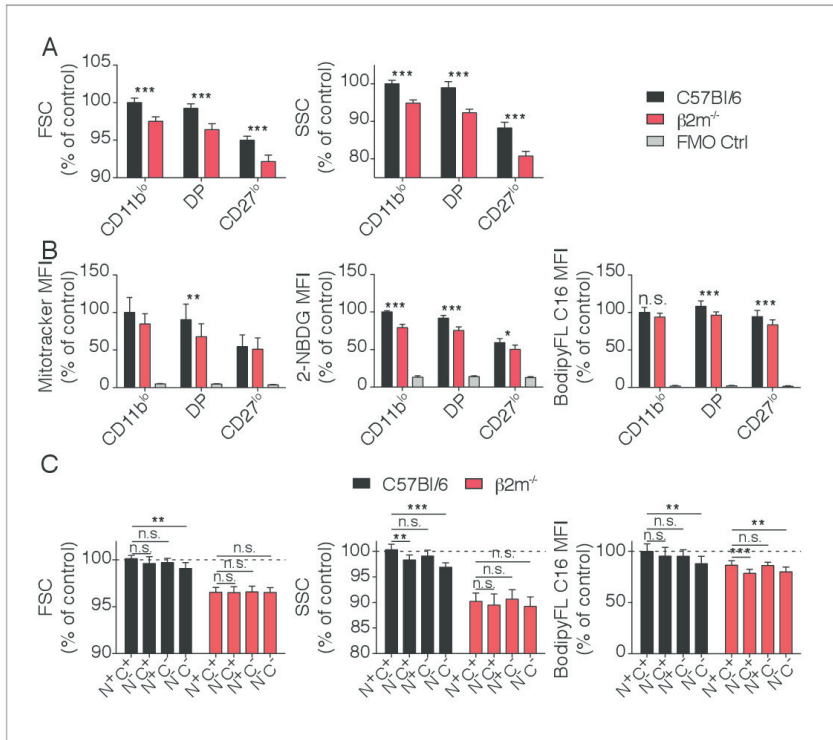


Figure 6. Metabolic parameters of reactive and hyporesponsive NK cells. (A) Bar graphs showing FSC and SSC values of splenic NK cell subsets from C57BL/6 or $B2m^{-/-}$ mice (mean \pm SD, $n = 6$ mice of each genotype in three independent experiments, t-test, $***p < 0.001$). MFI were normalized to the CD11b⁺ subset of C57BL/6 mice. (B) Bar graphs showing MFI of mitotracker staining, 2-NBDG or BodipyFL C16 incorporation of splenic NK cell subsets from C57BL/6 or $B2m^{-/-}$ mice (mean \pm SD, $n = 6-10$ mice of each genotype in three independent experiments, t-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n.s. non significant). MFI were normalized to the CD11b⁺ subset of C57BL/6 mice. (C) Bar graphs showing FSC and SSC values or BodipyFL C16 incorporation of splenic NK cell subsets (gated on CD27^{lo}) from C57BL/6 or $B2m^{-/-}$ mice (mean \pm SD, $n = 6-10$ mice of each genotype in three independent experiments, t-test, n.s. non significant, $*p < 0.05$, $***p < 0.001$). MFI were normalized to the N⁺C⁺ subset of C57BL/6 mice.

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15 instead of IL-2 (Figure 7—figure supplement 1). Acute IL-15 stimulation also restored the cytotoxic activity of hyporesponsive NK cells against YAC-1 cells while further enhancing cytotoxicity of C57BL/6 cells (Figure 7B). Again, this effect was completely reversed upon concomitant Torin2 treatment. Taken together, these results show that induction of responsiveness in NK cells upon cytokine exposure is a rapid phenomenon acting via mTOR activation.

In order to decipher the mechanism required for NK cell re-education, we next tested whether acute IL-15 treatment restored early signaling in hyporesponsive cells. We first investigated the impact of IL-15 treatment on the calcium flux triggered by NK1.1 stimulation in control or hyporesponsive NK cells. As expected, NK1.1 stimulation of hyporesponsive NK cells resulted in a very poor calcium flux compared to reactive NK cells (Figure 7C). Strikingly, treatment with IL-15 increased the calcium flux ability of reactive and hyporesponsive NK cells in an mTOR-dependent way (Figure 7C and Figure 7—figure supplement 2). We then measured the impact of IL-15 treatment on LFA-1 activation following NK1.1 stimulation. The presence of IL-15 in the assay rendered hyporesponsive NK cells able to activate LFA-1 upon NK1.1 stimulation and bind ICAM-1 coated beads (Figure 7D).

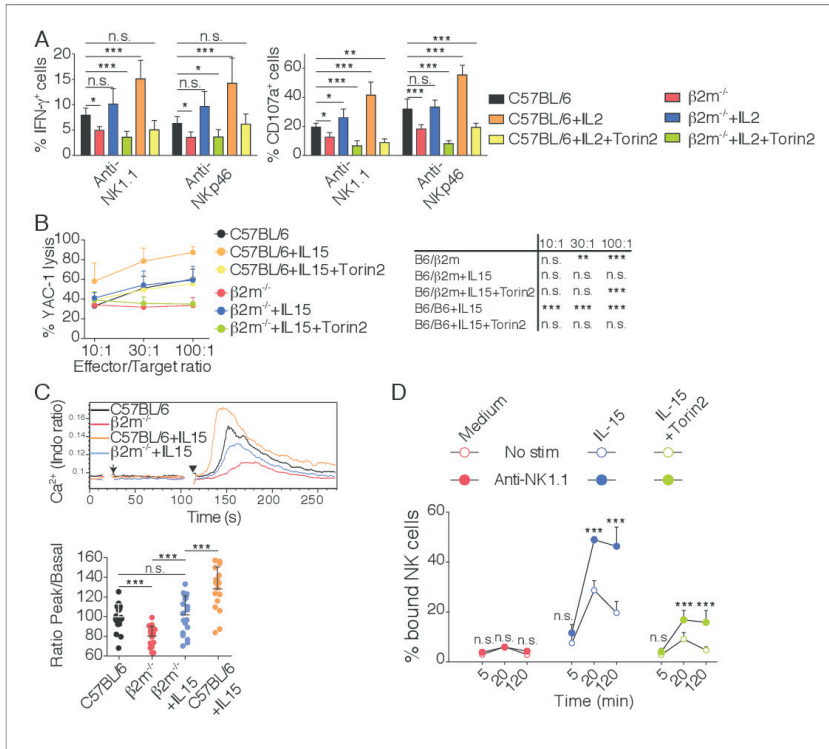


Figure 7. Cytokine stimulation overcomes NK cell education by inducing high mTOR activity that restores NKAr signaling. (A) Percentage (mean +SD) of IFN- γ or CD107a positive cells among splenic NK cells from C57BL/6 or $B2m^{-/-}$ mice following 4 hr stimulation with coated anti-NK1.1 or anti-NKp46 in the presence or absence of 200U/ml IL-2 and 250 nM Torin2 as indicated (n = 9–10 mice of each genotype in five independent experiments, one-way ANOVA comparing each condition to the C57BL/6 condition, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. non significant). (B) Percentage (mean +SD) of dead YAC-1 cells after a 4 hr co-culture with purified NK cells from C57BL/6 or $B2m^{-/-}$ mice at the indicated Effector/Target ratio in the presence or absence of 10 ng/ml IL-15 and 250 nM Torin2 as indicated (n = 7 mice of each genotype in three independent experiments, the table on the right presents the results of a two-way ANOVA comparing C57BL/6 with the other experimental conditions for the indicated Effector/Target ratio, ** $p < 0.01$, *** $p < 0.001$, n.s. non significant). (C) Top: Representative histogram overlay showing the Ca^{2+} flux intensity in splenic NK cells from C57BL/6 or $B2m^{-/-}$ mice with or without IL-15 (100 ng/ml). NK cells were activated following incubation with biotinylated anti-NK1.1 (Arrow) followed by cross-linking with streptavidin (Arrowhead). Down: Bar graph showing the Ratio Peak/basal normalized to the ratio of control NK cells (mean +SD of n = 17–20 replicates from 6 mice in six independent experiments, one-way ANOVA comparing the indicated conditions, ** $p < 0.01$, *** $p < 0.001$). (D) Percentage of splenic NK cells from $B2m^{-/-}$ mice bound to beads coated with ICAM-1 after the indicated incubation time with or without NK1.1 stimulation, in the presence or absence of 100 ng/ml IL-15 and 250 nM Torin2 (n = 6 mice in four independent experiments, two-way ANOVA comparing stimulated to non-stimulated conditions, n.s. non significant, *** $p < 0.001$).

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The following figure supplements are available for figure 7:

Figure supplement 1. Percentage (mean +SD) of IFN- γ or CD107a positive cells among splenic NK cells from C57BL/6 or $B2m^{-/-}$ mice following 4 hr stimulation with coated anti-NK1.1 or anti-NKp46 in the presence or absence of 10 ng/ml IL-15 and 250 nM Torin2 as indicated (n = 4 mice of each genotype in two independent experiments, one-way ANOVA comparing each condition to the C57BL/6 condition, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. non significant).

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Figure supplement 2. Left: Representative histogram overlay showing the Ca^{2+} flux intensity in splenic NK cells from C57BL/6 or $B2m^{-/-}$ mice with or without IL-15 (100 ng/ml) and Torin2 (500 nM).

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This effect was strongly decreased upon Torin2 treatment, underlying the non-redundant role of mTOR in this process.

Altogether, these results show that acute stimulation of the mTOR pathway can restore the ability of hyporesponsive NK cells to induce calcium flux and activate LFA-1 upon NK cell engagement, thereby re-establishing their reactivity.

Discussion

Here, to gain mechanistic insight into the phenomenon of NK cell education, we explored signal transduction pathways downstream NK cells in reactive and hyporesponsive NK cells. We found that the activity of the Akt/mTOR pathway was selectively higher in reactive NK cells. This was characterized by higher basal phosphorylation of direct and indirect targets of both mTOR complexes (mTORC1 and 2) in strict correlation with the reactivity level. In addition, this pattern was lost concomitantly with the loss of reactivity observed upon transfer of reactive cells in *B2m*^{-/-} hosts. Our screen also revealed that two out of the three NFκB p65 phosphorylations investigated (S468 and S529) correlated with reactivity. This could be the result of the heightened Akt/mTOR pathway activity, as mTORC2 has been involved in NFκB activation during CD4 T cell stimulation (Lee et al., 2010). Alternatively, this could reveal the involvement of other pathways in the control of NK cell reactivity.

What is the extracellular signal and the signaling pathway responsible for the maintenance of mTOR basal activity in reactive NK cells specifically? An obvious candidate would be IL-15 as this cytokine is a privileged activator of this pathway (Marçais et al., 2014). However, pSTAT5 levels were identical between reactive and hyporesponsive NK cells (data not shown). Moreover, in vivo treatment with antibodies blocking IL-15 signaling did not alter NK cell education (data not shown). Finally, it is difficult to envisage how reactive cells would get preferential access to IL-15. Instead, in line with the disarming hypothesis, we would favor a model in which basal mTOR activity is set independently of education signals. This initial activity would then be decreased by disarming signals. How mTOR activity is decreased by chronic NK cell stimulation is still an open question. We hypothesize that in the absence of surrounding MHC-I or in NK cells lacking functional NK receptors, unopposed NK cell signaling could lead to shut-down of the Akt/mTOR pathway due to depletion of necessary intermediates or establishment of negative feedbacks as it has been demonstrated in the case of induction of resistance to insulin (Um et al., 2006). Engagement of NK receptors would prevent this desensitization and maintain an optimal activity of the pathway. In favor of this hypothesis, we show that SHP-1, the phosphatase triggered by NK receptor ligation and necessary to maintain NK cell reactivity (Viant et al., 2014), was required to maintain an optimal activity of the mTOR pathway. Furthermore, transfer of *Nur77*^{GFP} cells into *B2m*^{-/-} hosts was accompanied by an increase in the GFP level, evidence of active NK cell signaling, and followed by the loss of mTOR basal activity concomitant with the loss of reactivity of NKG2A⁺Ly49C⁺ NK cells.

Previous studies have conclusively shown that NK cell education is not an on-off switch but rather a variation on a continuous axis (Brodin et al., 2009a; Joncker et al., 2009). We propose that the mTOR pathway is the long-sought molecular rheostat able to both respond to educating signals and control effector functions in return. Indeed, we showed that activity of the Akt/mTOR pathway is regulated commensurate with the level of NK receptor engagement by MHC-I molecules. Furthermore, we demonstrated that modulation of mTOR activity by exogenous cytokine or pharmacologic treatments was directly correlated with NK cell responsiveness. Furthermore, mTOR could also regulate NK cell responsiveness by integrating signals beyond NK receptor ligands. Considering the concept of the extended rheostat model as described initially by Höglund and colleagues (Brodin et al., 2009b), we envision extracellular inputs in an extended sense, including immunological as well as purely metabolic inputs. Interestingly, a number of environmental conditions, such as the presence of inflammatory (Sun and Lanier, 2008) or anti-inflammatory cytokines (Sungur et al., 2013), but also the presence of nutrients (Keppel et al., 2015), impact on NK cell responsiveness. All these stimuli positively or negatively affect mTOR activity (Efeyan et al., 2015; Marçais et al., 2014; Sinclair et al., 2013; Viel et al., 2016). mTOR activity could thus be the nexus targeted by these different stimuli which would explain their impact on NK cell responsiveness. Thus, considering mTOR as the rheostat of NK cell responsiveness would help to build a common conceptual framework in which these observations could be ordered.

Finally, we also present evidence on how mTOR activity affects NK cell effector functions. We demonstrated that mTOR activity controls two distinct events characterizing reactive NK cells and required for the triggering of effector functions: Ca^{2+} flux and integrin activation. How could mTOR activity control such apparently unrelated signaling events? Depending on the relative involvement of mTORC1 or mTORC2, several possibilities can be considered. First, the fact that Rapamycin which specifically inhibits mTORC1 is sufficient to decrease responsiveness unmasks the non-redundant role of this complex. In line with the role of mTORC1 in the control of cellular metabolism, we described that higher basal mTOR activity in educated cells translated into higher basal metabolism as measured by morphological parameters as well as glucose and fatty-acid uptake and mitochondrial content. We and others have described the necessary role of the mTORC1-dependent metabolism in the development of NK cell effector functions (Donnelly et al., 2014; Marçais et al., 2014). In addition to improving the cellular fitness, metabolism could directly modulate signaling by controlling the availability of key intermediates as recently described for Th17/Treg differentiation (Araujo et al., 2017). Another possibility would be through the regulation of the actin cytoskeleton. Indeed, an emerging mode of lymphocyte signaling regulation is through cytoskeleton-dependent regulation of membrane receptors compartmentalization (Mattila et al., 2016), a process that has been proposed to explain the reactivity of educated NK cells (Guia et al., 2011). mTORC2 has been shown to regulate the cytoskeletal organization (Huang et al., 2013; Sarbassov et al., 2004) and could therefore prime reactive NK cells by cytoskeletal modifications. An interesting parallel can also be drawn with T cell anergy. Indeed, TCR stimulation in the absence of CD28 co-stimulation results in T cell hyporesponsiveness to further re-stimulation. Numerous studies have shown that the precise control of mTOR activity is at the heart of this phenomenon (Chappert and Schwartz, 2010; Marçais et al., 2014; Zheng et al., 2007; 2009). Interestingly, this state is characterized by defective Ca^{2+} flux (Dubois et al., 1998). Further resembling hyporesponsive NK cells, treatment of anergic T cells with IL-2 restores their responsiveness, an event that relies on mTOR activation (Dubois et al., 1998; Zheng et al., 2007). Ca^{2+} flux is classically triggered by IP_3 -induced release of endoplasmic reticulum stores which, upon detection by the STIM1/2 sensors, leads to opening of the ORAI channels present on the plasma membrane and extracellular Ca^{2+} entry (Hogan and Rao, 2015). In addition, an underestimated Ca^{2+} store is the endo-lysosomal compartment (Morgan et al., 2011), which constitutes a further link with mTOR since mTORC1 is activated on the lysosomal surface and positively regulated by lysosomal nutrients (Efeyan et al., 2015) as well as by calcium release from lysosomal stores (Li et al., 2016). Concerning regulation of integrin activation, a putative link would be through the inhibition of GSK3 β . Indeed, this kinase is inhibited by Akt following mTORC2 activation (Hagiwara et al., 2012), and a recent study showed that its inhibition leads to better ability of NK cells to form conjugate via integrin activation (Parameswaran et al., 2016). In addition, PKC θ , a target of mTORC2 (Lee et al., 2010), activates WIP via S488 phosphorylation in lymphocytes (Fried et al., 2014). Since a macro-complex involving WIP, WASp, actin and myosin IIa has been defined in NK cells (Krzewski et al., 2006), WIP activation could explain better interaction with ICAM-1-coated beads in our assay and ultimately better docking to target cell.

In summary, these findings identify the activity of the mTOR pathway as the molecular rheostat responsible for the control of basal NK cell reactivity in response to NK cell ligation. In addition, this provides a molecular basis for a number of previous experiments showing that NK cell education can be overcome by cytokine treatment. Finally, our data underline the extreme versatility of the regulation of NK cell responsiveness and further point to mTOR as a valid target for the manipulation of NK cells for therapeutic purposes.

Materials and methods

Mice and adoptive transfers

Wild-type C57BL/6 mice were purchased from Charles River Laboratories (L'Arbresle). $B2m^{-/-}$ (Koller et al., 1990), $Ncr1^{\text{Cre/+}} Mtor^{\text{lox/lox}}$ (Marçais et al., 2014) and $Ncr1^{\text{Cre/+}} Ptpn6^{\text{lox/lox}}$ mice (Viant et al., 2014) were previously described, littermate control mice were used as controls. $Nur77^{\text{GFP}}$ mice were previously described (Moran et al., 2011). Female mice 8 to 24 week-old were used. $Nur77^{\text{GFP}}$ splenocytes were injected i.v. in C57BL/6 or $B2m^{-/-}$ host. Each host received 25×10^6 splenocytes labeled with CTV (1 μM , Molecular Probes) to allow subsequent identification. Host

mice were sacrificed one or 3 days after for analysis of the spleen. This study was carried out in accordance with the French recommendations in the Guide for the ethical evaluation of experiments using laboratory animals and the European guidelines 86/609/CEE. All experimental studies were approved by the bioethic local committee CECCAPP. Mice were bred in the Plateau de Biologie Expérimentale de la Souris, our animal facility.

Flow cytometry

Single cell suspensions of spleens were obtained and stained. Intracellular stainings for phosphorylated proteins were done using Lyse/Fix and PermlII buffers (BD Bioscience). Measurement of glucose uptake was performed as described (Marçais *et al.*, 2014). Mitochondrial content was measured using Mitotracker Green (Molecular Probes, 1 μ M) incubated for 10 min at 37°C in PBS. Lipid uptake was measured using BodipyFL C16 (Molecular Probes, 1 μ M) incubated for 30 min at 37°C in complete medium. Surface staining were then performed to identify the different populations. Flow cytometry was carried out on a FACS LSR II or on a FACS Fortessa (Becton-Dickinson). Data were analysed using FlowJo (Treestar). The following mAbs from eBioscience, BD Biosciences or Biolegend were used: anti-CD19 (ebio1D3), anti-CD3 (145-2 C11), anti-NK1.1 (PK136), anti-NKp46 (29A1.4), anti-CD49b (DX5), anti-CD11b (M1/70), anti-CD27 (LG.7F9), anti-Ly49I (YLI90), anti-NKG2A/C/E (20d5), anti-IFN- γ (XMG1.2), anti-CD107a (1D4B). The mAb 4LO3311 recognizing Ly49C was purified on protein A column from supernatant of the 4LO3311 hybridoma generously provided by Pr. Suzanne Lemieux (Institut Armand Frappier, Québec). NKG2A positive cells were identified using the 20d5 clone which also recognizes NKG2C and NKG2E, however, since mouse resting NK cells only express NKG2A, we considered 20d5 reactive cells as NKG2A positive (Vance *et al.*, 1998).

Cell culture and stimulation

1.5×10^6 splenocytes were cultured on antibody coated plates (anti-NKp46 (Goat polyclonal, R&D), anti-NK1.1 (PK136, BioXCell) at 10 μ g/ml on Immulon 2HB or Nunclon plates) with Golgi-stop (BD Biosciences) in the presence of anti-CD107a for 4 hr. Cytokines and mTOR inhibitors were used at the following concentrations unless otherwise stated: rmlL-15 (Peprotech; 100 ng/ml), IL-2 (muLL-2 supernatant; 200 U/ml), Rapamycin (Calbiochem; 25 nM), KU-0063794 (Stemgent; 3 μ M), AZD2014 (Selleckchem; 5 μ M) and Torin2 (Tocris; 250 nM). Surface and intracellular stainings were then performed and IFN- γ production as well as CD107a exposure was measured by flow cytometry. In some experiments, cell viability was determined using 7AAD (Invitrogen, 250 nM).

For phospho-flow stainings following short-term NK1.1 stimulation, 3×10^6 splenocytes were stimulated using biotinylated NK1.1 (PK136, 5 μ g/ml) followed 1 min 30 s later by streptavidin (Life Technologies, 10 μ g/ml) and fixed by addition of 10 volumes of Lyse/Fix at the indicated time point.

In vivo cytotoxicity assay

Recipient mice were treated by daily i.p. injection of Torin2 (10 mg/kg, vehicle: 40% H₂O, 40% PEG400 (Sigma), 20% N methyl two pyrrolidone (Sigma)) for 6 days prior to target transfer. Splenocytes from C57BL/6 or *B2m*^{-/-} mice were labeled respectively with CellTraceViolet (1 μ M) or CFSE (5 μ M) (both from Life Technologies), and 10×10^6 cells (5×10^6 of each genotype) were transferred by i.v. injection. 60 hr after transfer, splenocytes were isolated and analyzed by FACS. Percentage of remaining *B2m*^{-/-} cells was calculated using the following formula: % remaining cells = 100 x (number *B2m*^{-/-} cells/number C57BL/6 cells) at 60 h / (number *B2m*^{-/-} cells/number C57BL/6 cells) in input mix.

In vitro cytotoxicity assay

NK cells were first enriched by negative depletion prior to killing assay. Briefly, splenocytes suspension were incubated with biotinylated mAb against: CD3 (14-2 C11), TCR β (H57-597), TCR $\gamma\delta$ (GL3), CD19 (ebio1D3), TER-119 (ter119) (eBioscience), followed by incubation with anti-biotin microbeads (Miltenyi), and enrichment by magnetic separation on an AutoMACS. Enriched NK cells were co-cultured for 4 hr with YAC-1 cells labeled with CFSE (Life Technologies) at different Effector to target (E/T) ratios calculated based on the cell number and the percentage of NK cells after purification.

The percentage of dead cells within CFSE positive YAC-1 cells was measured by flow cytometry after staining with 7AAD.

Calcium flux

Calcium flux was measured essentially as described (Guia et al., 2011). Briefly, RBC-lysed splenocytes suspension in RPMI/0.2% BSA/25 mM HEPES were stained at RT with the following mAb: anti-CD3/CD19 PEeFluor610, anti-CD49b APC, anti-CD11b APCCy7, anti-CD27 PE. They were then stained at 1×10^7 cells/ml with Indo-1 (1 μ M, Life Technologies) for 30 min at 37°C and washed two times at 4°C. They were resuspended in the above medium and placed at 37°C for 30 min prior acquisition in the presence or absence of rmlL-15 (100 ng/ml) or Torin2 (250 nM). Samples were acquired on a LSRII (BD) as follow: 15 s baseline acquisition, addition of anti-NK1.1 biotin (PK136, 5 μ g/ml), acquisition for 1 min 30 s, addition of Streptavidin (Life Technologies, 10 μ g/ml) and, acquisition for another 3–5 min.

ICAM1 coated beads assay

One mg Protein G-coated 4–4.9 μ m beads (Spherotec) was incubated for 30 min with 3.5 μ g ICAM1-hlgG1Fc (R&D) on a rotating wheel at RT in PBS. Beads were then pelleted by centrifugation and washed two times with complete medium, counted on a FACS Accuri (BD) and resuspended at 1×10^7 beads/ml. In parallel, NK cells were purified (80–90% purity) using biotinylated antibodies directed against CD3, CD19, CD5, CD24, F4/80 and Ly6G and anti-biotin beads. They were then incubated with anti-NKp46-PE (29A1.4, BD) and purified anti-NK1.1 (PK136, BioXCell). 100,000 purified NK cells in 10 μ l were placed in a U-bottom well and 100,000 ICAM-1 coated beads were added. To cross-link NK1.1 and measure the effect of inside-out signaling, a Goat F(ab)₂ anti-mouse IgG (10 μ g/ml, Life Technologies) was added to the wells. Interaction was fixed at the indicated time-point by addition of 100 μ l Cytotfix/Cytoperm (BD). The percentage of interaction (i.e. percentage of NKp46 positive cells attached to beads) was measured by flow cytometry.

Statistical analysis

Statistical analyses were performed using Prism 5 (Graph-Pad Software). Two tailed unpaired t-test, and ANOVA tests with Bonferroni correction were used as indicated in the figure legends. Significance is indicated as follows: *p<0.05; **p<0.01; ***p<0.001. The heatmap presented in **Figure 1A** was established as follow: we first selected the phosphoepitopes for which the MFI (Mean Fluorescence Intensity) was significantly above the one of the FMO control (Student T-test). The MFI of the 15 selected phosphoepitopes for the 4 NC sub-populations defined in **Figure 1—figure supplement 2** was then normalized to the MFI value of the NKG2A⁺Ly49C⁺ populations in the C57BL/6 mice and the values obtained were averaged to calculate the means for each populations. These values were used to establish the Heatmap using the Multiple Experiment Viewer application. We used the R statistical language to manage our database and carry out the statistical analysis (R version 3.3.2). We splitted the database into six datasets (2 Mouse strains * Differentiation subsets), each containing the 15 phospho-epitopes. We performed an ANOVA for each phospho-epitope to test for the phosphorylation difference between the 4 NC sub-populations. The parameters of the ANOVA Type I SS were adapted to control for the experiment effect. The Bartlett Homogeneity of Variances Test was applied first, when it failed to reject its H0, then the phospho-epitope was retained for the ANOVA test. The normality of the residuals of the ANOVA model was checked graphically and numerically with the Shapiro-Wilk Normality Test. When this test failed to reject its H0 then the adjusted P values for multiple comparisons were extracted with the Tukey's 'Honest Significant Difference' method.

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Additional information

Competing interests

Mathieu Bléry: MB is employee of Innate-Pharma. Eric Vivier: EV is shareholder of Innate-Pharma. The other authors declare that no competing interests exist.

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Antoine Marçais, Conceptualization, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Methodology, Writing—original draft, Writing—review and editing; Marie Marotel, Alice Koenig, Sébastien Viel, Formal analysis, Investigation, Writing—review and editing; Sophie Degouve, Formal analysis, Investigation, Methodology, Writing—review and editing; Sébastien Fauteux-Daniel, Annabelle Drouillard, Laurie Besson, Formal analysis, Investigation; Heinrich Schlums, Investigation, Methodology, Writing—review and editing; Omran Allatif, Formal analysis, Validation, Methodology; Mathieu Bléry, Eric Vivier, Resources, Writing—review and editing; Yenan Bryceson, Supervision, Validation, Writing—review and editing; Olivier Thauinat, Supervision, Validation, Methodology, Writing—review and editing; Thierry Walzer, Conceptualization, Supervision, Funding acquisition, Project administration, Writing—review and editing

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Ethics

Animal experimentation: This study was carried out in accordance with the French recommendations in the Guide for the ethical evaluation of experiments using laboratory animals and the European guidelines 86/609/CEE. All experimental studies were approved by the bioethic local committee CECCAPP (Permit number: CECCAPP_ENS_2014_018).

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