Régulation des cellules NK par le TGF-β

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REGULATION DES CELLULES NK PAR LE TGF-β

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Résumé en français
Les cellules NK sont des lymphocytes de l'immunité innée impliqués dans la reconnaissance et l'élimination de cellules tumorales ou infectées par des pathogènes intracellulaires. La biologie des cellules NK est régulée par des facteurs intrinsèques comme les facteurs de transcription ainsi que par des facteurs environnementaux comme les cytokines, produites en condition homéostatique ou inflammatoire. Certaines cytokines, comme l'IL-15, l'IL-12 ou l'IL-18 sont connues pour potentialiser les fonctions effectrices des cellules NK. L'IL-15, en activant la voie STAT5 permet, d'une part, d'assurer la survie des cellules NK et, d'autre part *via* la kinase mTOR, d'induire leur prolifération, d'augmenter leur métabolisme ainsi que leurs fonctions effectrices. D'autres cytokines comme le TGF-β sont connues pour inhiber les fonctions des cellules NK. Le TGF-β1 est une des cytokines les plus immunosuppressives du système immunitaire et, en étant sécrété par différents types de cancers, il participe à l'échappement tumoral. Depuis longtemps, les effets du TGF-β *in vitro* sont connus pour contrer ceux de l'IL-15. L'objectif de ce travail a été d'étudier les effets du TGF-β sur la biologie des cellules NK. Nous avons observé que l'ajout de TGF-β, *in vitro*, induit un blocage rapide de l'activation de la voie mTOR par l'IL-15, que le TGF-β a des effets très proches de ceux de la rapamycine, un inhibiteur spécifique de mTOR et que, *in vivo* chez la souris, l'activation constitutive des voies de signalisation activées par le TGF-β induit un phénotype proche de celui de la délétion de mTOR dans les cellules NK.

Titre en anglais
Regulation of NK cell function by TGF-β

Résumé en anglais
NK cells are innate lymphocytes involved in the recognition and elimination of tumor or infected cells. The biology of NK cells is regulated by intrinsic factors such as transcription factors but also by cytokines produced at steady state or under inflammatory conditions. Some of these cytokines like IL-15, IL-12 or IL-18 are known to increase NK cells functions. IL-15 allows NK cell survival *via* STAT5 and, *via* mTOR, increase NK cell proliferation, metabolism and acquisition of functions. In the other hand, cytokines like TGF-β are known to inhibit NK cell function. TGF-β1 is a major immunosuppressive cytokine, often secreted by tumor cells and participates to tumor escape. The inhibitory effects of TGF-β *in vitro* on IL-2/15 mediated NK cell activation have long been shown, but the mechanism remains unknown. The objective of this work was to characterize the effects of TGF-β at a molecular level. We have observed that TGF-β induces a rapid blockade of IL-15 induced mTOR activation, *in vitro*. TGF-β and the mTOR inhibitor rapamycin have similar effects. Finally, using genetic mouse models *in viv*o, constitutive TGF-β signaling or mTOR deletion results in similar developmental arrests in NK cells.

Discipline
Immunologie

Mots-clés
Cellules Natural Killer, TGF-β, mTOR, Cancer

Intitulé et adresse du laboratoire
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A mes enfants, *Jules et Maëlle,*

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Liste des publications

Article 1 :

« TGF-β inhibits NK cell activation and functions through repression of the mTOR pathway »
S Viel, A Marçais, F Souza-Fonseca Guimaraes, R Loftus, J Rabilloud, M Grau, S Degouve, S Djebali, A Sanlavile, J Bienvenu, JC Marie, C Caux, J Marvel, L Town, N Huntington, L Bartholin, D Finlay, MJ Smyth, T Walzer
Science Signaling, accepté

Articles en annexe :

Article 2 : « Monitoring NK cell activity in patients with hematological malignancies »
S Viel, E Charrier, A Marçais, P Rouzaire, J Bienvenu, L Karlin, G Salles, T Walzer
Oncoimmunology, 2013

Article 3 : « Regulation of mouse NK cell development and function by cytokines »
A Marçais, S Viel, M Grau, T Henry, J Marvel, T Walzer
Frontiers in immunology, 2013

Article 4 : « T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow»
C Daussy, F Faure, K Mayol, S Viel, G Gasteiger, E Charrier, J Bienvenu, T Henry, J Marvel, K Yoh, S Takahashi, I Prinz, S de Bernard, L Buffat, T Walzer
Journal of Experimental Medicine, 2014

Article 5 : « The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells »
A Marçais, J Cherfils-Vicini, C Viant, S Degouve, S Viel, A Fenis, J Rabilloud, K Mayol, A Tavares, J Bienvenu, YG Gangloff, E Gilson, E Vivier T Walzer
Nature Immunology, 2014

Article 6 : « Natural Killer cells from Multiple Myeloma patients display an exhausted phenotype and their functions are not improved by lenalidomide treatment »
S Viel, E Charrier, O Allatif, L Besson, A Marçais, A Belot, L Karlin, G Salles, J Bienvenu, T Walzer
En préparation

Article 7 : « Alteration of NK Cell phenotype and function in obese individuals »
S Viel, L Besson, E Charrier, A Marçais, E Disse, J Bienvenu, T Walzer, C Dumontet
Clinical Immunology, accepté

Liste des abréviations

- ADCC : Cytotoxicité dépendante des anticorps (Antibody Dependant Cell Cytotoxicity)
- CD : Cluster of Differentiation
- CILP : Progéniteur commun aux ILC (Common ILC Progenitor)
- CLP : Progéniteur lymphoïde commun (Common Lymphoid Progenitor)
- CMH : Complexe Majeur d'Histocompatibilité
- CSH : Cellule Souche Hématopoïétique
- DC : Cellule dendritique (Dendritic Cells)
- EBV : Epstein Barr Virus
- EMT : Transition Epithelio-Mésenchymateuse
- FDA : Food and Drug Administration
- FKBP12 : FK506 Binding Protein 12
- GVL : Graft Vs Leukemia
- IgG : Immunoglobuline de type G
- IL : Interleukine
- ILC : Cellule lymphoïde innée (Innate Lymphoid Cells)
- IMiDs : Immunomodulatory Drugs
- iNK : cellules NK immature (immature NK cell)
- ITAM : Immunoreceptor-based activation motif
- ITIM : Immunoreceptor tyrosine-based inhibition motif
- JAK : Janus Kinase
- KIR : Killer cell Ig-like Receptors
- LAL : Leucémie Aigüe Lymphoïde
- LAM : Leucémie Aigüe Myéloïde
- LAP : Latency Associated Protein
- LCMV : Lymphocytic choriomeningitidis virus
- LLC : Large Latent Complex
- LMC : Leucémie Myéloïde Chronique
- LNH : Lymphome Non Hodgkinien
- LTBP : Large Latent TGF-β binding Protein
- LTi : Inducteurs de tissu lymphoïde (Lymphoïd Tissue inducer)
- MCMV : Murine Cytomegalovirus
- MDS : Syndrome Myélodysplasique (Myelodysplasic Syndrom)
- MICA/B : *major* histocompatibility complex class I chain–related molecules A/B
- mNK : cellules NK matures (Mature NK Cell)
- MM : Myélome Multiple
- mTOR : mammalian Target Of Rapamycin
- NCR : Récepteur de la cytotoxicité naturelle (Natural Cytotoxicity Receptor)
- NK : Natural Killer
- NKP : Précurseur des cellules NK (NK Precursor)
- PRR : Pattern Recognition Receptor
- RIRS : Récepteur Inhibiteur Reconnaissant le Soi
- ROR : Retinoid acid-related orphan receptor
- S1P : Sphingosin-1 Phosphate
- SAP : SLAM Associated Protein
- SLC : Small Latent Complex
- SMP : Syndrome Myéloprolifératif
- SNC : Système Nerveux Central

- TCR : T cell Receptor
- Tem : Lymphocytes T effecteurs mémoires
- TGF : Facteur de croissance transformant (Transforming Growth Factor)
- TLR : Toll-Like Receptor
- TLSP : Lymphopoïétine stromale thymique (Thymic Stromal Lymphopoietin)
- TNF : Facteur de nécrose tumorale (Tumor Necrosing Factor)
- Tox : Thymocyte selection-associated high mobility group box
- Treg : Lymphocytes T régulateurs
- ULBP : UL16 Binding Protein

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1. Les Cellules Innées Lymphoïdes

Les cellules Natural Killer (NK) sont des cellules lymphoïdes innées (ILC), découvertes grâce à leur capacité à éliminer spontanément des cellules tumorales. Considérant leur capacité à produire des cytokines de type Th1, et notamment de l'Interféron (IFN)-γ, les cellules NK ont été récemment reclassifiées comme des membres des cellules lymphoïdes innées de type 1 (Spits et al., 2013) (Figure 1).

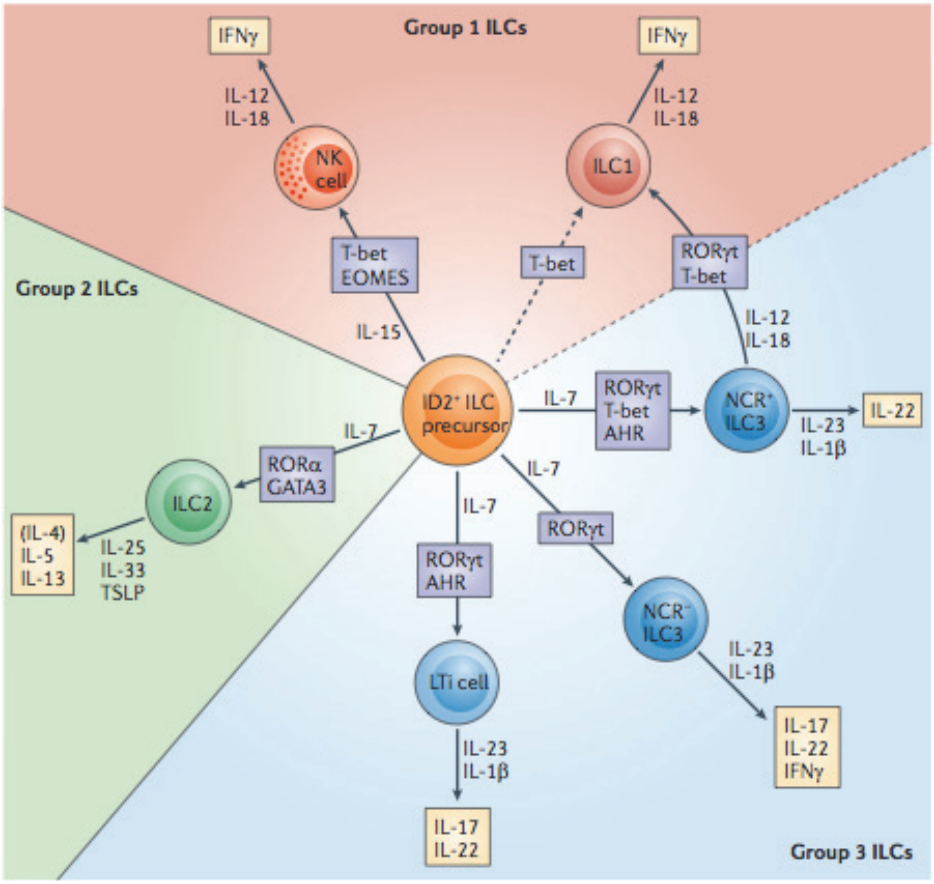

Figure 1: Classification des ILC
La classification des ILC est basée sur des critères fonctionnels. Les ILC1 sont définies par leur capacité à produire de l'IFN-γ. Les ILC2 sont capables de produire des cytokines de type Th2 (IL-4, IL-5, IL-13) tandis que les ILC3 produisent des cytokines de type Th17 (IL-17 et IL-22). (Spits et al., 2013)

Les principales caractéristiques des ILC de type 1 sont l'expression de T-bet et la capacité à produire de l'IFN-γ. Alors que les cellules NK conventionnelles forment une population plutôt homogène et sont essentiellement localisées dans le sang

et la rate, les ILC1 résident essentiellement dans les tissus. En fonction de leur tissu de résidence, les ILC1 présentent des caractéristiques phénotypiques particulières, expriment des facteurs de transcription différents et supportent des voies de différenciation distinctes. Les ILC1 se différencient donc des cellules NK conventionnelles mais forment également une population de cellules hétérogène entre elles.

Phénotypiquement, les cellules NK conventionnelles expriment classiquement CD49b, CD11b et KLRG1 (Figure 2), tandis que les ILC1 expriment CXCR6, CD127, CD160 et CD226. Les ILC1 du foie expriment en plus DX5 et TRAIL.

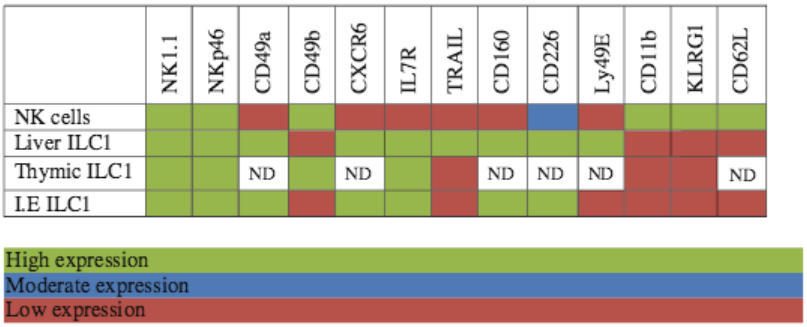

Figure 2: Principales caractéristiques phénotypiques des cellules NK conventionnelles et des ILC1.
(d'après (Seillet et al., 2015))

En ce qui concerne leurs fonctions effectrices, les ILC1 et les cellules NK sont capables de produire de l'IFN-γ. En revanche, les ILC1 sont moins cytotoxiques que les cellules NK. Les ILC1 hépatiques expriment plus faiblement granzyme B et perforine que les cellules NK conventionnelles (Daussy et al., 2014) tandis que les ILC1 des glandes salivaires expriment faiblement CD107a après une stimulation par des cytokines (IL-12/18) ou par des ionophores calciques (Cortez et al., 2014). Ces deux types d'ILC1 sont cependant douées de cytotoxicité impliquant d'autres voies que la voie perforine/granzyme puisqu'à la différence des cellules NK conventionnelles, elles expriment plus fortement TRAIL (Figure 2). En qui concerne les fonctions biologiques des ILC1, il est maintenant largement admis que les cellules NK jouent un rôle dans la réponse anti-tumorale, dans la réponse précoce contre les pathogènes intracellulaires et dans la régulation des réponses T. Du fait de la découverte plus récente de leur existence, le rôle des ILC1 est moins bien connu, mais il semble que ces cellules jouent essentiellement un rôle dans l'homéostasie des tissus qui les hébergent.

Chez des patients atteints de la maladie de Crohn, la fréquence des ILC1 augmentent d'ailleurs dans les tissus inflammés (Bernink et al., 2013). Si leur potentiel cytotoxique est moins développé que les cellules NK, les ILC1 semblent tout de même jouer un rôle dans la défense contre les pathogènes intracellulaires (Klose et al., 2014), *via* la production d'IFN-γ. Elles participent ainsi à l'immunité muqueuse.

Les autres membres des ILC sont les ILC2, qui se développent sous l'influence de l'IL-7 et produisent des cytokines de type Th2 (Interleukine (IL) -5, IL-13) en réponse à des stimulations par l'IL-25, l'IL-33 ou par la lymphopoïetine stromale thymique (TLSP) (Figure 1). Les ILC3 se caractérisent, quant à elles, par leur capacité à produire des cytokines IL-17 et/ou IL-22. Elles dépendent du facteur de transcription Retinoid acid-related orphan receptor (ROR)γt pour leur développement et leur fonction. Le groupe des ILC3 constitue un ensemble hétérogène de cellules comprenant les cellules « Lymphoid tissue-inducer » (LTi) ainsi que les ILC3 NCR^+ (Natural Cytotoxicity Receptor) et NCR^-.

Parmi les ILC qui sont toutes capables de produire des cytokines, seules les cellules NK possèdent une activité cytotoxique. Celle-ci est dirigée contre des cellules reconnues comme des cibles *via* un panel de récepteurs activateurs et inhibiteurs. C'est d'ailleurs cette propriété d'élimination spontanée de cellules tumorales qui a permis leur caractérisation en 1975 (Kiessling et al., 1975). Comme les autres cellules du système immunitaire, leur développement, maturation, prolifération et l'acquisition de leurs fonctions effectrices sont régulés par de nombreux signaux dont les cytokines. L'IL-15 est essentielle pour leur développement, maturation et activation. D'autres cytokines activatrices ou inhibitrices (IL-2, IL-4, IL-7, IL-10, IL-12, IL-18, Transforming Growth Factor (TGF)-β régulent la biologie des cellules NK à différentes étapes de la réponse immunitaire.

2. Les Cellules Natural Killer

2.1. Développement des cellules NK

2.1.1. Dans la moelle

Au cours de leur développement, les cellules NK passent par différents stades de maturation. S'il n'existe pas, à l'heure actuelle, de consensus qui définisse ces intermédiaires, certains auteurs s'accordent sur les marqueurs qui les caractérisent, chez la souris (Figure 3) et chez l'homme (Figure 4).

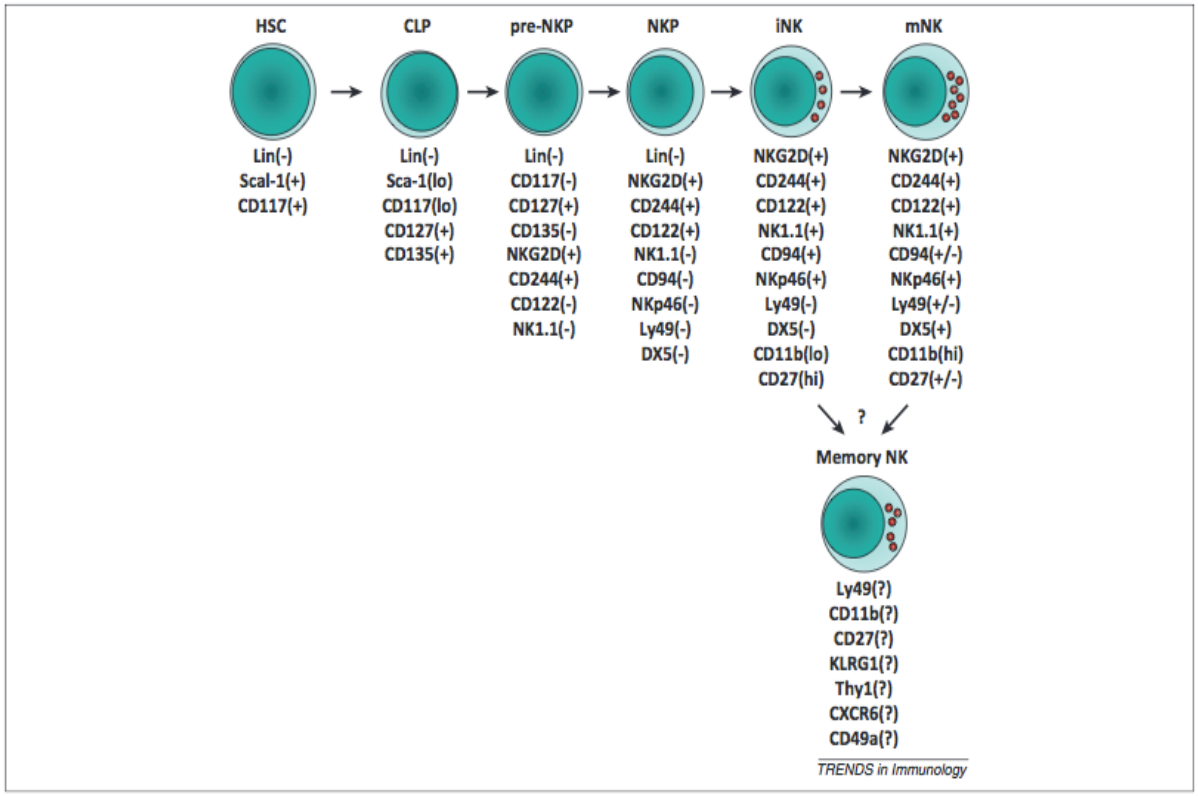

Figure 3: Développement des cellules NK murines
A partir de la cellule souche hématopoïétique, puis du progéniteur lymphoïde commun, les cellules NK se développent en 4 stades : pré-NKP, NKP, NK immatures puis NK matures. (Yu et al., 2013)

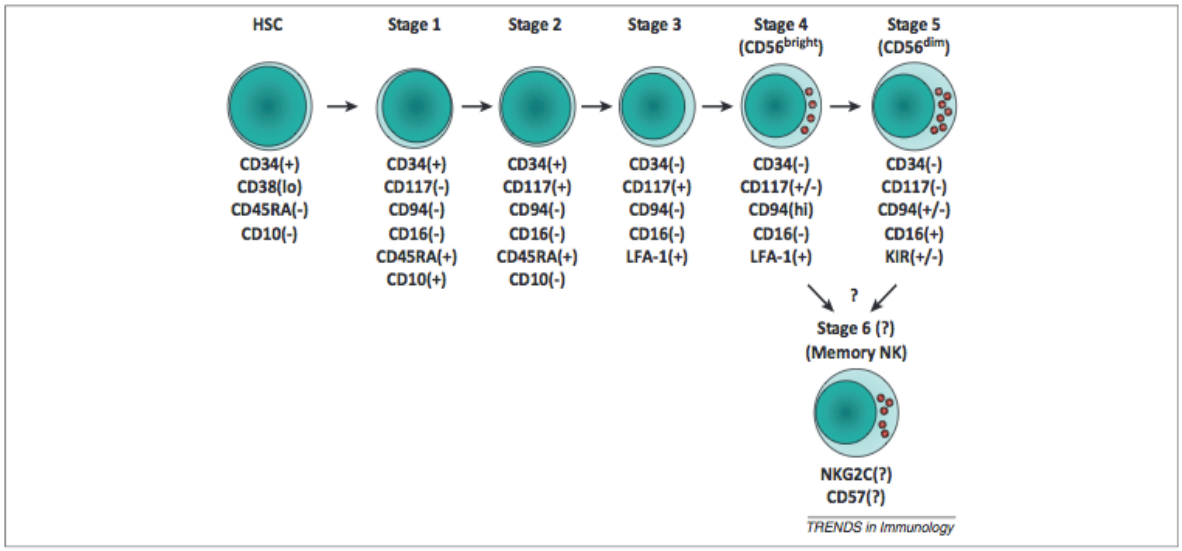

Figure 4: Développement des cellules NK humaines
Représentation linéaire du développement des cellules NK humaines en 5 étapes à partir de la cellule souche hématopoïétique. (Yu et al., 2013)

Les cellules NK se développent à partir d'une cellule souche hématopoïétique (CSH) de la moelle osseuse *via* un progéniteur lymphoïde commun (CLP) capable de donner naissance à tous les sous-types de lymphocytes (Figure 5).

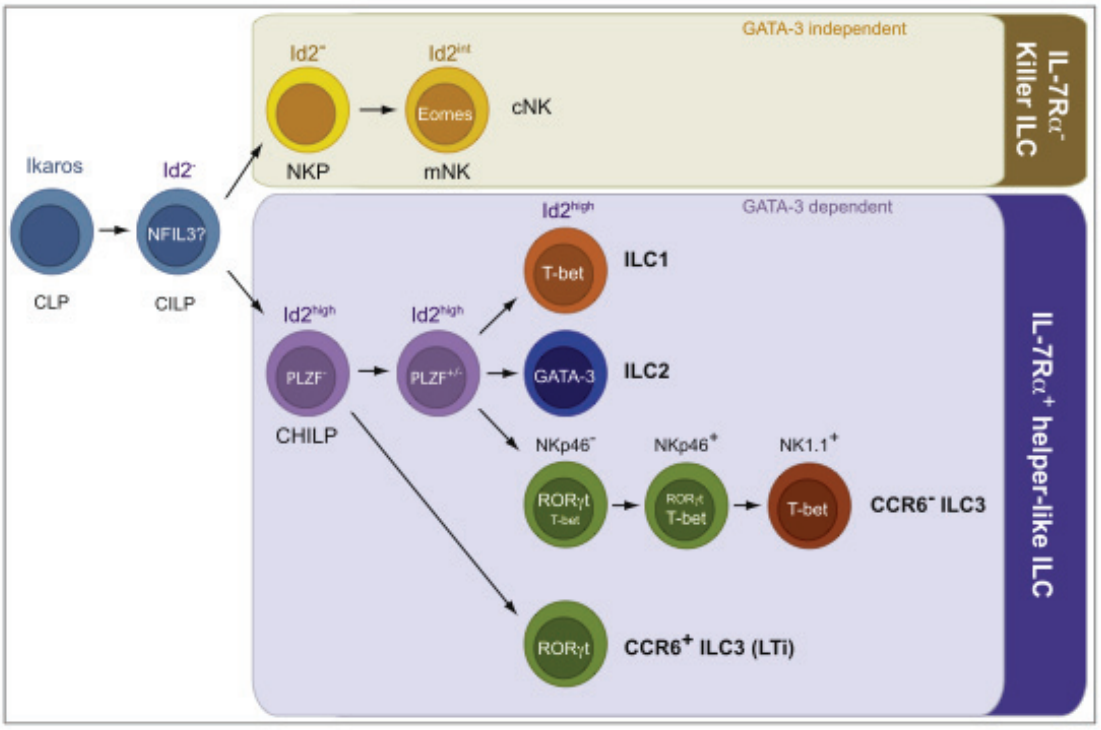

Figure 5: Développement des ILC
A partir du CLP, les ILC passent par un stade intermédiaire commun appelé CILP. Puis elles s'engagent dans deux voies différentes, la voie des ILC tueuses et la voie des ILC auxiliaires. (Diefenbach et al., 2014)

L'existence d'un progéniteur commun aux ILC (CILP), dérivant du CLP, a récemment été mise en évidence. Le développement de cette cellule progénitrice semble être sous le contrôle de la protéine de liaison à l'ADN Id2, les souris déficientes pour le gène ayant un déficit des 3 lignages d'ILC alors qu'elles ont un compartiment T et B normal (Diefenbach et al., 2014).

Les CSH et les CLP humains appartiennent respectivement aux fractions Lin^- $CD34^+CD38^{dim}CD45RA^-CD10^-$ et $CD34^+CD38^+CD45RA^+CD10^+$ du pool de cellules progénitrices hématopoïétiques (Galy et al., 1995) (Figure 4).

Les CSH murines sont, elles, définies comme Lin^-, $Sca\text{-}1^+$, $c\text{-}Kit^+$ alors que les CLP murines Lin^-,$Sca\text{-}1^{lo}$,$CD117^{lo}$,$CD127^+CD135^+$. De façon intéressante, des populations dont le phénotype ressemble à celui des CLP ont également été mises en évidence dans des tissus extra-médullaires suggérant l'existence d'un développement des cellules T et NK possible en dehors de la moelle osseuse (Res et al., 1996).

L'acquisition de la chaîne β du récepteur de l'IL-15 (CD122) constitue, ensuite, une étape importante dans la différenciation NK à partir du CLP.

On définit, en théorie, le précurseur NK (NKP) comme une cellule : (i) capable de se différencier en cellules NK sous l'influence d'IL-15 (expression des chaînes β (CD122) et γc (CD132) du récepteur de l'IL-15); (ii) qui n'a pas les caractéristiques phénotypiques et fonctionnelles des cellules NK matures (mNK) ; et (iii) qui n'exprime aucun autre antigène de surface spécifique de lignée comme CD3, CD14 ou CD19. En pratique, seulement une petite fraction des cellules qui répondent à cette définition (moins de 10%) sont capables de générer des cellules NK matures, *in vitro* (Rosmaraki et al., 2001). Il est donc très probable que seul un sous-type de ces cellules représente les réels progéniteurs des cellules NK.

Chez la souris, le stade NKP ($Lin^-CD122^+NK1^-DX5^-$) (Figure 3) a longtemps été considéré comme le stade le plus immature présent dans la moelle. Mais, en 2011, un stade plus précoce, dénommé « pré-NKP » a été mis en évidence (Carotta et al., 2011; Fathman et al., 2011).

Chez l'homme, il est nécessaire d'utiliser un grand nombre de marqueurs pour différencier les cellules du stade 2 (équivalent du NKP chez la souris) (Lin^- $CD34^{dim}CD45RA^+$ $\alpha4\beta7^{bright}$ $CD117^+CD161^{+/-}CD94^-$) des cellules du stade 3 (équivalent des iNK chez la souris) ($Lin^-CD34^-\alpha4\beta7^-CD117^+CD161^+CD94^-$) (Figure 4). Ces 2 stades répondent, cependant, tous les deux à l'IL-15 et donnent naissance

à des NK matures. Bien que les cellules du stade 3 représentent un stade intermédiaire de la lignée NK du fait de leur incapacité à se différencier en cellules B, T ou cellules dendritiques (DC) *in vitro*, cette population reste hétérogène en ce qui concerne l'expression de plusieurs antigènes de surface : CD7, CD56, CD121A, CD127, NKp44 et LFA-1 (Freud and Caligiuri, 2006). Des données récentes suggèrent qu'il existe, en réalité, des chevauchements de phénotype entre d'authentiques cellules du stade 3 et certains sous-types d'ILC.

L'acquisition des récepteurs fonctionnels apparaît dans le même ordre pendant le développement des cellules NK, *in vitro*, suite à un transfert adoptif ou dans le cadre de greffes de CSH, chez l'homme.

Chez la souris, les précurseurs, acquièrent d'abord NK1.1, CD94/NKG2A et NKp46 suivis par les récepteurs Ly49, DX5 et finalement CD11b (Kim et al., 2002) (Figure 3). Chez l'homme, une acquisition progressive et ordonnée apparaît également dans l'ordre suivant : (i) : CD161, (ii) CD56, CD94, NKp46 et NKG2D (iii) KIRs (killer cell Ig-like receptors) et CD16. L'expression de certains antigènes, comme CD56 chez l'homme et NKp46, CD94 et CD27 dans les deux espèces est diminuée, voire disparaît, dans les stades finaux du développement NK.

La maturation s'accompagne de l'acquisition progressive des fonctions effectrices, cytotoxicité et production d'IFN-γ. Chez la souris, on peut détecter la cytotoxicité et la production de cytokines dès le stade $CD11b^-$, mais ces fonctions sont plus marquées après l'acquisition de CD11b et de CD94 (Kim et al., 2002). Ces cellules NK immatures (iNK) ont été désignées: $Lin^-CD122^+NK1.1^+CD94^+Ly49^-DX5^-CD11b^-$ (Loza and Perussia, 2001) (Figure 3). Chez l'homme, les capacités fonctionnelles des cellules NK ne sont également essentiellement détectées qu'à partir du stade 4 (Figure 4) (Lin^- $CD34^-$ $CD117^{+/-}$ $CD94^+$ $CD56^{bright}$ $CD16^-$) indiquant que l'expression de CD94 précède la maturation fonctionnelle. Les cellules intermédiaires du stade 3 humaines ressemblent aux iNK murines, mais diffèrent par leur absence d'expression de CD94.

Avant de terminer leur maturation (stade $CD56^{dim}$), les cellules NK humaines passent par un dernier stade intermédiaire, « $CD56^{bright}$ » (Figure 4). Les cellules NK $CD56^{bright}$ sont essentiellement présentes dans les organes lymphoïdes secondaires. Elles ont d'importantes capacités de production de cytokines, mais des capacités cytotoxiques plus faibles que les cellules NK $CD56^{dim}$ qui sont localisées essentiellement dans le sang périphérique. Il est aujourd'hui admis que les cellules

NK CD56^{bright} sont des précurseurs des cellules NK CD56^{dim} (Chan et al., 2007), d'où leur désignation comme cellules intermédiaires du stade 4. Les cellules CD56^{bright} sont majoritaires dans les tissus néonataux, dans le sang de cordon et sont les premières à apparaître après une greffe de CSH. Les cellules NK CD56^{dim} (stade 5) apparaissent plus tardivement dans la vie, ou après greffe de CSH.

Chez la souris, le marqueur CD11b (ou Mac1) a d'abord été utilisé pour séparer les cellules NK (Kim et al., 2002). Les cellules NK CD11b^{-} sont localisées essentiellement dans la moelle osseuse, les ganglions lymphatiques et le foie. Considérées comme les plus immatures, elles possèdent d'importantes propriétés prolifératives et d'auto-renouvellement, mais ont des capacités fonctionnelles plus limitées. Les cellules CD11b^{+} sont localisées en périphérie (rate, sang). Ces cellules, plus matures, ont perdu leur capacité de prolifération, mais expriment davantage de récepteurs NK et ont des capacités fonctionnelles plus développées que les cellules CD11b^{-}. Plus récemment, il a été défini un programme de maturation des cellules NK murines en 4 étapes (Chiossone et al., 2009), en fonction de l'expression membranaire de CD27, protéine de la famille des récepteur du TNF (Tumor Necrosis Factor) et de CD11b (Figure 6) : CD11b^{-}CD27^{-} (DN : doubles négatives), CD11b^{-} CD27^{+} (aussi appelées CD11b^{-}) CD11b^{+}CD27^{+} (DP : doubles positives) et CD11b^{+}CD27^{-} (CD27^{-}).

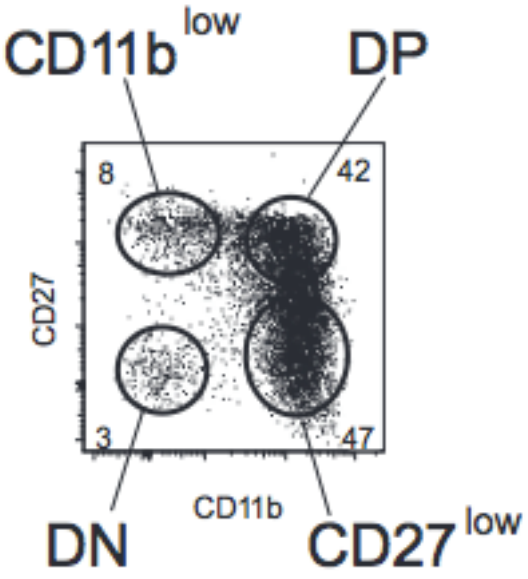

Figure 6: Différenciation terminale des cellules NK murines
Les cellules NK murines passent par 4 stades de maturation en fonction de l'expression membranaire de CD27 et CD11b : CD11b^{-}, DP et CD27^{-}, stade de différenciation terminal. (Chiossone et al., 2009)

La population DN a longtemps été considérée comme un stade précédant le stade CD11b$^-$. En effet, elles sont les premières à réapparaitre après la déplétion des cellules exprimant NKp46 (Chiossone et al., 2009). Cependant, ces cellules ont des caractéristiques inattendues par rapport aux autres cellules de la lignée NK. Tout d'abord, elles n'expriment pas CD27, alors que cette molécule est exprimée par toutes les cellules des stades plus précoces (iNK, NKP et pré-NKP) (Carotta et al., 2011; Fathman et al., 2011; Klose et al., 2014), mais également par les cellules des stades plus matures, CD11b$^-$ et DP. De plus, alors que le stade CD11b$^-$ est enrichi dans la moelle osseuse, les cellules NK DN sont présentes à la même fréquence dans la moelle et la rate. Il est maintenant suggéré que ces cellules DN sont plutôt des ILC1 Eomes$^-$ (Daussy et al., 2014).

Les cellules NK CD11b$^-$ sont considérées comme des précurseurs, capables de se différencier. Elles sont peu présentes en périphérie et se localisent essentiellement dans la moelle et les ganglions. Les DP et CD27$^-$ sont les formes effectrices, ayant perdu leur capacité d'auto-renouvellement, même si, dans certains contextes, elles peuvent participer à l'expansion du pool de cellules NK lors d'une réponse immunitaire. Elles représentent la majorité des cellules NK dans les organes périphériques et dans le sang. Chez la souris, la proportion relative de ces 4 sous populations varie donc en fonction des organes mais évolue également au cours de la vie de ces animaux.

2.1.2. Dans le foie

Le foie est le principal lieu de l'hématopoïèse pendant la phase embryonnaire du développement. A la naissance, une population de cellules NK particulières, exprimant Trail, est majoritaire dans le foie. Avec l'âge, les cellules NK conventionnelles, en migrant dans le foie, deviennent majoritaires.

Chez l'adulte, les cellules NK conventionnelles (CD49b$^+$, Trail$^-$) coexistent donc avec les cellules NK résidentes ou ILC1 du foie, CD49a$^+$Trail$^+$ (Takeda et al., 2005). Par des analyses transcriptomiques, nous avons montré que ces deux groupes ont des profils d'expression génique différents suggérant qu'ils proviennent d'une lignée distincte (Daussy et al., 2014). A la différence des NK conventionnelles, les ILC1 du foie n'expriment pas Eomes, ni CX3CR1, S1P1 et S1P5, ce qui semble expliquer leur incapacité à recirculer. Le rôle exact de ces cellules n'est pas totalement compris, mais comme les cellules NK, les ILC1 du foie sont cytotoxiques et expriment la perforine et les granzymes. Elles semblent également jouer un rôle régulateur,

directement (Terme et al., 2012) ou indirectement par des interactions avec les lymphocytes T. Certains auteurs, dans des modèles d'hypersensibilité aux haptènes (Paust et al., 2010; Peng et al., 2013; Rouzaire et al., 2012a), leur confèrent même des capacités de mémorisation. Ces propriétés n'ont, néanmoins, été observées que dans des modèles de transfert adoptif.

2.1.3. Autres sites de différenciation des cellules NK

Les cellules NK du thymus ont été décrites en 2006 (Vosshenrich et al., 2006). A la différence des cellules NK conventionnelles, elles expriment CD127, la chaîne α du récepteur de l'IL-7 et dépendent de GATA-3 pour leur développement. Ces ILC1 thymiques peuvent se développer *in vitro* et *in vivo* à partir d'un sous-type de thymocytes immatures (Vargas et al., 2011) suggérant qu'elles ne dérivent pas d'un précurseur de la moelle osseuse (Klose et al., 2014). Du fait de leur très faible nombre dans le thymus, ces cellules sont peu étudiées et leur rôle, mal connu.

Chez la souris, des populations d'ILC1 ont également été décrites dans l'intestin (Fuchs et al., 2013). Elles dépendent de T-bet, mais, à la différence des cellules NK conventionnelles, elles ne nécessitent pas l'expression d'IL-15Rα. Leurs équivalents chez l'homme ont également été identifiés. Comme les ILC1 du foie, elles se caractérisent par une expression de CD49a, CXCR6 et CD69 (Fuchs et al., 2013). Une dernière population de cellules NK intra-épithéliales, les ILC1 des glandes salivaires sont caractérisées par une expression de T-bet et Eomes ainsi que de faibles capacités de production d'IFN-γ (Cortez et al., 2014). Du fait de leur découverte récente, leur rôle n'est pas connu.

2.1.4. Facteurs impliqués dans la différenciation des cellules NK

a. Facteurs intrinsèques: rôle des facteurs de transcription

Le réseau de facteurs de transcription qui régule le développement et la maturation des ILC et plus particulièrement des cellules NK est complexe. D'une part, certains facteurs de transcription agissent à différents niveaux de la différenciation. D'autre part, ils ont parfois des rôles redondants, antagonistes ou séquentiels ce qui rend la compréhension de leur rôle intrinsèque difficile à appréhender. Depuis quelques années et la découverte des ILC, ce champ d'application connaît des découvertes importantes même si certains points restent encore à élucider. Les principaux

facteurs de transcription impliqués dans la différenciation et la maturation des cellules NK sont représentés sur la Figure 7.

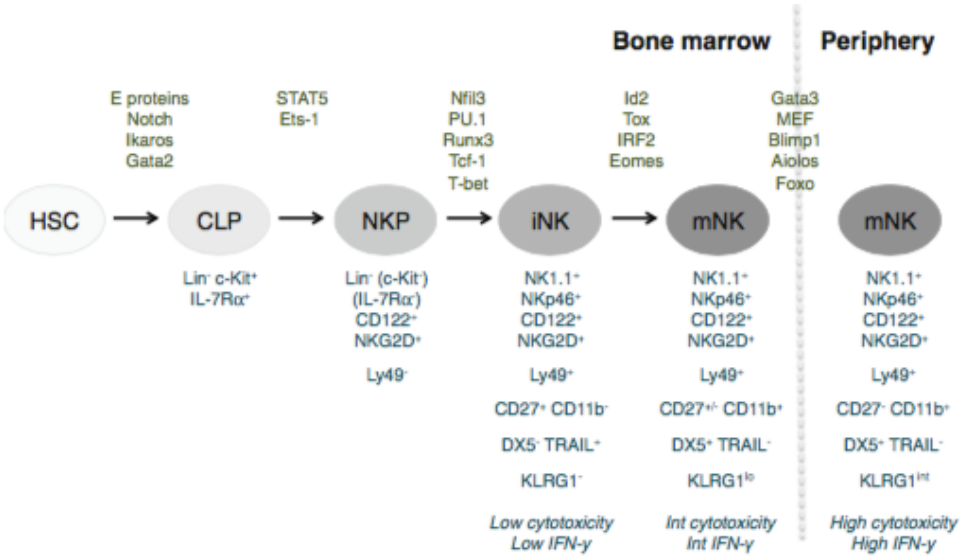

Figure 7: Facteurs de transcription impliqués dans le développement des cellules NK
Les facteurs de transcription ainsi que leurs cibles potentielles sont indiqués pour chaque stade de développement des cellules NK conventionnelles.(Sun, 2015)

Alors que le stade NKP représente un stade précurseur distinct des précurseurs des cellules B et T, il n'est pas encore certain que ce précurseur ne puisse s'engager dans d'autres lignées d'ILC. Cependant, différents facteurs de transcription sont indispensables au développement des ILC, sans affecter l'activité des précurseurs des cellules B et T. C'est le cas d'Id2, qui agit principalement en bloquant l'activité des facteurs de transcription à « E box ». En l'absence d'Id2, le développement NK est altéré ainsi que celui des ILC2 et 3 alors que le système immunitaire adaptatif n'est pas touché (Diefenbach et al., 2014). Id2 agit donc très tôt dans le développement des ILC et semble participer à la génération du précurseur commun aux ILC, le CILP (Common ILC progenitor). Un autre exemple de facteur de transcription qui affecte essentiellement les ILC est celui de Tox (Thymocyte selection-associated high mobility group box). L'absence de Tox induit des perturbations importantes du compartiment NK (Aliahmad et al., 2010) et des autres ILC (Seehus et al., 2015). Comme Id2, Tox est essentiel pour le développement du CILP *in vitro* et *in vivo* (Seehus et al., 2015).

Le facteur de transcription Nfil3 joue un rôle essentiel dans le développement des cellules NK. En l'absence de Nfil3, la différenciation est bloquée au stade iNK (Gascoyne et al., 2009; Kamizono et al., 2009). Nfil3 semble également agir plus précocement et de manière indirecte, en régulant l'expression de Tox (Yu et al.,

2014) impactant ainsi le développement global de tous les types d'ILC (Geiger et al., 2014).

Le facteur de transcription T-bet a d'abord été identifié comme un facteur de polarisation des lymphocytes T $CD4^+$ vers le profil Th1 (Szabo et al., 2000), mais la majorité des ILC1s, dont les cellules NK conventionnelles, dépendent au moins en partie de T-bet pour leur développement (Daussy et al., 2014). T-bet est donc devenu le facteur de transcription qui définit les ILC de type 1 (Figure 5). Dans les cellules NK, T-bet agit conjointement avec Eomes pour promouvoir la maturation et la fonction des cellules NK (Gordon et al., 2012), mais à des stades différents. En accord avec la dépendance des cellules NK vis à vis de l'IL-15 pour leur développement, cette cytokine est connue pour induire T-bet (Townsend et al., 2004) qui, à son tour, favorise la sécrétion d'IFN-γ (Szabo et al., 2000). Les cellules NK déficientes pour T-bet présentent des défauts de leurs fonctions effectrices, cytotoxicité et sécrétion d'IFN-γ (Townsend et al., 2004). La double déficience T-bet/Eomes conduit à une absence de cellules NK matures, mais n'altère pas le développement du précurseur NKP (Gordon et al., 2012). Ceci montre que T-bet et Eomes ne sont pas requis pour la formation du NKP, mais sont nécessaires pour l'engagement stable des cellules NK après ce stage. Dans les cellules NK conventionnelles, Eomes est requis pour la maturation et notamment pour le passage du stade $CD11b^-$ au stade $CD11b^+$. L'expression de T-bet, qui augmente après la sortie des cellules NK de la moelle, permet la différenciation terminale des cellules NK conventionnelles (Figure 8). Dans le foie, T-bet est finalement requis pour le développement et la maturation des cellules NK résidentes, mais pas Eomes (Daussy et al., 2014).

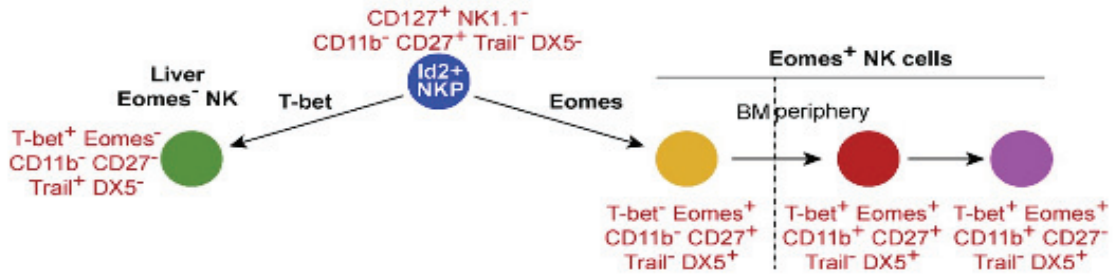

Figure 8: Rôle de T-bet et Eomes dans la maturation des cellules NK conventionnelles et du foie.
Dans la moelle osseuse, Eomes permet la maturation des cellules NK et notamment l'acquisition de l'expression de CD11b. T-bet, dont l'expression augmente en périphérie, permet la maturation terminale des cellules NK conventionnelles. Dans le foie, le développement des cellules NK hépatiques est dépendante de T-bet, mais pas d'Eomes. (Daussy et al., 2014)

Le facteur de transcription GATA-3 est connu pour contrôler différents aspects de la différenciation des cellules NK (Samson et al., 2003; Vosshenrich et al., 2006). Le fait que la déficience totale de GATA-3 soit létale, chez la souris, a rendu complexe l'étude de son rôle dans le développement des cellules NK. Pour y remédier, les auteurs ont transféré du foie fœtal d'embryons murins dans des souris $Rag2^{-/-}$. Dans les souris receveuses, le nombre de cellules NK est normal dans la moelle suggérant que ce facteur de transcription joue un rôle dans la maturation terminale des cellules NK, mais pas dans leur différenciation. Les cellules NK déficientes pour GATA-3 ont des défauts de maturation et des défauts de production d'IFN-γ. Si ce facteur de transcription ne semble pas indispensable dans les phases précoces du développement des cellules NK conventionnelles, il semble l'être, en revanche, pour les cellules NK thymiques. La génération d'animaux sélectivement déficients pour GATA-3 dans les cellules NK, en utilisant le système Cre-lox, permettrait de revisiter le rôle exact de ce facteur de transcription dans la biologie des cellules NK.

b. Facteurs extrinsèques: rôle de l'environnement

Une des étapes clés dans la différenciation des cellules NK est l'acquisition de la réponse à l'IL-15. Certaines molécules comme l'IL-3, l'IL-7, c-kit ligand et flt3 ligand, sécrétées par les cellules stromales, favorisent la transition, des précurseurs non répondeurs aux stades intermédiaires répondeurs à l'IL-15, *in vitro* (Yu et al., 1998). Ces cytokines semblent agir de façon indirecte en facilitant le développement des DC qui sont une source majeure d'IL-15 (Guimond et al., 2010).

Les autres régulateurs connus du développement des cellules NK incluent l'IL-1β qui diminue la sensibilité des cellules NK à l'IL-15 (Hughes et al., 2010), le TGF-β qui inhibe la différenciation des cellules progénitrices hématopoïétiques en cellules NK (Allan et al., 2010) et la voie Axl/Gas6 qui facilite le développement des cellules NK induit par l'IL-15 et les ligands de c-kit et flt3 (Park et al., 2009).

2.2. Circulation des cellules NK

L'export des cellules NK matures, de la moelle osseuse vers la périphérie, est dépendante de S1P5 (Sphingosine-1 phosphate receptor 5) (Walzer et al., 2007a) et de CXCR4 (Mayol et al., 2011). S1P5 est un récepteur de S1P, lipide pour lequel il existe un gradient de concentration entre les tissus et le sang. Les concentrations de S1P sont, en fait, élevées dans le sang et la lymphe et faibles dans les organes lymphoïdes. L'existence de ce gradient, couplé au fait qu'au cours de la maturation des cellules NK, l'expression de S1P5 augmente, favorise la sortie des cellules NK des organes lymphoïdes *via* les canaux lymphatiques efférents (Mayol et al., 2011). Parallèlement à l'augmentation de l'expression de S1P5, l'expression de CXCR4 diminue au cours de la maturation. CXCR4 est le récepteur de CXCL12, chimiokine sécrétée, entre autre, par les cellules stromales de la moelle (Tokoyoda et al., 2004) et qui permet la rétention des cellules souches hématopoïétiques, mais également des cellules NK immatures qui expriment CXCR4. La sortie homéostatique des cellules NK de la moelle dépend donc, au moins, de ces deux signaux : engagement de S1P5 et diminution de l'expression de CXCR4 (Figure 9).

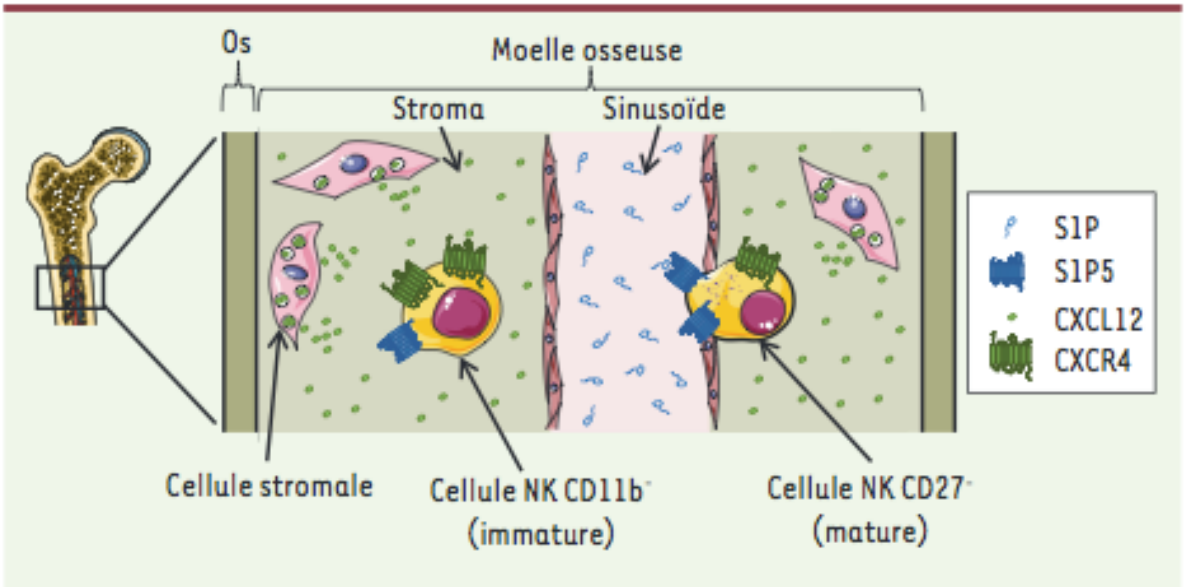

Figure 9: Sortie des cellules NK de la moelle vers la périphérie
Lors de la maturation, l'expression de S1P5 augmente en même temps que celle de CXCR4 diminue permettant aux cellules NK de suivre le gradient de S1P et de sortir de la moelle. (Rouzaire et al., 2012b)

Une fois en périphérie, certaines cellules NK sont capables de recirculer en condition homéostatique. Des travaux utilisant des souris parabiontes, ont montré que les cellules NK matures de la rate et du poumon étaient capables de migrer d'un organe à l'autre *via* la circulation sanguine alors que les cellules NK des ganglions, du foie et de la moelle osseuse étaient plutôt résidentes (Thomas et al., 2011).

En condition inflammatoire, les cellules NK sont également capables de migrer dans différents organes comme les ganglions, les poumons, le foie ou le système nerveux central (SNC) (Trinchieri, 1989), grâce notamment à des récepteurs chémotaxiques. Ces principaux récepteurs sont CCR2, CCR5, CXCR3 et CX3CR1 qui permettent aux cellules NK de répondre à un large panel de chémokines pro-inflammatoires (CCL2-3, CCL5, CCL7-9, CCL11, CCL13, CXCL9-11 et CX3CL1) (Grégoire et al., 2007). Cette large réactivité pourrait permettre de garantir le recrutement dans les tissus des cellules NK dans les situations pathologiques où seul un nombre limité de chémokines est exprimé. L'interaction des chémokines avec leurs récepteurs permet donc la migration des cellules NK dans différents tissus comme dans le foie *via* CCL2/CCL3 (Hokeness et al., 2005; Salazar-Mather et al., 1998), dans le poumon (Morrison et al., 2003; Shang et al., 2000; Zeng et al., 2003), dans le SNC (Thapa et al., 2007; Trifilo et al., 2004) ou la rate (Grégoire et al., 2008). Elle permet de

mobiliser les cellules NK dans des contextes infectieux (viraux, bactériens et fongiques), tumoraux, ou après injection de ligands de TLR (Toll Like Receptors).

2.3. Récepteurs NK

En l'absence d'un répertoire de récepteurs générés par la recombinaison d'ADN, les cellules NK doivent compter sur des récepteurs innés, codés par la lignée germinale, pour distinguer les cellules saines de cellules qui doivent être éliminées. Pour cela, les cellules NK disposent d'un grand nombre de récepteurs, chacun ayant une spécificité unique pour ses ligands et ses propres propriétés de signalisation. Certains de ces ligands sont exprimés largement par de nombreux types cellulaires, d'autres, principalement par les cellules du système hématopoïétique ou alors par des cellules soumises à un stress (infectieux ou tumoral, par exemple). Une partie des récepteurs des cellules NK ont des fonctions inhibitrices qui servent à protéger les cellules saines *via* la reconnaissance des molécules de classe I du complexe majeur d'histocompatibilité (CMH).

2.3.1. Récepteurs activateurs

Les récepteurs activateurs des cellules NK reconnaissent essentiellement des ligands sur des cellules infectées ou transformées et permettent aux cellules NK de reconnaître et d'éliminer ces cellules. Certains auteurs soutiennent que les cellules NK quiescentes ne peuvent être activées que par l'engagement d'au moins 2 récepteurs activateurs ou plus (Bryceson et al., 2006) à l'exception du récepteur FCγRIIIa (CD16) qui, à lui seul, suffirait à activer des cellules NK au repos. L'activation des voies de signalisation en aval des récepteurs activateurs conduit à une réorganisation du cytosquelette, à la dégranulation des cellules NK et à la libération des protéines cytotoxiques contenues dans les granules de sécrétion ainsi qu'à la production et à la sécrétion de cytokines.

a. Récepteurs de la cytotoxicité naturelle (NCR)

Les NCR NKp30, NKp44 et NKp46 ont été les premiers récepteurs activateurs identifiés dans les cellules NK (Cantoni et al., 1999; Pende et al., 1999). Tandis que NKp30 et NKp44 peuvent être exprimés par certains sous types de lymphocytes T, NKp46 a longtemps été considéré comme spécifique des cellules NK (Sivori et al., 1997; Walzer et al., 2007b). On sait, aujourd'hui, que son expression est également partagée avec certaines ILC3 (Reynders et al., 2011). Ces récepteurs sont des

protéines transmembranaires de la super famille des immunoglobulines. NKp46 et NKp30 se couplent aux chaînes FCRγ et/ou CD3ζ alors que NKp44 s'associe à DAP12. Toutes ces protéines adaptatrices contiennent un motif ITAM (immunoreceptor-based activation motif), impliqué dans la transduction du signal, dans leur partie intracytoplasmique. Alors que certains ligands d'origine virale ont été identifiés (Arnon et al., 2006), les ligands cellulaires des NCR sont, en revanche, moins connus (Byrd et al., 2007). Parmi eux, on peut citer les protéines BAT3 ou B7-H6, ligands de NKp30 (Brandt et al., 2009; Pogge von Strandmann et al., 2007). L'activation des cellules NK médiée par les NCR est dépendante du motif ITAM des protéines adaptatrices. L'engagement des NCR induit la phosphorylation du motif ITAM par une kinase de la famille Src (Figure 10). Cette étape permet la fixation des protéines Syk et ZAP70 aux motifs ITAM conduisant à l'activation de différentes voies de signalisation incluant PI3K, PLCγ et Vav.

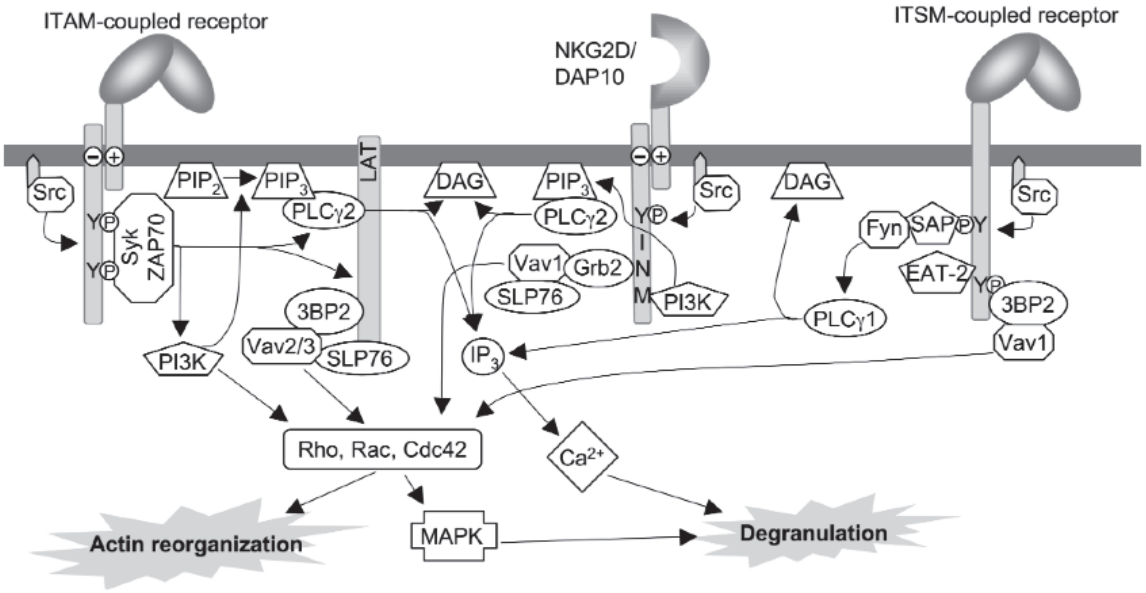

Figure 10: Signalisation des récepteurs activateurs
L'activation des récepteurs à ITAM (NCRs, CD16) induit la phosphorylation du motif ITAM par la kinase Src qui permet la fixation de protéines adaptatrices Syk et ZAP70 activant les voies Vav, PI3K et PLCγ et entrainant la réorganisation de l'actine et la dégranulation. Les récepteurs couplés à DAP-10 (NKG2D) ou à motif ITSM (2B4) agissent en synergie avec les récepteurs à ITAM pour activer des cellules NK quiescentes. (Watzl and Long, 2010)

b. NKG2D

NKG2D est un récepteur de la famille des lectines de type C, exprimé à la surface des cellules NK, des lymphocytes T γδ et des T CD8 (Bauer et al., 1999). NKG2D

porte un résidu arginine dans la partie centrale de sa région transmembranaire permettant son association avec la protéine adaptatrice DAP-10 (Wu et al., 1999). NKG2D reconnaît différents ligands comme MICA/B (major histocompatability complex class I chain–related molecules A and B) et les protéines ULBPs (UL16 binding protein) chez l'homme et les membres de la famille Rae1 (Retinoic Acid Early inducible proteins*),* les glycoprotéines du groupe H60 (Histocompatibility antigen 60) et la molécule Mult1 (Mouse UL16-binding protein-like transcript *1*) chez la souris. Le fait que ces ligands soient surexprimés par des cellules cancéreuses fait de ce récepteur un important co-activateur dans la reconnaissance des tumeurs (Guerra et al., 2008). La signalisation de NKG2D dépend de DAP10. La partie intracytoplasmique terminale de DAP10 contient un domaine YINM qui, une fois phosphorylé par les kinases Src, peut fixer la sous-unité p85 de PI3K ou la protéine adaptatrice Grb2 (Upshaw et al., 2006). Le recrutement et l'activation de ces deux éléments permettent le déclenchement des cascades de signalisation aboutissant à une réorganisation de l'actine et à la dégranulation de la cellule NK (Figure 10).

c. 2B4

2B4 est un membre des récepteurs de la famille des récepteurs SLAM (Claus et al., 2008) dont l'expression n'est pas limitée aux cellules NK et dont le ligand est CD48, protéine largement exprimée par les cellules du système hématopoïétique. Le domaine intracellulaire de ce récepteur contient des motifs ITSM qui sont phosphorylées par des kinases Src (Figure 10). La phosphorylation du motif ITSM permet le recrutement de protéines adaptatrices comme SAP (SLAM Associated Protein) (Sayos et al., 1998), EAT-2 ou ERT (Ma et al., 2007). SAP recrute alors la kinase Fyn dont l'interaction avec SAP est indispensable à l'activation des cellules NK médiée par 2B4.

Dans certaines circonstances, 2B4 peut également inhiber les fonctions des cellules NK (Veillette, 2006). Chez l'homme, ce phénomène n'a, cependant, été rapporté que dans des cas de déficiences pour la protéine SAP, comme chez les patients atteints de Syndrome Lymphoprolifératif lié à l'X (Bottino et al., 2001) ou dans des cellules NK immatures qui n'expriment la protéine SAP qu'à bas niveau (Sivori et al., 2002).

d. DNAM-1

DNAM-1 (ou CD226) est un récepteur exprimé par les cellules NK, les lymphocytes T et les monocytes. Ses ligands sont le récepteur du poliovirus (CD155) et Nectin-2

(CD112), exprimés par les cellules épithéliales et endothéliales et qui sont surexprimés par certaines tumeurs (Bottino et al., 2003). DNAM-1 joue, d'ailleurs, un rôle important dans l'immunité anti-tumorale des cellules NK (Chan et al., 2014; Gilfillan et al., 2008; Iguchi-Manaka et al., 2008). Alors que chez l'homme, toutes les cellules NK expriment ce récepteur, chez la souris, DNAM-1 identifie deux sous-types fonctionnels de cellules NK (Martinet et al., 2015). Les cellules NK DNAM-1^+ ont d'importantes capacités de prolifération, produisent plus de cytokines pro-inflammatoires comme l'IFN-γ, l'IL-6 ou le GM-CSF et ont des propriétés antitumorales plus importantes que les cellules NK DNAM-1^-, qui dérivent des cellules NK DNAM-1^+ et qui produisent essentiellement des chimiokines.
De façon intéressante, la production supérieure de cytokines pro-inflammatoires par les cellules NK DNAM-1^+ est conservé pendant la maturation suggérant que la perte de DNAM-1 correspond à une différenciation fonctionnelle distincte de la maturation. L'engagement du récepteur induit sa phosphorylation dans sa partie cytoplasmique par des kinases Src et permet le recrutement de protéines de liaisons à l'actine.

e. NKp80

NKp80 est un récepteur de la famille des lectines de type C. Il est exprimé à la surface des cellules NK et de certains lymphocytes T CD8 (Vitale et al., 2001). Son ligand est AICL (activation-induced C-type lectin) (Welte et al., 2006), exprimé par les cellules myéloïdes et dont l'interaction avec son récepteur contribue à l'activation réciproque des cellules NK et des cellules myéloïdes *in vitro*.

f. KIR/Ly49

Certains récepteurs de la famille des KIR chez l'homme, Ly49 chez la souris et le complexe CD94/NKG2C ont des fonctions activatrices. En dehors de Ly49H qui intervient dans la reconnaissance du cytomégalovirus murin (Arase et al., 2002), leur rôle est mal connu.

g. CD16

Les cellules NK $CD56^{dim}$ expriment le récepteur de faible affinité pour la fraction constante des Immunoglobulines de type G (IgG) FcγRIIIA (ou CD16) qui permet le phénomène de cytotoxicité dépendante des anticorps (ADCC). Comme pour NKp46 et NKp30, CD16 est couplé avec les chaînes CD3ζ et/ou Fc$R\gamma$, son engagement

induisant l'activation de la voie de signalisation *via* la phosphorylation des motifs ITAM.

h. Synthèse

La multiplicité des récepteurs activateurs sur la cellule NK leur permet probablement de répondre à un grand nombre de situations pathologiques différentes. Ainsi, les souris ou patients déficients pour l'un ou l'autre de ces récepteurs semblent avoir des phénotypes plus ou moins spécifiques (Table 1) et des susceptibilités aux infections, tumeurs ou même maladies auto-immunes/métaboliques. La signalisation des récepteurs semble par contre relativement stéréotypée même si des études fines manquent.

Espèce	Gène déficient	Susceptibilité
Souris		
	Ly49H	MCMV
	NKG2D	Tumeurs
	NKp46	Virus de la grippe, diabète, obésité
	DNAM-1	Myélome
Homme		
	SAP(2B4)	EBV
	CD16 (polymorphisme)	Réponse aux anticorps thérapeutiques (ADCC)

Tableau 1: Principales susceptibilités associées à des déficiences des récepteurs activateurs chez la souris et chez l'homme
(MCMV: Murine Cytomegalovirus, EBV: Epstein Barr Virus, ADCC: Antibody Dependant Cell Cytotoxicity)

2.3.2. Récepteurs inhibiteurs

a. Les récepteurs de la famille des Ly49/KIRs

L'existence de mécanismes qui régulent l'activité des cellules NK vis à vis des cellules du soi a été envisagée très tôt après la découverte de ces cellules. On a compris, plus tard, que l'expression normale de molécules de classe I du CMH protège les cellules saines d'une élimination par les cellules NK (Ljunggren and Kärre, 1985). La découverte des récepteurs inhibiteurs, chez la souris et chez l'homme, a ensuite permis de définir les bases du mécanisme de cette protection (Ciccone et al., 1992; Karlhofer et al., 1992). La description du motif ITIM (immunoreceptor tyrosine-based inhibition motif) (Long, 2008), qui sert à la fixation de la phosphatase SHP-1 aux récepteurs Ly49 et KIRs, a conduit à l'identification d'un grand nombre de récepteurs à motifs ITIM présents dans de nombreux types

cellulaires. Bien que ce motif soit ancien et conservé au cours de l'évolution, certains de ces récepteurs ont évolué rapidement. Les récepteurs inhibiteurs pour les molécules de classe I du CMH chez la souris, de la famille Ly49, sont, en effet, très différents de leurs équivalents humains de la famille des KIR. D'autres récepteurs, comme le complexe CD94/NKG2A signalent également *via* un motif ITIM.

Les cellules NK humaines expriment les récepteurs de la famille des KIR (ou CD158) qui comprend des récepteurs inhibiteurs pour HLA-B et HLA-C. Chez la souris, les membres de la famille Ly49 reconnaissent les molécules H-2 de classe I du CMH avec différents degrés de spécificité. NKG2A, lui, est conservé entre les deux espèces, mais pas ses ligands. La forme humaine reconnaît la molécule HLA-E alors que le ligand de la forme murine est Qa1. CD85 appartient à la troisième catégorie de récepteurs à motif ITIM qui reconnaît les molécules de classe I du CMH, chez l'homme. Enfin, les cellules NK expriment différents autres récepteurs à motifs ITIM mais dont les ligands ne sont pas des molécules du CMH, c'est le cas de CD161b, Siglec-7 ou CD300a.

Une fois phosphorylés, les motifs ITIM recrutent les phosphatases SHP-1 et SHP-2 qui induisent la déphosphorylation de Vav1 conduisant à l'inhibition des signaux induits par les récepteurs activateurs (Figure 11).

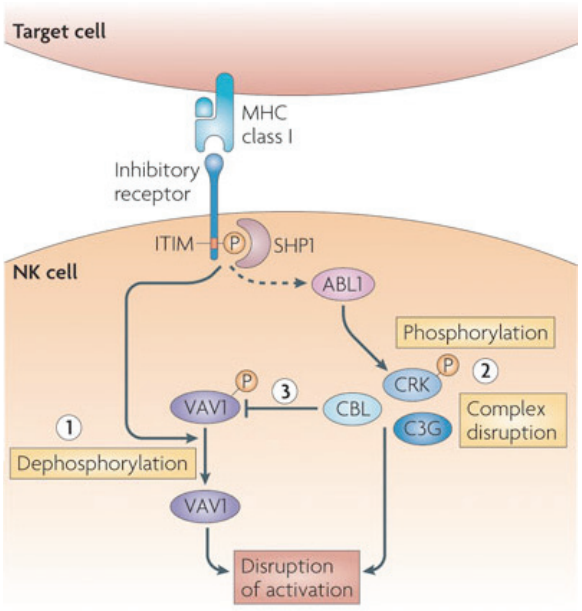

Figure 11 : Signalisation des récepteurs inhibiteurs
L'activation des récepteurs inhibiteurs à ITIM a 2 conséquences : la phosphorylation de SHP1 qui déphosphoryle VAV1 et la phosphorylation de CRK qui induit la perturbation d'un complexe d'activation formé par CRK, CBL et C3G. (Höglund and Brodin, 2010)

b. KLRG1

KLRG1 (Killer cell lectin-like receptor G1) est un récepteur inhibiteur de la famille des lectines de type C, contenant un motif ITIM dans sa partie intracytoplasmique (Guthmann et al., 1995; Hanke et al., 2001). Il est exprimé par certains sous-type de cellules NK et de lymphocytes T effecteurs mémoires (Tem) et est considéré comme un marqueur de différenciation terminale. Son expression par les cellules NK lors d'infections virales est inversement corrélée à leur capacité de produire de l'IFN-γ (Robbins et al., 2002) et est sous la dépendance de l'IL-15 (Huntington et al., 2007). Plus précisément, nous avons récemment montré que l'induction de l'expression de KLRG1 nécessite la kinase mTOR (Marçais et al., 2014).

Si son rôle exact n'est pas entièrement connu, il a été proposé que KLRG1 agisse en élevant le seuil d'activation des cellules NK et en atténuant ainsi les réponses effectrices pro-inflammatoires. Cependant, les interactions entre KLRG1 et ses ligands (E-,N- et R-cadhérines) sont faibles et semblent nécessiter la formation de complexes multimériques pour délivrer un signal physiologiquement pertinent (Rosshart et al., 2008). De plus, l'absence de KLRG1 n'affecte pas ni la différenciation, ni la fonction des cellules NK et des lymphocytes T $CD8^+$. De plus, les animaux déficients pour KLRG1 développent des réponses antivirales normales à la fois lors de la phase aigüe et chronique de l'infection (Gründemann et al., 2010).

Adaptateur	Récepteur activateurs	Ligands connus	Récepteur inhibiteur	Ligands connus
Humain/murin				
FcRγ, CD3ζ	CD16	IgG	CD94-NKG2A	HLA-E ($Qa1^b$)
	NKp46	HA	KLRG1	Cadhérines
DAP12	CD94-NKG2C	HLA-E ($Qa1^b$)	LAIR-1	Collagène
DAP10	NKG2D	MIC, ULBP		
SAP	2B4	CD48		
-	DNAM-1	CD112, CD155		
Humain				
FcRγ, CD3ζ	NKp30	B7-H6	KIR2DL1-3	HLA-Cw4/3
DAP12	NKp44		KIR3DL1	HLA-Bw4
	KIR2DS1	HLA-Cw3	KIR3DL2	HLA-A3/11
	KIR2DS2	?	KIR2DL5	?
	KIR3DS1	HLA-Cw4	CD161b	LLT1
	KIR2DL4	HLA-G		
	NKp80	AICL		
Murin				
DAP12	Ly49D	H2	Ly49a/c/e/f/g/i	H2
	Ly49H	m157		
FcRγ	NK1.1			

Tableau 2: Principaux récepteurs activateurs/inhibiteurs et leurs ligands chez la souris et chez l'homme

AICL : activation-induced C-type lectin, HA : Hémagglutinine, MIC : major histocompatability complex class I chain–related molecules, ULBP : UL16 binding protein (adapté d'Anel et al., 2012)

2.4. Education des cellules NK

La fonction des récepteurs inhibiteurs n'est pas seulement de contrer l'activité des récepteurs activateurs. Ils servent également à « éduquer» les cellules NK matures vis-à-vis des cellules du soi. Les études, chez la souris, soutiennent un modèle de régulation séquentielle dans lequel les gènes Ly49 sont exprimés de façon stochastique pendant la maturation des cellules NK jusqu'à ce qu'un récepteur reconnaisse des molécules du CMH de classe I du soi. Cette théorie doit toutefois être relativisée puisque certaines cellules NK, en condition homéostatique, n'expriment pas de récepteur au CMH (Fernandez et al., 2005). Cependant, et pour la majorité des autres cellules NK, qui expriment des récepteurs inhibiteurs qui reconnaissent les molécules de classe I du CMH, la reconnaissance du soi induirait la transduction de signaux négatifs qui restreignent l'expression de récepteur inhibiteur additionnel (Hanke et al., 2001; Schönberg et al., 2011). Ce phénomène serait à l'origine de la création d'un répertoire de cellules NK, chacune d'entres elles

exprimant une combinaison distincte de récepteurs inhibiteurs. L'expression d'un récepteur inhibiteur reconnaissant le soi (RIRS) permet également la maturation fonctionnelle des cellules NK par un processus appelé éducation.

Pendant longtemps, il a été considéré que toutes les cellules NK matures exprimaient au moins un RIRS. On peut imaginer, qu'en l'absence totale de ce type de récepteur, les cellules NK seraient en permanence stimulées *via* leurs récepteurs activateurs, auraient un phénotype de cellules activées et seraient auto-réactives. En fait, dans les modèles de défaut de fonction des récepteurs inhibiteurs (délétion des gènes codant pour SHP-1 ou pour la b2-microglobuline, chez la souris, déficiences en TAP-1 et TAP-2, chez l'homme), le phénotype obtenu est tout autre. Ces cellules NK non éduquées sont hypo-réactives, en terme de cytotoxicité et de sécrétion de cytokines (Elliott et al., 2010; Furukawa et al., 1999; Liao et al., 1991; Viant et al., 2014; Wickström et al., 2014). Pour expliquer le phénomène d'éducation, 3 modèles ont été proposés (Figure 12). Le 1er modèle, ou « armement », soutient que les cellules NK sont hypo-réactives par défaut et c'est l'engagement de leurs RIRS, au cours du développement, qui permet leur éducation (Kim et al., 2005). A l'inverse, dans le 2ème modèle (« désarmement »), il est proposé que les cellules NK, au cours de leur développement, engagent leurs récepteurs activateurs et inhibiteurs. En l'absence de stimulation de leurs récepteurs inhibiteurs, les cellules NK subiraient, alors, une stimulation chronique conduisant à un état d'épuisement et se traduisant par une hypo-réactivité. Ces cellules NK, anergiques, deviendraient, alors, tolérantes au soi (Fernandez et al., 2005). Le 3ème modèle (modèle du rhéostat) propose que l'éducation des cellules NK ne suive pas un modèle « on/off », mais qu'elle intègre l'intensité des signaux inhibiteurs à travers le nombre de RIRS engagés, mais également la qualité des signaux, dépendant de l'affinité des ligands avec leurs récepteurs (Brodin et al., 2009; Joncker et al., 2009).

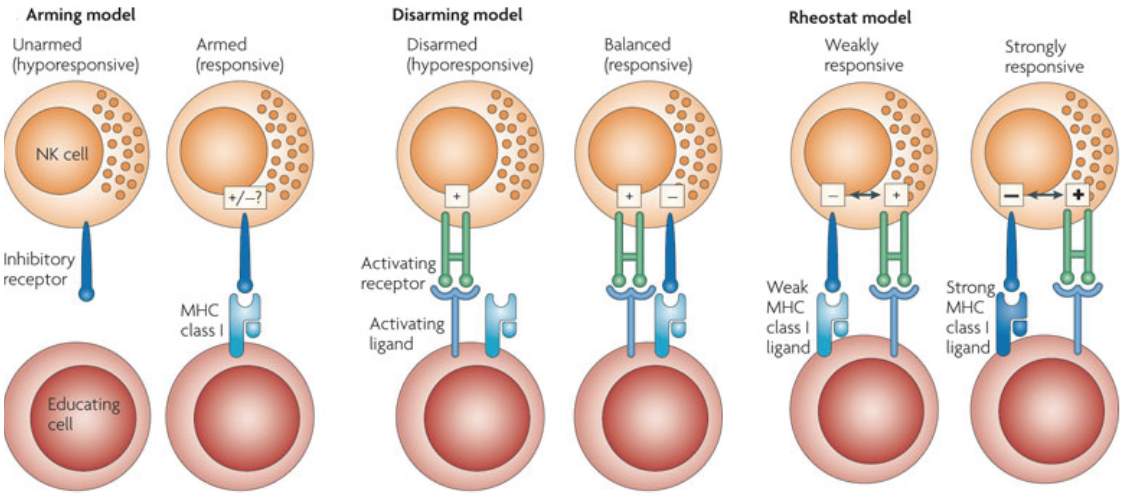

Figure 12: Education des cellules NK
Présentation des 3 modèles d'éducation des cellules NK. Dans le « arming model », il est considéré que les cellules NK non-éduquées sont hypo-répondeuses par défaut et c'est l'engagement de leurs RIRS qui permet leur éducation. Dans le « disarming model », les cellules NK recevraient, au cours de leur développement, des signaux activateurs et inhibiteurs et l'absence de stimulation de leurs RIRS conduiraient à un état d'épuisement. Enfin, dans le 3ème modèle, il est proposé que l'éducation prenne en compte des critères qualitatifs (affinité des ligands avec les récepteurs inhibiteurs) et quantitatifs (nombre de RIRS engagés) (Höglund and Brodin, 2010)

2.5. Fonction des cellules NK

2.5.1. Voies d'activation des cellules NK

a. Activation non dépendante des anticorps

Lorsqu'une cellule NK éduquée rencontre une cellule saine ou normale, les signaux inhibiteurs délivrés grâce à l'interaction des récepteurs inhibiteurs avec les molécules de classe I du CMH l'emportent sur les signaux activateurs. C'est, en partie, ce qui explique que les cellules NK soient tolérantes aux cellules saines (Figure 13-A). Historiquement, le premier modèle proposé pour expliquer l'élimination de cellules tumorales par les cellules NK est celui du « missing self » (ou « soi manquant ») (Kärre et al., 1986; Ljunggren and Kärre, 1985). En condition de pression de sélection, il est fréquent que des cellules transformées ou infectées par des virus diminuent leur expression de molécule de classe I du CMH. Ce phénomène induit une levée d'inhibition des cellules NK, qui s'activent et éliminent ces cellules reconnues comme des cibles (Figure 13-B). En situation pathologique, la cellule cible peut également induire l'expression de ligands des récepteurs activateurs. Il peut s'agir de « non-soi d'origine infectieuse » (cas des cellules infectées) ou de « soi induit par le stress » (cas des cellules tumorales, par exemple). Dans ce cas, les

signaux activateurs sont supérieurs aux inhibiteurs et la cellule NK s'active. (Figure 13-C).

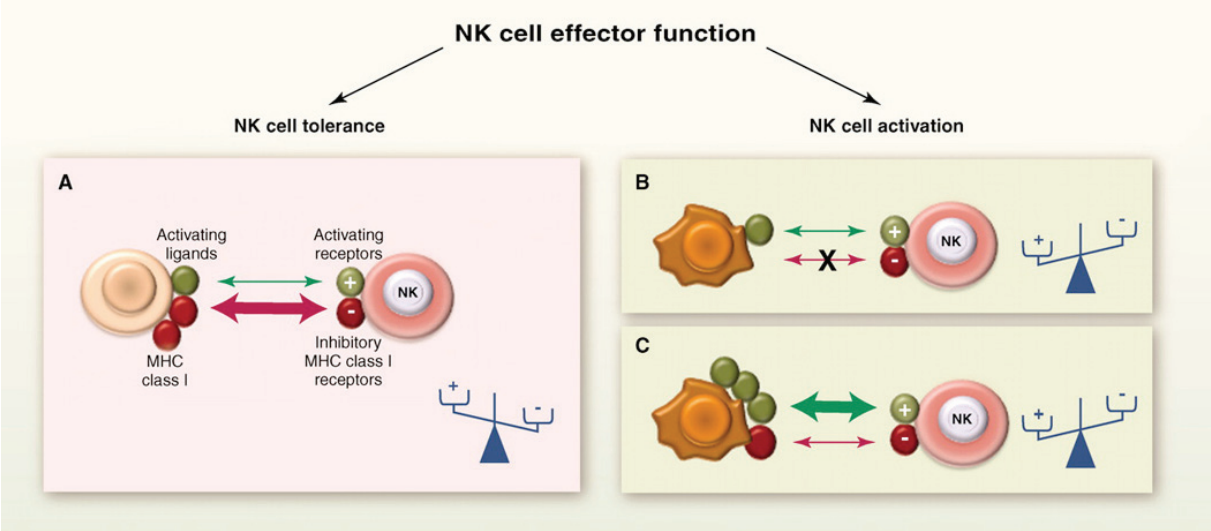

Figure 13: Reconnaissance des cellules cibles par les cellules NK
Les cellules NK sont capables de mesurer les niveaux d'expression de nombreuses molécules exprimées à la surface des cellules qu'elles rencontrent. Elles épargnent les cellules saines qui expriment des niveaux élevés de molécules de classe I du CMH (A). En revanche, elles reconnaissent et éliminent les cellules qui diminuent l'expression de molécules de classe I du CMH (B) ou augmentent l'expression de ligands activateurs (C). (Vivier et al., 2011)

b. Activation dépendante des anticorps

Les cellules NK sont également capables de reconnaître des cellules recouvertes d'IgG *via* les récepteurs des fragments Fc des IgG, les FcγRs. Ces récepteurs appartiennent à la super famille des immunoglobulines et sont divisés en 3 classes chez l'homme FcγRI, FcγRIIA/B/C et FCγRIIIA/B (correspondant respectivement à CD64, CD32 et CD16) et 4 chez la souris puisque FcγRIV n'existe que chez les rongeurs (Bruhns, 2012).Les cellules NK humaines expriment essentiellement FcγRIIC/CD32c (Morel et al., 1999) et FCγRIIIA/CD16A (Lanier et al., 1988). Comme les autres récepteurs activateurs, CD16A s'associe aux chaînes FCRγ et CD3ζ qui contiennent des motifs ITAM tandis que CD32c possède son propre motif ITAM dans sa partie cytoplasmique. Leur activation conduit à la dégranulation des cellules NK, à la production de cytokines et finalement à la mort de la cellule opsonisée. *In vitro*, il a été montré que les cellules NK FCgRIIIA^{+} sont les principales cellules effectrices de l'ADCC induite par des anticorps thérapeutiques (Cooley et al., 1999; Dall'Ozzo et al., 2004; Manches et al., 2003). Chez la souris, les cellules NK ne semblent par

contre pas essentielles à l'ADCC (Nimmerjahn and Ravetch, 2008) alors que les macrophages notamment hépatiques seraient requis (Montalvao et al., 2013)

Chez l'homme, il existe des polymorphismes pour les gènes codants CD16A et CD32C. Ces variations peuvent influencer l'interaction de ces récepteurs et conduire à une réponse thérapeutique dépendante du génotype de l'individu. De plus, les différents isotypes d'IgG ainsi que les modifications post-traductionnelles qu'elles subissent (glycosylation et fucosylation) influencent également l'affinité des IgG pour leurs récepteurs (Koene et al., 1997; Niwa et al., 2004; Shields et al., 2002; Shinkawa et al., 2003). Ainsi, de nombreuses études en cancérologie ont montré que la réponse clinique à différents anticorps monoclonaux dépendait du polymorphisme affectant ces deux récepteurs (comme le remplacement de la valine par une phénylalanine en position 158 de CD16A) (Bibeau et al., 2009; Calemma et al., 2012; Cartron et al., 2002; Musolino et al., 2008; Treon et al., 2005; Varchetta et al., 2007; Weng, 2003). Cependant, ces résultats n'ont pas été confirmés, notamment dans des études qui associent des chimiothérapies classiques aux anticorps monoclonaux (Ghesquières et al., 2012; Hurvitz et al., 2012; Paez et al., 2010).

2.5.2. Conséquences de l'activation

a. Formation de la synapse immunologique

L'induction des fonctions effectrices des cellules NK nécessite un contact étroit de la cellule NK avec sa cible, ceci pour assurer un ciblage précis du processus cytolytique vers une cellule anormale, souvent à l'intérieur d'un tissu et sans affecter les cellules avoisinantes. La spécificité d'action des cellules NK est assurée grâce à la formation d'une synapse immunologique. Ce phénomène, dont le mécanisme est comparable en de nombreux points à celui mis en jeu par les lymphocytes T CD8 comporte 3 étapes (Orange, 2008).

La première étape est l'initiation, qui commence par la reconnaissance de la cible. Puis, a lieu, la phase d'adhésion qui est facilitée par des protéines de la famille des intégrines comme LFA-1 et CD11b. Ces récepteurs vont se concentrer au niveau de la synapse et sont capables de transduire les premiers signaux activateurs, qui seront amplifiés par la suite par les authentiques récepteurs activateurs.

La deuxième étape est la phase effectrice, qui commence par la réorganisation des polymères d'actines dépendante de l'activation de Vav1 en aval des récepteurs activateurs et de la protéine WASP. La phase effectrice se poursuit par la

polarisation des granules lytiques. Elle débute par le mouvement des granules le long des microtubules à partir du MTOC, centre d'organisation des microtubules cellulaires et sont réorientées vers la synapse. Alors que l'actine est nécessaire pour la polarisation des granules, il semble qu'une partie du réseau d'actine soit ensuite désassemblée pour permettre la création d'un conduit à travers lequel les granules pourront accéder à la membrane plasmique. Après une phase d'arrimage, les granules vont fusionner et libérer leur contenu.

La troisième et dernière étape est la phase de terminaison. Elle débute par la diminution progressive de l'expression des récepteurs et des intégrines au niveau de la synapse pour permettre la dissociation entre la cellule NK et sa cible.

b. Cytotoxicité

Les deux voies principales impliquées dans la cytotoxicité, aboutissant à la mort de la cellule cible, sont la voie perforine/granzyme, dont le relargage est assuré par la dégranulation et la voie impliquant les récepteurs de mort (Fas, TNFR, TrailR) (Figure 14). Ces deux voies sont complémentaires, mais il est probable que la voie perforine/granzyme soit la plus importante pour assurer la cytotoxicité.

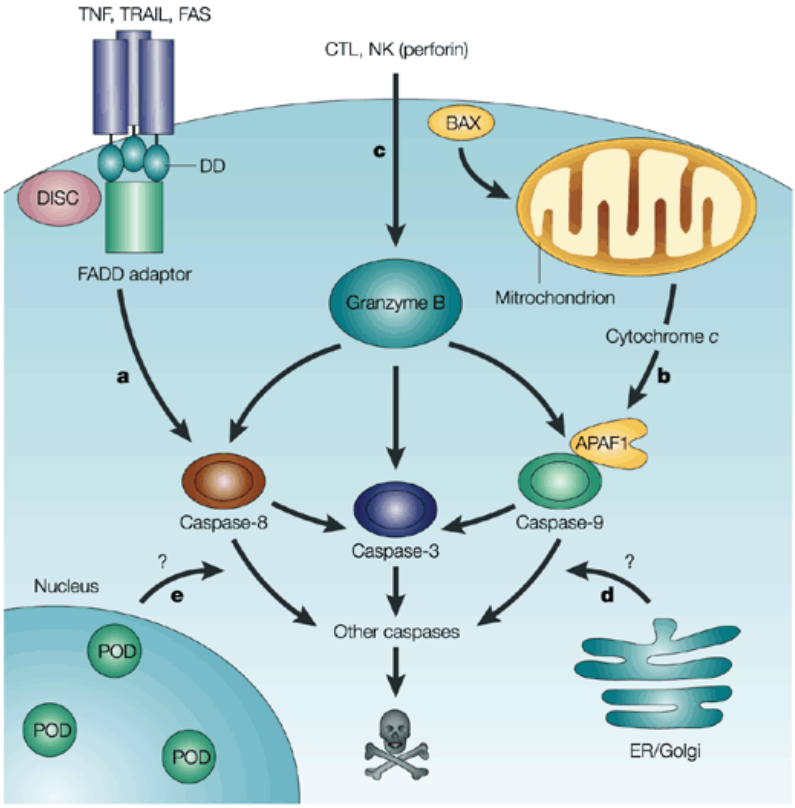

Figure 14: Voies de cytotoxicité des cellules NK.
Les cellules NK mettent en jeu deux types de mécanismes de cytotoxicité: l'engagement de récepteurs de mort (Fas, TNFRI, Trail) exprimés à la surface de la cellule et la voie perforine/granzyme. (Reed, 2002)

La perforine est une protéine de 67kDa capable de créer des pores dans les membranes cellulaires (Podack et al., 1985). Elle est stockée à l'intérieur des cellules cytotoxiques dans des granules de sécrétion. Suite à la reconnaissance d'une cible, le contenu de ces granules incluant la perforine et les granzymes est libéré dans la synapse immunologique. La perforine, en se polymérisant, forme des pores dans la membrane de la cellule cible et permet notamment l'entrée des granzymes. Les granzymes sont des sérines protéases qui induisent la mort cellulaire par différents mécanismes. Les plus abondantes chez l'homme et la souris sont les granzyme A et B. Comme les protéines de la famille des caspases, granzyme B clive ses substrats comme la protéine Bid (BH3 Interacting Domain) (Barry et al., 2000) qui initie la perforation de la membrane externe de la mitochondrie libérant le cytochrome c dans le cytoplasme. Le cytochrome c va permettre la formation de l'apoptosome et l'activation de la caspase-9 qui va activer à son tour la pro-caspase-3, capable d'hydrolyser un grand nombre de protéines essentielles à l'homéostasie cellulaire. Granzyme A induit également l'apoptose, mais de façon indépendante des caspases (Beresford et al., 1999). L'ensemble de ces mécanismes induit donc la mort de la cible, par un mécanisme d'apoptose.

La deuxième voie permettant la lyse de la cellule cible est la voie Fas-Fas ligand. Fas, ou CD95, est un membre de la superfamille des récepteurs du TNF. La liaison de Fas ligand à son récepteur entraîne le recrutement de la protéine adaptatrice FADD (Chinnaiyan et al., 1995), qui, à son tour, recrute la procaspase-8. Deux voies de signalisation sont ensuite décrites. En effet, si la quantité de caspase-8 est suffisante dans la cellule, son activation permet le clivage direct de la procaspase-3. Si la quantité de caspase-8 est insuffisante, une boucle d'amplification mitochondriale médiée par le clivage de la protéine Bid est alors nécessaire.

Il faut, enfin, noter que d'autres membres de la famille du TNF comme le TNF lui-même ou TRAIL (TNF-alpha-Related Apoptosis-Inducing Ligand) sont capables d'induire la mort par apoptose de cellules portant des récepteurs pour ces molécules et ainsi de contribuer à la cytotoxicité des lymphocytes NK.

c. Production de cytokines

En dehors de la cytotoxicité, la deuxième fonction des cellules NK est la production de cytokines et de chimiokines. Après une stimulation par différentes cibles, permettant l'engagement de récepteurs activateurs différents, les cellules NK sont

capables de produire de l'IFN-γ, du TNF-α et les chimiokines RANTES (CCL5), MIP1-α (CCL3) et MIP1-β (CCL4) (Fauriat et al., 2010). Dans ce même travail, il n'a pas été mis en évidence de production d'IL-5, IL-10, IL-13 et GM-CSF, pourtant également proposées comme étant sécrétées par les cellules NK. Il a donc été proposé que la synthèse de ces cytokines était plutôt indirecte et induite par d'autres cytokines. En effet, chez l'homme, la production d'IL-10 est induite par les cytokines IL-2, IL-12 ou IL-15 (Fehniger et al., 1999; Wolk et al., 2002) tandis qu'elle requiert STAT4, en aval du récepteur de l'IL-12 chez la souris (Grant et al., 2008). Néanmoins et si les cellules NK sont capables de produire un panel varié de cytokines, celle qui les caractérise le mieux est l'IFN-γ, propriété qui leur a valu leur reclassement au sein des ILC de type 1. Les cellules NK sont, en effet, capables de produire des quantités importantes d'IFN-γ et dans des temps très précoces au cours de la réaction immunitaire. L'IFN-γ joue, d'ailleurs, un rôle important dans le contrôle des infections virales et bactériennes chez la souris (Orange et al., 1995; Soudja et al., 2014) et chez l'homme (Novelli and Casanova, 2004). Cette cytokine a un rôle pléïotropique puisque beaucoup de types cellulaires expriment son récepteur. Elle augmente la clairance virale, *via* notamment l'induction de l'expression des molécules du CMH de classe I et II, permet un contrôle de la réplication virale, favorise la polarisation de la réponse immunitaire vers une réponse Th1 et active les macrophages et les DC.

2.6. Rôles des cellules NK dans l'immunité

Les cellules NK sont capables de reconnaître différents types de cellules stressées. Nous détaillerons ici leur rôle dans l'immunité infectieuse ainsi que dans la régulation de la réponse immunitaire. Leurs fonctions dans l'immunité anti-tumorale seront discutées dans le troisième chapitre de cet exposé.

2.6.1. Immunité anti-infectieuse

Bien que le modèle viral reste le plus utilisé pour étudier les fonctions des cellules NK, elles sont capables de répondre à des infections contre tous les types de pathogènes intracellulaires (bactéries, parasites), mais également extracellulaires par des mécanismes indirects (Horowitz et al., 2012).

a. Immunité antivirale

Les réponses des cellules NK ainsi que leur fonction ont été évaluées dans de nombreux modèles d'infection virale. L'activation de la cytotoxicité médiée par les cellules NK ainsi que leur production d'IFN-γ sont observées après infection par LCMV (lymphocytic choriomeningitidis) (Biron et al., 1996; Welsh, 1978), MCMV (Murine Cytomegalovirus) (Grundy et al., 1982; Orange and Biron, 1996a, 1996b; Orange et al., 1995), HSV (Herpes Virus Simplex) (Ching and Lopez, 1979) ou le virus de la grippe (Santoli et al., 1978). En général, le pic de la réponse des cellules NK s'observe dans les heures ou jours qui suivent une primo-infection alors que la réponse adaptative met plus d'une semaine à apparaître. L'importance des cellules NK dans la défense antivirale précoce a été démontrée chez la souris par la mise en évidence d'une plus grande susceptibilité à de nombreux virus comme MCMV (Orange et al., 1995), HSV (Habu et al., 1984) ou Influenza (Stein-Streilein and Guffee, 1986) après déplétion des cellules NK. La contribution des cellules NK dans la défense contre les infections virales, chez l'homme, est également documentée. Une faible activité cytotoxique des cellules NK est, en effet, associée à une sensibilité accrue aux infections par HSV (Biron et al., 1989; Ching and Lopez, 1979), EBV (Epstein-Barr Virus) (Joncas et al., 1989) ou CMV (Biron et al., 1989; Quinnan et al., 1982). Dans les phases tardives d'infection par le VIH (virus de l'immunodéficience humaine), des défauts de l'activité des cellules NK ont été mises en évidence (Bonavida et al., 1986; Katz et al., 1987). Ces anomalies sont associées à une réduction importante des cellules NK $CD3^{-}CD56^{+}$ et plus particulièrement de la sous-population de cellules NK $CD56^{bright}$ (Alter et al., 2005; Brenner et al., 1989). Cette observation a d'ailleurs conduit certains auteurs à émettre l'hypothèse que cette réduction du nombre de cellules NK serait liée à l'émergence d'une population de NK rare chez les individus $CD3^{-}CD56^{-}CD16^{+}$ (Mavilio et al., 2005). Des déficits en cellules NK sont également associées à des formes sévères d'infection par le papillomavirus (Ballas et al., 1990) tandis que la survenue d'infections chroniques par EBV chez des patients ayant une altération de la fonction NK suggèrent que les cellules NK contribuent au contrôle de l'EBV (Joncas et al., 1989). Les cellules NK semblent également jouer un rôle dans les infections par le VHC (virus de l'hépatite C). En effet, lors de la phase aigüe, le rapport du nombre de cellules NK $CD56^{bright}/CD56^{dim}$ augmente ainsi que l'expression de NKG2D et les capacités fonctionnelles des cellules NK (cytotoxicité et production d'IFN-γ) (Amadei et al.,

2010). A l'inverse, lors d'un défaut d'élimination du virus, en phase chronique, l'expression de NKG2D et des NCR (NKp30 et NKp46) diminue (Alter et al., 2011). La distribution des cellules NK est également affectée puisque leur nombre dans le sang périphérique est réduit, mais est rétabli par une thérapie antivirale efficace (Dessouki et al., 2010).Enfin, chez les patients répliquant le VHB (virus de l'hépatite B), il a également été mis en évidence des défauts de coopération entre les pDC et les cellules NK (Martinet et al., 2012).

Si de nombreux mécanismes peuvent conduire indirectement à l'activation des cellules NK lors d'infections virales (soi manquant, non soi, ADCC ou activation par des cytokines comme l'IL-15, l'IL-12 ou les interférons de type I), les cellules NK sont également capables de reconnaître certains ligands microbiens de façon directe. Les premiers indices d'une reconnaissance directe de déterminants viraux par les cellules NK dans les infections virales viennent de la découverte de l'interaction entre l'hémagglutinine du virus de la grippe avec NKp46 (Mandelboim et al., 2001). La découverte de l'importance du récepteur Ly49H dans le contrôle des infections par MCMV (Cytomégalovirus Murin), *via* sa liaison avec la protéine virale m157, a eu lieu à peu près à la même période (Arase et al., 2002). Cette reconnaissance est d'ailleurs essentielle dans la réponse contre MCMV puisque la mutation de m157 bloque l'activation des cellules NK $Ly49H^+$ et augmente la susceptibilité des souris à l'infection (Bubić et al., 2004).

b. Immunité contre les autres types de micro-organismes

Si les cellules NK sont capables d'éliminer des cellules infectées par des bactéries intracellulaires (Griggs and Smith, 1994; Katz et al., 1990; Klimpel et al., 1986), *in vitro*, leur rôle *in vivo* est essentiellement d'activer les macrophages qui, à leur tour, assureront l'élimination du pathogène. En effet, dans cette fonction, leur capacité de production de cytokines semble plus importante que leur capacité cytotoxique. Dans les souris SCID, l'infection par *Listeria monocytogenes* induit la sécrétion d'IL-12 et de TNF-α par les macrophages qui activent la production d'IFN-γ par les cellules NK (Tripp et al., 1994). La neutralisation de l'IL-12 produite diminue la résistance à Listeria, mettant en évidence un rôle important de l'IFN-γ dans le contrôle de ce type d'infection. De façon surprenante, certains auteurs ont observé que la délétion des cellules NK augmentait la résistance à certains pathogènes comme Listeria (Schultheis and Kearns, 1990, 1990; Takada et al., 1994), *Escherichia coli* (Badgwell

et al., 2002), *Streptococcus pneumoniae* (Christaki et al., 2015; Kerr et al., 2005) ou *Pseudomonas aeruginosa* (Newton et al., 1992). Les mécanismes qui expliquent ces phénomènes ne sont pas identifiés, mais il est important de noter que la majorité des expériences visant à dépléter les cellules NK utilisent soit un anti NK1.1 soit un anti-asialo GM1. Ces deux anticorps peuvent donc cibler d'autres populations lymphoïdes comme les NKT (anti NK1.1) ou certains lymphocytes T (anti-asialo GM1).

2.6.2. Régulation de la réponse immunitaire

Les cellules NK sont capables d'interagir avec de nombreux types cellulaires différents, comme les DC et les macrophages, qui participent à l'activation des cellules NK au cours de la réponse antimicrobienne, mais également avec les lymphocytes T et B. Concernant l'interaction avec les DC, les cellules NK participent à leur homéostasie, en éliminant les DC immatures *in vitro* et en facilitant leur maturation mais également en favorisant la mise en place d'une réponse spécifique humorale et cellulaire (Walzer et al., 2005).

Les cellules NK sont également capables d'interagir avec les lymphocytes T. L'élimination de lymphocytes T activés par les cellules NK a d'abord été mise en évidence *in vitro*, chez la souris (Rabinovich et al., 2003). Dans ce travail, les auteurs ont montré que cette élimination impliquait l'interaction de NKG2D avec ses ligands exprimés par les cellules T. Chez l'homme, l'activation des cellules T *via* leur TCR (T Cell Receptor) augmente l'expression des ligands de NKG2D, ce qui rend les cellules T sensibles à une élimination par les cellules NK (Cerboni et al., 2007). *In vivo*, il a été plus récemment montré que les cellules T activées sont aussi la cible des cellules NK d'une manière dépendante de NKG2D et de perforine, chez la souris (Soderquest et al., 2011). L'élimination des lymphocytes T a également été observée dans des contextes d'infection virale, notamment dans les infections à LCMV, pendant laquelle les cellules NK sont capables d'éliminer les lymphocytes T spécifiques. Un travail récent a montré que la déplétion des cellules NK pendant les phases précoces de l'infection permettait un meilleur contrôle de l'infection. Les auteurs ont suggéré l'existence d'un système de régulation en 3 voies dans lequel les cellules NK sont capables d'éliminer les lymphocytes T CD4 activés, qui, à leur tour, régulent la fonction des lymphocytes T CD8 (Waggoner et al., 2011). Des travaux soutenant cette hypothèse ont également montré que la déplétion des cellules NK réduit les phénomènes immunopathologiques et prévient la chronicité des infections (Lang et al., 2012).

De façon intéressante, des travaux suggèrent que les lymphocytes T ont développé des mécanismes de résistance à l'élimination par les cellules NK. Lors d'infections virales, les lymphocytes T incapables de répondre aux IFN de type I ($Ifnar^{-/-}$) sont plus sensibles à l'élimination des cellules NK (Xu et al., 2014). Cette sensibilité augmentée s'explique par l'augmentation de l'expression de ligands de récepteurs activateurs (Crouse et al., 2014) tout comme la diminution de l'expression de ligands de récepteurs inhibiteurs (Xu et al., 2014) par les lymphocytes T $Ifnar^{-/-}$.

3. Régulation des cellules NK par les cytokines

3.1. Régulation par l'IL-15

L'IL-15 a un rôle central dans le développement, la survie et l'activation des cellules NK. Découverte en 1994, elle se fixe à des récepteurs ayant des sous-unités communes avec le récepteur de l'IL-2. En conséquence, ces deux cytokines partagent un nombre important de fonctions biologiques (Ring et al., 2012).

3.1.1. Préambule

L'IL-15 murine est une protéine de 14-15kDa avec une similarité de séquence nucléique de seulement 19% avec l'IL-2, mais une structure tridimensionnelle très proche. Ces deux cytokines interagissent avec des récepteurs possédant la chaîne gamma commune (γc) (CD132) et la chaîne β du récepteur de l'IL-2/15 (CD122). La chaîne γc est aussi une sous-unité des récepteurs d'autres cytokines (IL-4/7/9/21) alors que la chaîne β est seulement impliquée dans la signalisation induite par l'IL-2 et l'IL-15. Les récepteurs de l'IL-2 et de l'IL-15 ne diffèrent que par leur chaîne α, IL-2Rα (CD25) étant spécifique de l'IL-2 et IL-15Rα de l'IL-15. A la différence d'IL-2Rα, IL-15Rα possède une très forte affinité pour son ligand (constante d'affinité de l'ordre de 10^{-11}M contre 10^{-8}M pour l'IL2Rα). Cette propriété, couplée au fait que l'IL-15 et l'IL-15Rα sont co-exprimés par les mêmes cellules, permet leur liaison intracellulaire dans le réticulum endoplasmique. Ce complexe est ensuite transporté à la surface de la cellule et présenté aux cellules avoisinantes qui expriment les chaînes β et γc du récepteur de l'IL-2/15. Ce mécanisme, appelé *trans*-présentation a permis d'expliquer le fait que l'expression d'IL-15Rα par les cellules NK n'était pas nécessaire au maintien de leur homéostasie. Un tel phénomène permet une délivrance précise du stimulus en provoquant une activation locale des cellules NK.

En effet, une disponibilité systémique de cette cytokine semble être néfaste comme en témoigne le développement de leucémies dans les souris transgéniques pour l'IL-15 (Fehniger et al., 2001; Mishra et al., 2012). L'existence d'une voie de présentation en *cis*, dans laquelle l'IL-15 est co-exprimée avec ses récepteurs à la surface des cellules NK a également été décrite (Figure 15). Certains auteurs suggèrent même qu'elle contribue autant que la présentation en *trans* à l'activation des cellules NK (Zanoni et al., 2013).

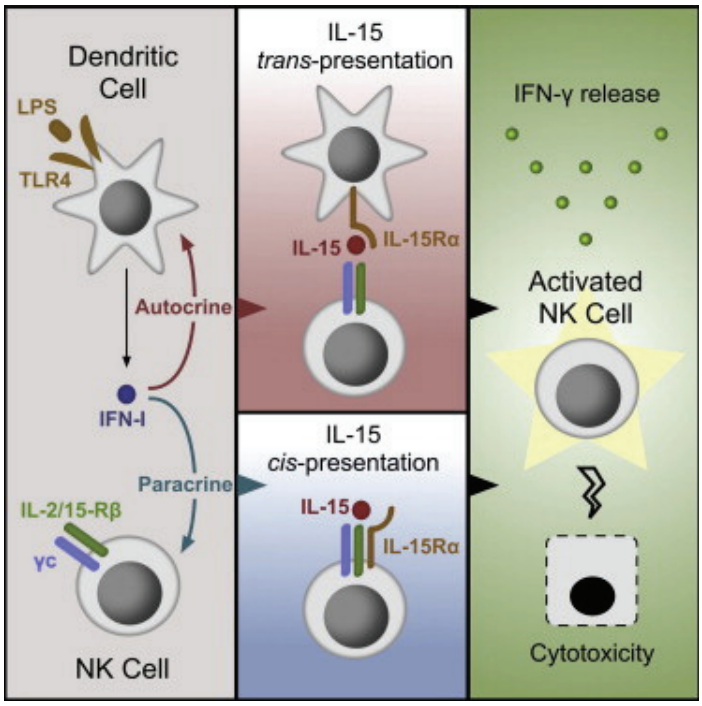

Figure 15: Modes de présentation du complexe IL-15/IL-15Rα
Le complexe IL-15/IL-15Rα peut être présenté par les cellules dendritiques (trans) ou par les cellules NK elles-mêmes (cis). (Zanoni et al., 2013)

3.1.2. Rôle

Le rôle clé de l'IL-15 dans la biologie des cellules NK est illustré par l'absence totale de cellules NK dans les souris déficientes pour les composants de la voie de l'IL-15(Kennedy et al., 2000; Lodolce et al., 1998). Son rôle ne se limite cependant pas au développement. Cette cytokine participe également à la survie des cellules NK matures en périphérie, en modulant l'expression de protéines pro- et anti-apoptotiques ainsi qu'au phénomène de « priming ». Les cellules NK quiescentes sont en effet peu cytotoxiques et nécessitent d'être activées pour exprimer leur réel potentiel cytotoxique. Cette étape est dépendante de l'IL-15, produite par les DC et les monocytes (Lucas et al., 2007). L'IL-15 agit en augmentant l'expression, par les

cellules NK, des protéines effectrices comme granzyme B et perforine qui sont peu ou pas exprimées par les cellules NK non-primées (Fehniger et al., 2007). L'IL-15 agit également en synergie avec l'IL-12 pour induire l'expression d'IFN-γ par les cellules NK (Koka et al., 2004). Enfin, l'IL-15 contrôle la prolifération homéostatique des cellules NK (Prlic et al., 2003; Castillo et al., 2009; Mortier et al., 2009) aussi bien que la prolifération induite par des infections virales, bactériennes ou fongiques (Lucas et al., 2007; Mortier et al., 2008; Nguyen et al., 2002; Tran et al., 2003).

3.1.3. Régulation

Le mécanisme de la régulation de l'expression de l'IL-15 au niveau basal n'est pas complètement élucidé. Le facteur de transcription IRF1, impliqué dans la production d'IL-15 dépendante des IFN de type I, joue probablement un rôle dans ce processus. En fait, l'expression de ce facteur est nécessaire à la fois par les cellules hématopoïétiques et non hématopoïétiques pour la génération des cellules NK (Ogasawara et al., 1998). L'ARN messager (ARNm) de l'IL-15 est exprimé, *in vivo*, par de nombreux tissus et types cellulaires d'origine hématopoïétique ou non (Burkett et al., 2003; Koka et al., 2003; Schluns et al., 2004a). Le rôle de l'origine (hématopoïétique ou non) de l'IL-15 dans l'homéostasie des cellules NK n'est pas complètement compris. Des travaux avaient suggéré que la trans-présentation de l'IL-15 par le système hématopoïétique était plus efficace puisque le fait de limiter l'expression d'IL-15Rα au système hématopoïétique suffisait à générer un nombre normal de cellules NK dans la moelle et n'induisait qu'une légère diminution de leur nombre en périphérie (Castillo et al., 2009; Schluns et al., 2004b). Mais, la délétion spécifique du gène codant pour l'IL-15 dans certains tissus comme le tissus adipeux a des conséquences sur le nombre des cellules NK non seulement au niveau local mais aussi au niveau systémiques (Liou et al., 2014). Néanmoins, la synthèse locale d'IL-15 dans les différents tissus non hématopoïétiques semble réguler essentiellement le développement des cellules NK résidentes.

En lien avec sa dualité d'action dans le contrôle de l'homéostasie et de l'activation des cellules NK, l'IL-15 est exprimée à bas niveau par les monocytes/macrophages et les DC en condition homéostatique, mais son expression peut-être considérablement augmentée par des agents pro-inflammatoires comme le LPS bactérien (Doherty et al., 1996), le poly(I:C) ou les Interférons de type I (Zhang et al., 1998). Plus récemment, en utilisant des souris transgéniques exprimant la protéine

GFP sous le contrôle de l'IL-15 endogène, il a été confirmé que l'IL-15 était fortement exprimée par les cellules de la lignée myéloïde (polynucléaires neutrophiles et éosinophiles, monocytes, et cellules dendritiques), les polynucléaires basophiles étant les cellules avec les niveaux les plus élevés (Colpitts et al., 2013) (Figure 16).

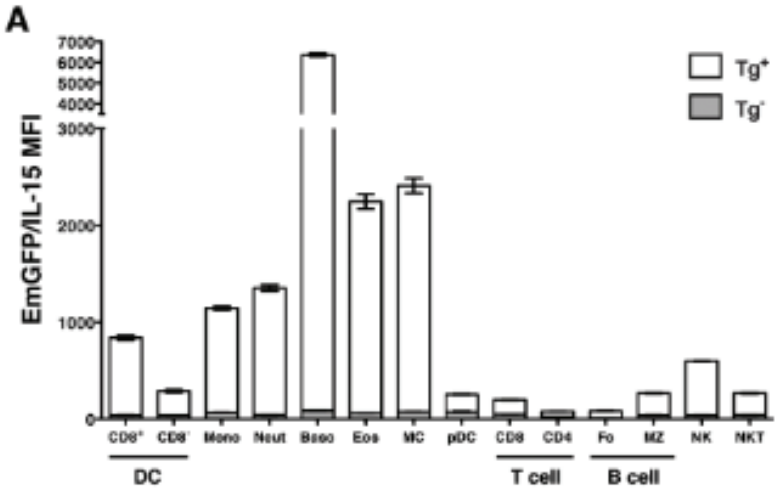

Figure 16: Niveau d'expression de l'IL-15 par les cellules du système immunitaire
L'IL-15 est exprimée à des niveaux plus élevés dans les cellules de la lignée myéloïde (polynucléaires, monocytes/macrophages et cellules dendritiques) et à des niveaux plus faibles dans les cellules NK et NKT (Colpitts et al., 2013).

3.1.4. Signalisation

Du fait de récepteurs communs et de similarités d'effets entre l'IL-2 et l'IL-15 *in vitro*, il a été suggéré que l'IL-15 active les mêmes voies de signalisation. Mais ces deux cytokines ne sont pas redondantes. Au cours de traitements par IL-2 et IL-15, les effets immunologiques observés sont différents (Oh et al., 2003). Ces données ont été confirmées plus récemment, puisque, dans les lymphocytes T CD8,l'analyse de la transcription de gènes induits par l'IL-2 et l'IL-15 a également montré des différences.

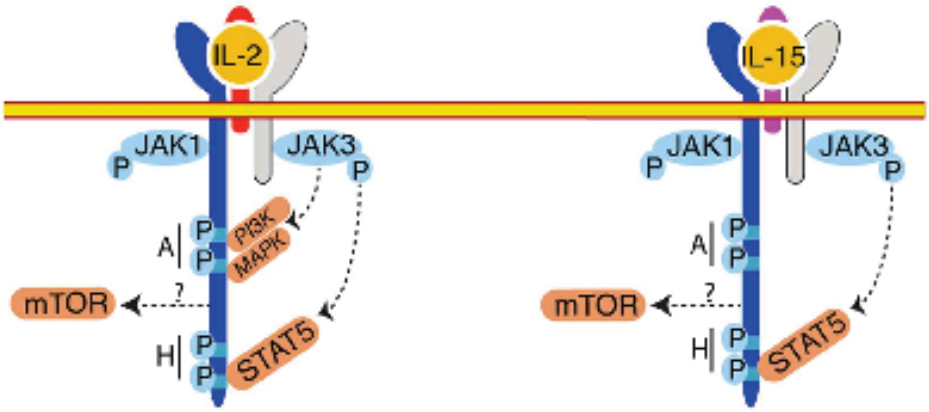

Figure 17: Signalisation en aval des récepteurs de l'IL-2/15
Suite à l'engagement du récepteur, des résidus tyrosines sont phosphorylés au niveau des régions « A » et « H » et permettent le recrutement et la phosphorylation de STAT5 par JAK1/3.L'engagement du récepteur active également mTOR, ainsi que les voies PI3K et MAPK (Marçais et al., 2013).

Suite à la fixation de l'IL-2 sur son récepteur, le signal est enclenché par la phosphorylation des protéines JAK (Janus Kinase) 1 et 3, fixées sur les chaînes IL-15Rβ et IL-15Rγc (Boussiotis et al., 1994; Miyazaki et al., 1994; Russell et al., 1994; Zhu et al., 1998). Ces kinases phosphorylent les résidus tyrosine d'IL-15Rβ (Figure 17), qui servent de site d'ancrage à des protéines comme la protéine adaptatrice Shc, les protéines IRS (Insulin Receptor Substrate) et les facteurs de transcription STAT5a et b, dont l'activation conduit à l'activation de 3 voies de signalisation : la voie Jak-STAT, la voie PI3K-Akt et la voie MAPK. La voie STAT5 est très importante dans le développement des cellules NK car les animaux déficients pour STAT5 présentent une diminution du nombre de NK associée à des défauts de réponse à l'IL-2 et l'IL-15 (Imada et al., 1998). La délétion NK-spécifique de STAT5a et b conduit à une disparition quasi complète des cellules NK (Imada et al., 1998), probablement liée à un blocage de la différenciation au stade précurseur NKP (Eckelhart et al., 2011). Nous avons récemment montré, que l'IL-15 active également mTOR (mammalian Target Of Rapamycin) (Marçais et al., 2014), kinase qui contrôle la maturation et l'activation des cellules NK par des signaux pro-inflammatoires, d'une façon qui pourrait être dépendante de PI3K (données non publiées).

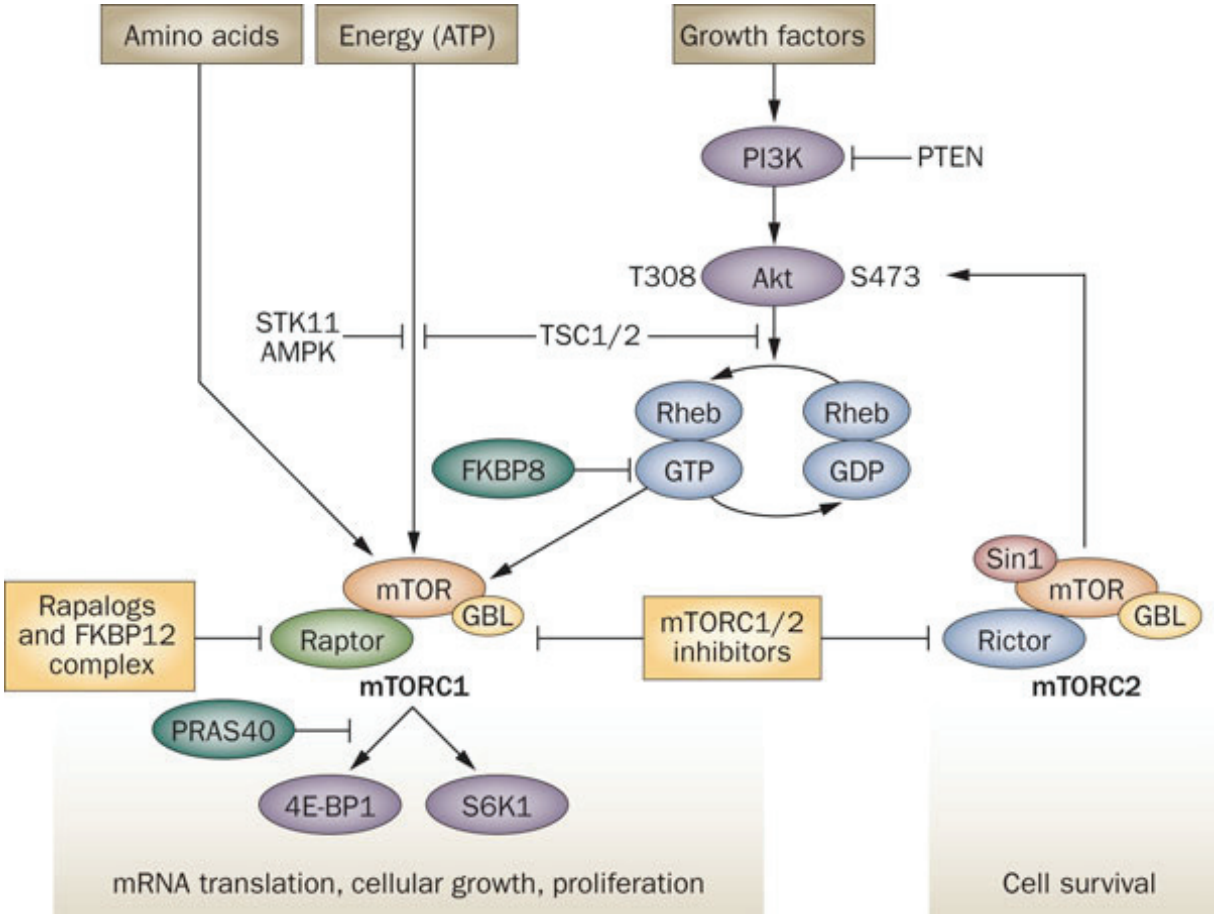

Figure 18: Voie de signalisation mTOR
La protéine mTOR est présente sous forme de deux complexes, associée à Raptor (mTORC1) et Rictor (mTORC2). mTOR est activé par l'IL-15, dans les cellules NK, mais son activation est également sensible à des signaux métaboliques (concentration en glucose, O2, acides aminés, ratio ADP:ATP). L'activation de mTORC1 conduit à la phosphorylation des protéines S6 et 4E-BP1 permettant la croissance cellulaire, la prolifération et l'acquisition des fonctions effectrices. (Dancey, 2010)

mTOR est une sérine/thréonine kinase qui peut être activée par différents signaux extracellulaires : ratio AMP/ATP, concentration en glucose, en O2 ou en acides aminés, facteurs de croissance (Figure 18), mais également des signaux inflammatoires comme il a récemment été décrit dans les lymphocytes T (Zeng and Chi, 2013). mTOR est une protéine présente au sein de 2 complexes, mTORC1 et mTORC2 qui diffèrent par les protéines qui les composent et par les cibles qu'ils phosphorylent. En suivant la phosphorylation des cibles clés de mTOR (la protéine ribosomale S6, 4EBP-1), nous avons observé que l'activité mTOR est régulée lors du développement des cellules NK avec une diminution progressive au cours de la différenciation et de la sortie des cellules NK de la moelle. En revanche, l'activité de mTOR est fortement induite lorsque les cellules NK sont exposées à des signaux pro-inflammatoires comme le poly(I:C). Enfin, nous avons également montré que

l'activation de mTOR nécessite des concentrations élevées d'IL-15 alors que de faibles concentrations de cette cytokine suffisent pour induire la phosphorylation de STAT5. Cette observation pourrait expliquer la double activité de l'IL-15 sur les cellules NK.

mTOR contrôle également le développement des cellules NK, puisque la délétion sélective de mTOR dans ces cellules induit un blocage de la prolifération et de la différenciation au stade $CD11b^-$. Cependant, la survie de ces cellules NK déficientes n'est pas affectée. L'envoi des signaux de survie est plutôt assuré par STAT5 (Eckelhart et al., 2011). L'IL-15 contrôle également l'activité de mTOR *in vitro* en induisant la prolifération des cellules NK, en augmentant leur métabolisme et en induisant l'expression de récepteurs métaboliques comme le récepteur de la transferrine (CD71) et CD98, un transporteur d'acides aminés. mTOR contrôle finalement l'acquisition des fonctions effectrices en induisant l'expression de perforine et granzyme B et participe au priming des cellules NK en augmentant leur capacité de cytotoxicité ou de production d'IFN-γ. Dans un article récent, les auteurs ont montré que mTOR module le protéome des lymphocytes T cytotoxiques en contrôlant l'expression de protéines impliquées dans le métabolisme, l'adhésion et les fonctions effectrices de ces cellules (Hukelmann et al., 2015). La kinase mTOR semble réguler négativement (CD62L, enzymes de la phosphorylation oxydative, ribosomes) et positivement (granzymes, perforine, IFN-γ) un nombre équivalent de protéines ce qui suggère qu'elle régule à la fois leur production et leur dégradation. Si de façon générale les auteurs ont observé une bonne corrélation entre la quantité de transcrits et la quantité de protéines régulés par mTORC1, certaines protéines comme les protéines ribosomales ne semblent être régulées qu'au niveau traductionnel.

Certains inhibiteurs de mTOR ont des applications thérapeutiques. C'est le cas de la rapamycine, inhibiteur spécifique de mTORC1, qui en s'associant avec des protéines de la famille des FKBP (FK506 Binding Proteins) bloque l'activation du complexe formé par mTOR et Raptor. La rapamycine est utilisée dans deux contextes : pour éviter les rejets de greffes ainsi qu'en cancérologie pour diminuer le métabolisme des cellules cancéreuses.

3.2. Régulation par l'IL-2

Les effets de l'IL-2 sont proches de ceux de l'IL-15. L'IL-2 permet, notamment, de générer des cellules NK activées à partir de culture de cellules NK immatures de la moelle (Grimm et al., 1983). Une des principales différences entre ces deux cytokines, est le fait que l'IL-15 est active à des doses 10 à 50 fois moins importantes que l'IL-2 pour activer les cellules NK. Ceci résulte de l'absence d'expression de CD25 (IL-2Rα) par les cellules NK en condition homéostatique. Les effets de mutations dans la voie de l'IL-2 sur les cellules NK sont difficiles à interpréter, car ces mutations conduisent à des syndromes d'auto-immunité (Dauphinee et al., 1981), liés au rôle de l'IL-2 dans le développement des Lymphocytes T régulateurs (Treg). Pour éviter ce phénomène, des souris déficientes à la fois en IL-2 et en lymphocytes T ont été créées. Dans ces souris $Rag^{-/-}$ $Il2^{-/-}$, le nombre et le développement des cellules NK est normal indiquant que l'IL-2 n'est pas nécessaire au maintien de l'homéostasie des cellules NK en l'absence de lymphocytes T et B (Vosshenrich et al., 2005). Ces observations sont corrélées au fait qu'à la différence de l'IL-15, produite en condition homéostatique, l'IL-2 est principalement sécrétée lors d'une stimulation du système immunitaire. Cette IL-2, produite en condition inflammatoire, semble néanmoins avoir un rôle dans l'activation des cellules NK suite à des infections bactériennes (Granucci et al., 2004) ou parasitaires (Bihl et al., 2010). Des travaux anciens avaient également montré que les cellules NK entraient en compétition avec les lymphocytes T vis-à-vis de l'IL-2 (Henney et al., 1981; Su et al., 1994). Chez l'homme, la production d'IL-2 par les lymphocytes T spécifiques d'antigène, suite à une vaccination, active également les cellules NK périphériques (Horowitz et al., 2010). L'intérêt de l'IL-2 dans la biologie des cellules NK a récemment été renouvelé par une série d'articles proposant que l'activité des cellules NK était inhibée par les Treg, captant l'excès d'IL-2 produits par les lymphocytes T activés (Gasteiger et al., 2013a; Sitrin et al., 2013). En supprimant la population de Treg, il a été mis en évidence l'émergence d'une population de cellules NK immatures, qui sur-expriment CD25 en présence d'IL-12 (Gasteiger et al., 2013b), les rendant plus sensibles à l'IL-2.

3.3. Régulation par le TGF-β

3.3.1. Préambule

Le génome des vertébrés contient plus de 30 ligands appartenant à la superfamille du TGF-β: les TGF-β1, 2 et 3, les activines, les inhibines, Nodal, les “Bone Morphogenetic proteins” (BMPs), l’hormone de Regression Mullerienne (HRM) ainsi que les facteurs de différenciation et de croissance (GDFs) (Figure 19).

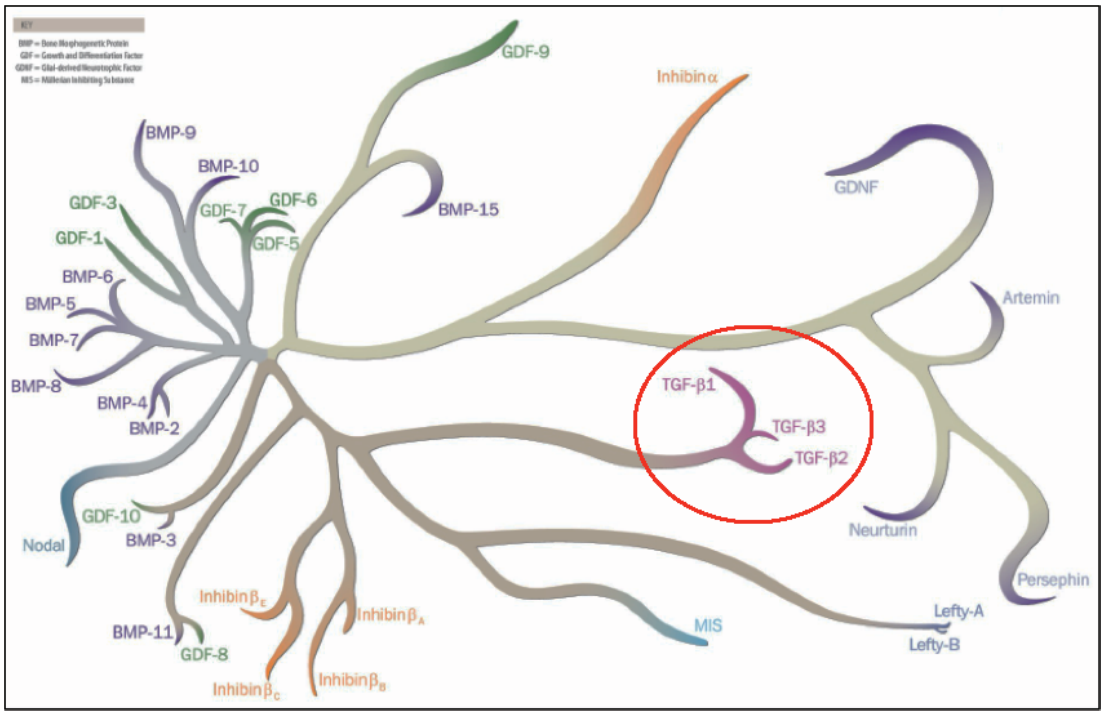

Figure 19: Arbre phylogénétique de la superfamille du TGF-β
(R&D Systems, 2007).

Les isoformes du TGF-β sont impliquées dans de nombreux processus cellulaires : inhibition de la croissance, migration cellulaire, invasion, transition épithélio-mésenchymateuse (EMT) ou l’immunosuppression. Dans le système immunitaire, l’isoforme la plus exprimée est le TGF-β1 (Li et al., 2006). L’importance de cette cytokine est illustrée par le fait que les souris déficientes pour le TGF-β1 ou son récepteur, meurent *in-utero* ou, que si elles survivent, elles développent une inflammation systémique fatale en quelques semaines (Kulkarni et al., 1993; Shull et al., 1992). Ces données suggèrent que le TGF-β1 a un rôle primordial dans le contrôle des réponses auto-immunes.

3.3.2. Structure et sécrétion

Les trois isoformes humaines du TGF-β sont codées par trois gènes distincts mais présentent une structure très proche (9 résidus cystéines dont 8 formant un « nœud

à cystéine », le 9ème résidu étant crucial pour l'homodimérisation), partagent le même récepteur et signalent de la même façon. En revanche, leur niveau d'expression est très variable en fonction des tissus (Millan et al., 1991). Les souris déficientes pour chacune des 3 isoformes, ont en effet, des phénotypes bien distincts (Kulkarni et al., 1993; Proetzel et al., 1995; Sanford et al., 1997).

Ces isoformes sont synthétisées sous la forme d'un précurseur qui doit être protéolysé pour être actif. D'abord, le peptide signal est retiré du pré-pro-TGF-β pendant le transit dans le réticulum endoplasmique. Puis, après une étape de dimérisation, des endoprotéases, de la famille des convertases, clivent le précurseur en peptide mature en C-terminal, la partie N-terminale formant le LAP (Latency Associated Protein) (Figure 20).

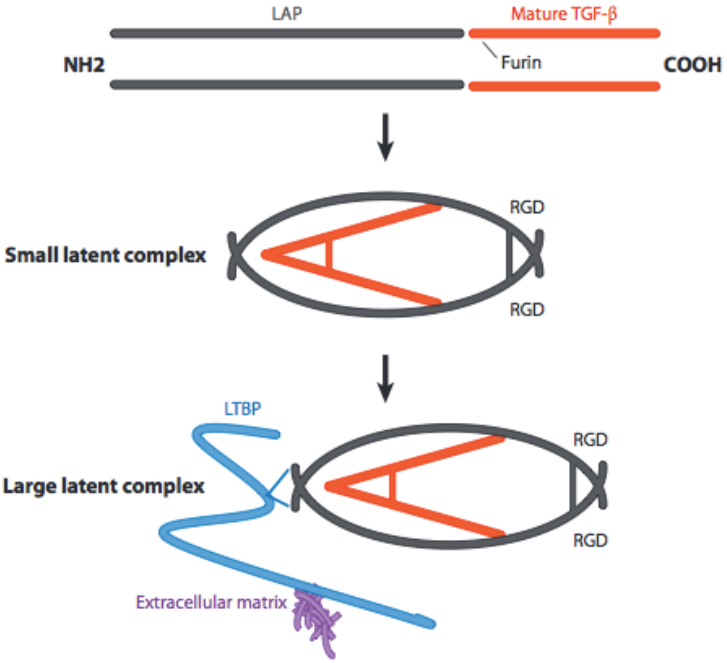

Figure 20 : Synthèse et sécrétion du TGF-β
Le TGF-β est synthétisé de façon associée au peptide LAP formant un complexe latent appelé SLC. L'association avec LAP permet de maintenir le TGF-β sous une forme inactive, empêchant sa liaison à ses récepteurs. Dans certains types cellulaires, le LAP peut se fixer à la protéine de liaison LTBP pour former un autre complexe (LLC) capable de se fixer aux protéines de la matrice extracellulaire. (Travis and Sheppard, 2014)

Après clivage, l'association, grâce à des liaisons non covalentes, du TGF-β mature et du LAP forme le SLC « Small Latent Complex » (Figure 20). Le LAP, en protégeant les épitopes d'interaction du TGF-β avec son récepteur maintient le TGF-β sous une forme latente.

Le SLC peut circuler librement ou se lier de façon covalente au LTBP (large Latent TGF-β binding Protein) et forme, alors, le LLC (Large Latent Complex). Les peptides LAPs confèrent la latence du complexe alors que les LTBPs permettent la fixation du TGF-β à la matrice extracellulaire et son stockage.

Pour se fixer à son récepteur, le TGF-β doit être libéré du LAP. Les mécanismes conduisant à la formation du TGF-β actif varient selon le type cellulaire, mais ciblent tous directement le LAP (Annes, 2003). *In vitro*, il a été montré que des conditions extrêmes (de pH ou de température) dénaturent les LAP, mais pas le TGF-β mature. *In vivo*, c'est la fixation de la thromobospondin-1 qui perturbe les liaisons non covalentes entre les LAP et le TGF-β mature. L'activation peut également être réalisée par des protéases (plasmine, thrombine, transglutaminases plasmatiques) qui clivent le LAP en TGF-β actif. Un autre mécanisme important d'activation est celui médié par certaines intégrines comme αvβ6 et αvβ8 qui reconnaissent une séquence particulière, la séquence RGD des LAP (Sheppard, 2005; Worthington et al., 2015). L'activation du TGF-β par les intégrines ne concerne que le TGF-β1 et TGF-β3 puisque le TGF-β2 ne contient pas cette séquence RGD.

3.3.3. Récepteurs du TGF-β

Une fois sous forme active, le TGF-β peut se fixer à ses récepteurs spécifiques qui ont une activité de type serine/thréonine kinase.

La famille des récepteurs du TGF-β est composée de deux sous-catégories de récepteurs transmembranaires, les récepteurs de type I (dont TGFbRI ou ALK5) et de type II (dont TGFbRII). Il existe 7 récepteurs de type I: ALK1-7 et 5 de type II. Ces deux types de récepteurs comportent tous une région extracellulaire riche en cystéine et un domaine intracellulaire dans lequel est situé leur domaine kinase (Massagué, 1998). TGFbRII a une activité kinase intrinsèque tandis que TGFbRI doit être phosphorylé pour être actif. Alors que les membres des BMP peuvent se fixer directement au récepteur de type I (Dijke et al., 1994; Koenig et al., 1994), les TGF-β (Franzén et al., 1993; Wrana et al., 1992) et les activines (Attisano et al., 1993) ont une affinité uniquement pour les récepteurs de type II. En se fixant aux domaines extracellulaires des récepteurs de type I et II, le TGF-β forme un complexe hétérodimérique qui permet une conformation productive des domaines à activité kinase facilitant la phosphorylation et l'activation de TGFbRI. Pour une signalisation optimale de TGFbRII, un récepteur additionnel, le betaglycane (connu aussi sous le

nom de TGFbRIII), permet la fixation du TGF-β à TGFbRII avec une meilleure affinité (Brown et al., 1999). Dans les cellules non stimulées, TGFbRI est maintenu inactif par son association avec la protéine FKBP12 (FK506-binding protein *12*) (Wang et al., 1996).

3.3.4. Signalisation

a. Voies canoniques

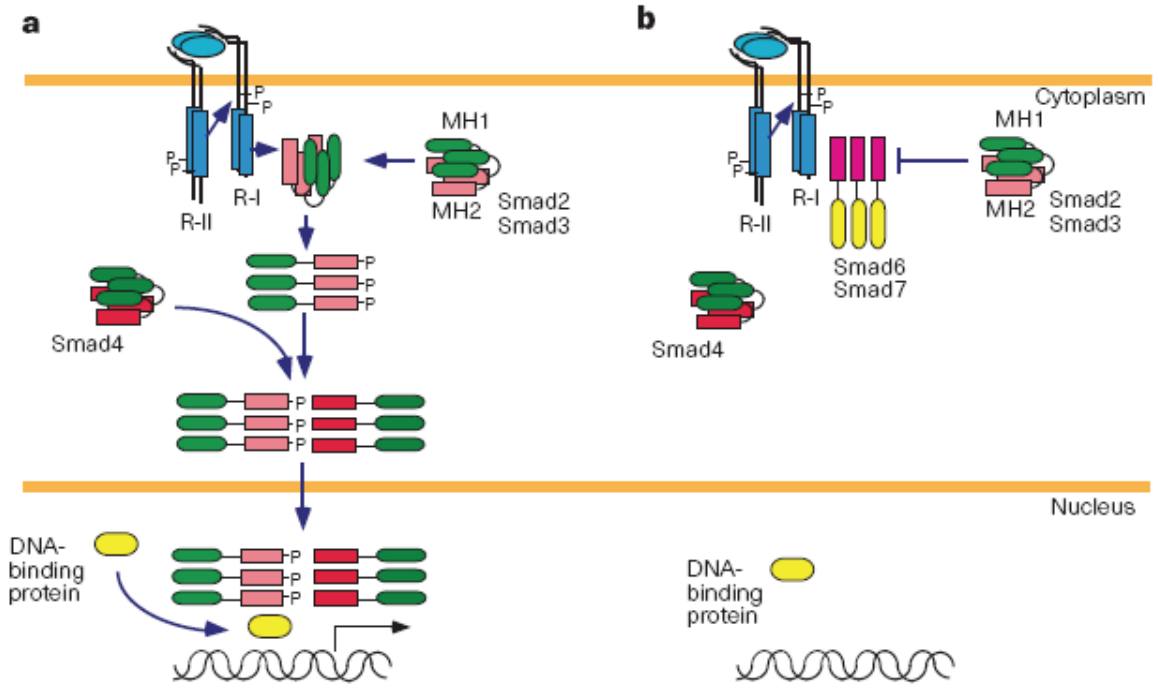

Figure 21: Signalisation du TGF-β *via* les protéines smad.
TGFbRII phosphoryle TGFbRI qui phosphoryle à son tour les R-Smad qui s'associent à Smad4 pour former un complexe qui migre dans le noyau (a). Les I-Smad inhibent cette voie de signalisation en se fixant à TGFbRI et empêche la phosphorylation des Smad 2 et 3 (b). (Heldin et al., 1997)

La fixation du TGF-β à ses récepteurs déclenche le recrutement des protéines Smad associées au récepteur (R-Smad), au niveau du domaine cytoplasmique du récepteur TGFbRI activé. Les R-Smad, Smad2 et Smad3 possèdent deux régions hautement conservées au cours de l'évolution, les domaines MH1 (MAD homology domain 1) et MH2 (MAD homology domain 2) (Figure 21-a), reliés par une région intermédiaire variable riche en proline. MH1 est responsable de la liaison à l'ADN, tandis que MH2 permet l'activité transcriptionnelle ainsi que les interactions entre les protéines Smad. L'extrémité C-terminale de MH2 contient la séquence SSXS, cible de la phosphorylation de TGFbRI. Cette phosphorylation induit un changement de

conformation qui libère le domaine MH2 du domaine MH1, permettant d'initier la transduction du signal. L'interaction des Smad avec les récepteurs activés est assurée par une protéine d'ancrage : SARA (Smad Anchor for Receptor Activation). D'autres protéines, comme Hrs, Dab2 et Axin peuvent faciliter l'interaction des R-Smad avec les récepteurs activés et amplifier le signal (Moustakas et al., 2001).

La phosphorylation des R-Smad par TGFbRI permet leur dissociation du récepteur et leur oligomérisation avec le médiateur commun (co-Smad), Smad 4, pour former un complexe hétérodimérique qui est transloqué dans le noyau. Le complexe R-Smad/Smad4 est connu pour interagir directement avec des régions particulières de l'ADN, les SBE (Smad-Binding Elements). Ces régions, riches en GC, sont localisées dans le promoteur des gènes cibles du TGF-β. Comme pour les facteurs de transcription, il existe des co-activateurs ou des corépresseurs qui régulent la transcription des gènes cibles de façon spécifique du type cellulaire (Heldin et al., 1997).

Il est connu que Smad 2 et 3 peuvent migrer dans le noyau en l'absence de Smad4 (Fink et al., 2003). Cependant, en l'absence de Smad4, ni Smad2 ni Smad3 ne peuvent avoir une activité transcriptionnelle, ce qui suggère que la fonction principale de Smad4 est de réguler la transcription plutôt que de transmettre les signaux d'activation du cytoplasme au noyau.

La régulation des voies de signalisation médiée par les protéines Smad est assurée par différents mécanismes. L'ubiquitination des protéines et leur dégradation par le protéasome est un mécanisme commun de régulation. La protéine Smurf peut interagir avec les protéines Smad et est impliquée dans leur ubiquitination. Smurf régule la quantité de Smad1 (un R-Smad impliqué dans la transduction du signal médié par les BMP) dans le cytoplasme de cellule non stimulée et cible également la région phosphorylée en C-terminal de Smad2. Une fois engagée dans un complexe transcriptionnel, Smad2 peut également être ubiquitiné et dégradé par le protéasome sous l'influence de Smurf2 (Kiessling et al., 1975; Lin et al., 2000). La forme nucléaire et phosphorylée de Smad3 est également ubiquitinée après l'achèvement de son activité transcriptionnelle. Dans ce cas, l'ubiquitination est médiée par le complexe SCF/Roc1. Enfin, et même si le mécanisme d'ubiquitination de Smad 4 est mal connu, la protéine Jab1 (Jun-activating domain binding protein 1) semble être impliquée dans sa dégradation (Wan et al., 2002).

Un autre mécanisme de régulation de la voie Smad implique les Smad inhibitrices (Smad-I), notamment Smad6 et Smad7 (Figure 21-b), qui agissent comme des inhibiteurs des voies de signalisation de tous les membres de la superfamille du TGF-β. Ces protéines ont une structure légèrement différente des autres Smad : elles partagent des similarités dans leur domaine C-terminal, mais leur partie N-terminale n'est pas homologue. Elles agissent essentiellement en entrant en compétition avec les R-Smad vis-à-vis de leur fixation au récepteur TGFbRI activé. Elles forment, en effet, avec TGFbRI, des liaisons plus stables que les R-Smad. En conséquence, elles empêchent la formation du complexe hétéromérique actif. La transcription de l'ARNm des I-Smad est induite par différents stimuli dont le TGF-β lui même, ainsi, ces protéines agissent comme des autorégulateurs de la transduction du signal des membres la superfamille du TGF-β.

b. Voies non canoniques

La voie de transcription Smad, qui contrôle la réponse transcriptionnelle de tous les membres de la superfamille du TGF-β est ubiquitaire et fonctionnelle dans tous les types cellulaires. Cependant, un certain nombre de voies de signalisation indépendantes des Smad, activées en fonction du contexte et qui contribuent à des réponses biologiques spécifiques d'un type cellulaire, ont également été mises en évidence. Les voies de signalisation indépendantes des Smad qui peuvent être activées par les membres de la superfamille du TGF-β sont les voies des petites GTPases, plusieurs branches de la voie MAP kinase comme Erk, Jnk ou p38 et la voie PI3 Kinase (Figure 22).

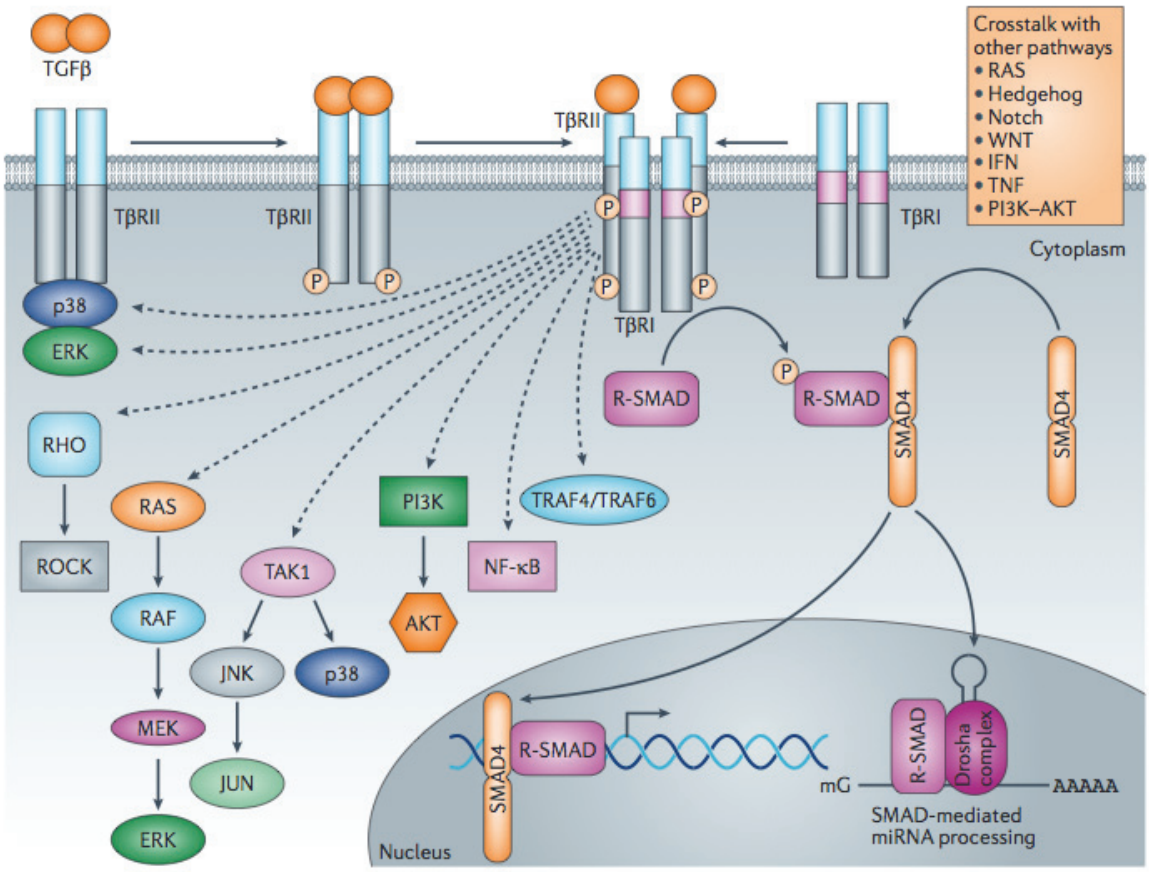

Figure 22: Voies d'activation non canoniques du TGF-β
En fonction du type cellulaire et du contexte, le TGF-β peut activer de nombreuses voies de signalisation comme TRAF4/TRAF6, NF-κB, AKT, p38, Jun ou ERK. (Akhurst and Hata, 2012)

Le TGF-β peut induire la transition épithélio-mésenchymateuse (EMT), processus par lequel les cellules épithéliales, formant un bloc serré, perdent leur polarité apico-basale et acquièrent un phénotype migratoire, dit mésenchymateux. L'EMT est un processus nécessaire au développement précoce, à la cicatrisation des plaies et est également impliqué dans la formation des métastases à partir d'une tumeur solide. Ce phénomène est initié par la dissolution des jonctions serrées situées au pôle apical des cellules épithéliales. On connaît, aujourd'hui le rôle clé des voies non canoniques activées par le TGF-β dans la réalisation de ce processus (Kiessling et al., 1975).

Plus précisément, le récepteur TGFbRII peut phosphoryler la protéine PAR6, protéine qui régule la polarité cellulaire et qui induit le recrutement de Smurf1. L'ubiquitination de la GTPase RhoA par Smurf1 favorise alors la perte des jonctions serrées. Bien que la dissolution des jonctions serrées soit indépendante des Smad, les évènements de transcription médiés par Smad restent nécessaires pour

compléter l'EMT induite par le TGF-β. Néanmoins, dans des modèles de cancer du sein, le blocage de PAR6 bloque le développement de métastases rénales et inhibe l'EMT des cellules tumorales primaires, ce qui illustre bien l'importance de cette voie dans la formation des métastases. (Viloria-Petit et al., 2009). De plus, les TGF-β et les BMPs sont aussi connues pour activer les GTPases Rho dans différents types cellulaires (Kardassis et al., 2009).

Les voies non canoniques peuvent aussi conduire à l'activation de plusieurs branches de la voie MAP (Mitogen-activated protein) kinase comme Erk, Jnk ou p38. En plus d'agir comme des Sérine/thréonine kinases, les récepteurs de type I et II sont connus pour avoir une double spécificité et pour phosphoryler des résidus Tyrosine (Mu et al., 2012; Zhang, 2009). La phosphorylation de la protéine Shc1 par les récepteurs de type I ou la phosphorylation de la tyrosine de TGFbRII peut induire le recrutement d'autres protéines à domaines SH2 comme Grb2 ou Shc1 et permettre l'activation des voies de signalisation Erk ou p38. Le TGF-β peut également stimuler la voie p38/Jnk en induisant l'activation de TAK1 (TGF-β Activated Kinase 1) *via* TRAF6 (Sorrentino et al., 2008; Yamashita et al., 2008). Dans certains contextes, le TGF-β induit également l'activation de la voie PI3K/AKT *via* les protéines Smad (Conery et al., 2004) mais également de manière directe et indépendante des Smad (Lamouille and Derynck, 2011; Mu et al., 2012; Zhang, 2009). L'activation de PI3K conduit à l'activation des voies AKT et mTOR pour réguler le métabolisme, la croissance cellulaire et la traduction. PI3K est également activé dans les cellules en EMT, sans doute pour faciliter leur migration et l'invasion cellulaire (Lamouille and Derynck, 2011). Au niveau moléculaire, le mécanisme d'action du TGF-β qui contrôle l'activation de PI3K reste inconnu.

3.3.5. Effets du TGF-β

Le TGF-β, et plus précisément, le TGF-β1, est considéré comme l'une des cytokines les plus immunosuppressives du système immunitaire (Li et al., 2006). Chez la souris, la délétion du gène codant pour la cytokine, ou son récepteur, induit un syndrome auto-immun systémique, médié par les lymphocytes T, conduisant à une mort rapide des animaux (Kulkarni et al., 1993; Shull et al., 1992). Le même phénotype est observé lorsque le gène codant pour TGFbRII est supprimé sélectivement dans les lymphocytes T (Gorelik and Flavell, 2000; Marie et al., 2006) ou dans les DC (Ramalingam et al., 2012). Ceci est essentiellement dû au fait que le TGF-β joue un rôle important dans le contrôle de la tolérance des lymphocytes T, en

promouvant le développement des Treg, mais aussi en freinant l'activation et la différenciation des lymphocytes T CD4 et CD8 (Li et al., 2006). Le TGF-β joue également un rôle important dans l'inhibition des cellules innées, comme les cellules NK (Ghiringhelli et al., 2005; Smyth et al., 2006). L'ajout de TGF-β dans des cultures de cellules NK contenant de l'IL-2 a, depuis longtemps, montré des effets inhibiteurs sur la prolifération, la cytotoxicité et la sécrétion d'IFN-γ des cellules NK (Espevik et al., 1988; Grimm et al., 1988; Malygin et al., 1993; Ortaldo et al., 1991; Yamamoto et al., 1994). Un article de 2005 s'est intéressé aux effets de la réduction des voies de signalisation dépendantes du TGF-β *in vivo* (Laouar et al., 2005). Pour cela, les auteurs ont généré des souris exprimant un récepteur transgénique capable de fixer le TGF-β mais incapable de transduire le signal (dominant négatif), sous la dépendance du promoteur de CD11c (Figure 23).

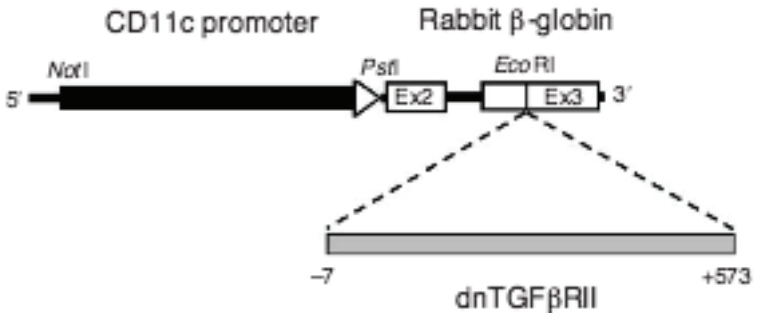

Figure 23: Construction du transgène CD11c-TGFbRIIdn
(d'après (Laouar et al., 2005))

Dans ce modèle, les auteurs ont montré que l'abrogation des signaux induits par le TGF-β dans les cellules NK induit leur prolifération, favorise leur maturation et augmente leur capacité de production d'IFN-γ. Il est, cependant, important de noter que, dans ce modèle, l'effet ne se limite pas qu'aux cellules NK puisque CD11c est exprimé par les cellules dendritiques, les cellules NK et certains monocytes.
La production de TGF-β par les cellules tumorales ou dans des contextes d'infections virales chroniques a un impact négatif sur les fonctions des cellules NK, notamment en diminuant l'expression des récepteurs activateurs NKp30 et NKGD (Castriconi et al., 2003; Lee et al., 2004) ou en modulant l'expression de récepteurs aux chimiokines (Castriconi et al., 2013). A l'inverse, la délétion des récepteurs du TGF-β sur les lymphocytes T conduit à l'activation incontrôlée des lymphocytes T CD8 qui sur-expriment différents récepteurs activateurs des cellules NK (Marie et al., 2006). Ces éléments suggèrent que le TGF-β est un important répresseur de l'expression

des récepteurs NK (Ruiz et al., 2014). Le mécanisme moléculaire par lequel le TGF-β régule l'homéostasie des cellules NK n'est pas totalement élucidé. Les protéines Smad sont connues pour inhiber l'expression de T-bet, en se fixant directement sur son promoteur, limitant ainsi la production d'IFN-γ par les cellules NK (Trotta et al., 2008; Yu et al., 2006). Il n'est, en revanche, pas certain que l'inhibition de T-bet explique, à elle seule, tous les effets du TGF-β sur les cellules NK.

3.4. Régulation par l'IL-12

L'IL-12 a été découverte en 1989 (Kobayashi et al., 1989) et fût initialement dénommée NKSF (NK Cell Stimulating Factor) par sa capacité à potentialiser les fonctions effectrices des cellules NK (cytotoxicité et sécrétion d'IFN-γ). Elle est composée de 2 sous-unités p35 et p40 qui se fixent à un récepteur composé de 2 chaînes β1 et β2, toutes deux exprimées par les cellules NK (Hyodo et al., 1999).

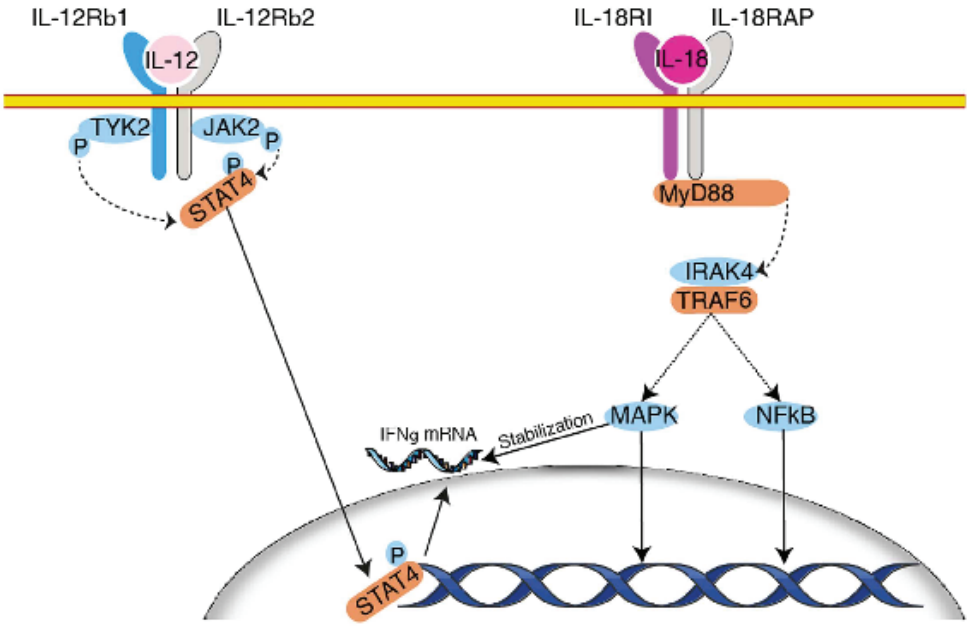

Figure 24: Synergie d'action entre l'IL-12 et l'IL-18 pour la production d'IFN-γ par les cellules NK
L'engagement du récepteur de l'IL-12 conduit à la phosphorylation de STAT4, qui active la transcription d'IFN-γ. La fixation de l'IL-18 sur son récepteur induit, quant à elle, l'activation de MAPK en aval de MyD88 aboutissant à la stabilisation de l'ARNm de l'IFN-γ mais également à l'amplification de sa production. (Marçais et al., 2013)

Cette cytokine est essentiellement produite par des cellules inflammatoires activées (monocytes, macrophages, neutrophiles et DC). Une fois la cytokine fixée sur son récepteur, la sous unité β2 est phosphorylée et sert de site d'ancrage aux kinases tyk2 et Jak2 conduisant à la phosphorylation de STAT4 (Bacon et al., 1995;

Jacobson et al., 1995; Shimoda et al., 2000) (Figure 24). L'importance de cette voie dans les cellules NK est illustrée par le fait que les souris déficientes dans les voies tyk2 et STAT4 ont des défaut de production d'IFN-γ (Nguyen et al., 2002; Shimoda et al., 2000). Chez l'homme, des mutations affectant STAT4 se caractérisent également par des défauts de production d'IFN-γ ainsi que par une diminution des capacités cytotoxiques des cellules NK(Gollob et al., 2000).

L'effet principal de l'IL-12, *in vivo*, est d'induire la production d'IFN-γ, son rôle dans la potentialisation de la cytotoxicité est plus controversé. En effet, la délétion du gène codant pour p35, ou l'utilisation d'anticorps bloquant, réduisent de 20 fois la production d'IFN-γ sans affecter la cytotoxicité (Nguyen et al., 2002; Orange and Biron, 1996a). Au niveau moléculaire, l'induction de l'IFN-γ par l'IL-12 est du à une augmentation de sa transcription, sous la dépendance de tyk2 (Shimoda et al., 2000). Comme beaucoup d'ARNm codant pour les cytokines, l'ARNm de l'IFN-γ est instable et essentiellement régulé au niveau post-transcriptionnel. L'IL-12 semble agir en le stabilisant, d'une façon dépendante de p38 (Mavropoulos et al., 2005). Ceci explique en partie la synergie d'action entre l'IL-12 et de l'IL-18 dans la production d'IFN-γ par les cellules NK (Mavropoulos et al., 2005) (Figure 24). L'IL-18 n'est pas la seule cytokine à agir en synergie avec l'IL-12. Des études plus anciennes ont mis en évidence une synergie d'action entre l'IL-2 et l'IL-12 (Kobayashi et al., 1989) impliquant également la régulation de la demi-vie de l'ARNm de l'IFN-γ (Chan et al., 1992).

3.5. Régulation par l'IL-18

Nommée initialement IGIF (IFN-γ Inducible Factor) (Okamura et al., 1995), l'IL-18 est une cytokine qui partage des fonctions biologiques avec l'IL-12, notamment la potentialisation des fonctions effectrices des cellules NK (Takeda et al., 1998) (Figure 24). Son absence conduit à une réduction des fonctions des cellules NK dans différents modèles (Wang et al., 2009; Wei et al., 1999). Comme l'IL-1 qui appartient à la même famille, l'IL-18 est produite sous la forme d'un précurseur, la proIL-18. Mais, à la différence des autres membres de la famille de l'IL-1, la forme proIL-18 est exprimée de façon constitutive (Gracie et al., 2003), par les cellules myéloïdes (macrophages activés, cellules dendritiques, polynucléaires neutrophiles), mais également par des cellules non hématopoïétiques (Pizarro et al., 1999). Ce précurseur nécessite un clivage par la caspase-1 au sein de l'inflammasome pour

générer la forme active (Ghayur et al., 1997; Gu et al., 1997). La sécrétion d'IL-18 par les cellules dendritiques apparaît au niveau de la synapse immunologique formée entre les cellules dendritiques et les cellules NK (Semino et al., 2005). C'est ce contact étroit qui permet, d'ailleurs, la production d'IFN-γ par les cellules NK (Bellora et al., 2010; Gerosa et al., 2002; Humann and Lenz, 2010; Lapaque et al., 2009; Piccioli et al., 2002). La régulation de la voie de l'IL-18 met en jeu des protéines régulatrices comme l'IL-18BP (Novick et al., 1999) ou une forme membranaire de l'IL-18 (Bellora et al., 2012) qui limitent les effets systémiques de cette cytokine.

Une fois fixée à son récepteur, l'IL-18RI, celui-ci se dimérise avec une seconde sous-unité, l'IL-18 RAP (IL-18R Accessory Protein). Ce complexe permet le recrutement de Myd88 qui induit l'activation des voies MAPK et NFκB *via* IRAK4 et TRAF6. Les cellules NK sont les seules cellules du système immunitaire qui expriment des niveaux élevés du récepteur de l'IL-18, ce qui en fait des cellules très sensibles à l'IL-18 (Kastenmüller et al., 2012). La délétion sélective de l'IL-18RI dans les cellules NK induit une diminution de leur capacité à produire de l'IFN-γ (Hoshino et al., 1999). Un tel phénotype est également observé dans les cellules NK déficientes pour IRAK4 (Suzuki et al., 2003). L'IL-18 est essentielle pour la production d'IFN-γ par les cellules NK dans de nombreux contextes : infections bactériennes (Rowland et al., 2006), virales (Pien et al., 2000), fongiques (Kawakami et al., 2000) et parasitaires (Haeberlein et al., 2010). En plus de réguler la production d'IFN-γ, l'IL-18 participe également au priming des cellules NK (Chaix et al., 2008; Zanoni et al., 2013) et à l'acquisition de fonctions migratrices *via* la surexpression de CCR7 (Bellora et al., 2010; Mailliard et al., 2005). Enfin, le rôle de l'IL-18 dans la réponse anti-tumorale est incertain. Certains auteurs avancent un effet antitumoral protecteur (Allen et al., 2010; Salcedo et al., 2010) alors que d'autres un effet plutôt immunosuppresseur *via* l'élimination, par les cellules NK, de cellules dendritiques participant à l'immunosurveillance tumorale (Terme et al., 2012)

3.6. Régulation par l'IL-10

L'IL-10 est une cytokine connue pour inhiber la sécrétion des cytokines de type Th1 (Fiorentino et al., 1989). Elle est produite par les macrophages, les cellules dendritiques, les lymphocytes B, certains sous-types de lymphocytes T et les cellules NK elles-mêmes (Kamanaka et al., 2006; Lee et al., 2009; Moore et al., 2001;

Perona-Wright et al., 2009), qui expriment les deux chaînes du récepteur de l'IL-10. Si ses effets sur les cellules NK semblent plutôt indirects, le traitement *in vitro* de cellules NK par de l'IL-10 ayant peu d'effet (Brady et al., 2010), la production d'IL-10 par les cellules NK semble jouer un rôle dans la régulation de la réponse des lymphocytes T CD8 (Tarrio et al., 2014). La génération de souris déficientes pour la voie de l'IL-10, sélectivement dans les cellules NK, devrait apporter plus d'information sur son rôle exact dans la biologie des cellules NK.

3.7. Régulation par l'IL-7

L'IL-7 est, comme l'IL-2 et l'IL-15, une cytokine de la famille γc, connue pour son rôle dans le développement des lymphocytes T dans le thymus et B dans la moelle. Le fait que les précurseurs NK et les cellules NK immatures expriment fortement CD127 (IL-7Rα) est plutôt inattendu puisqu'en l'absence d'IL-7, le développement et les fonctions des cellules NK sont complètement normaux (He and Malek, 1996; Vosshenrich et al., 2005). Une population particulière de cellules NK dépendantes de l'IL-7 a été identifiée dans le thymus et les ganglions (Vosshenrich et al., 2006). Ces cellules sont cytotoxiques et dépendent de l'IL-15. Cependant, elles diffèrent des cellules NK conventionnelles du fait de leur expression caractéristique de CD127, d'un développement dans le thymus et de leur dépendance au facteur de transcription GATA-3 (Ribeiro et al., 2010; Vargas et al., 2011; Vosshenrich et al., 2006). Du fait de leur rareté (environ 1000 cellules dans un thymus murin), ces cellules restent peu étudiées. De plus, dans le thymus, il est très difficile de distinguer les lymphocytes T γδ, des authentiques cellules NK (Stewart et al., 2007). Enfin, leur origine est controversée puisque les cellules NK $CD127^+$ sont en nombre normal dans les ganglions de souris athymiques (Stewart et al., 2007).

3.8. Régulation par l'IL-4

L'IL-4 est un autre membre des cytokines de la famille γc connu pour ses propriétés proTh2. Son absence n'a pas d'effet sur l'homéostasie des cellules NK (Vosshenrich et al., 2005), cependant, les cellules NK expriment le récepteur de l'IL-4 comme en témoigne leur grande sensibilité au traitement par l'IL-4 *in vitro* (Brady et al., 2010). Ses effets principaux sont d'inhiber les fonctions clés des cellules NK comme la cytotoxicité et la sécrétion de cytokines. En effet, l'IL-4 inhibe la production d'IFN-γ, de TNFα et GM-CSF induite par l'IL-12, chez l'homme (Marcenaro et al., 2005). Des effets similaires ont été observés chez la souris, l'IL-4 inhibant la cytotoxicité

dépendante des cytokines des cellules NK. L'IL-4 agit directement en inhibant l'expression de récepteurs activateurs comme NKG2D (Brady et al., 2010; Marcenaro et al., 2005) et indirectement en inhibant la maturation des DC induite par les cellules NK (Agaugué et al., 2008). Dans les lymphocytes T CD8, l'IL-4 induit fortement l'expression d'Eomes (Weinreich et al., 2010). Compte tenu du rôle majeur de ce facteur de transcription dans la maturation des cellules NK, on peut imaginer l'importance des effets de l'IL-4 dans la biologie des cellules NK. Paradoxalement, à côté de ses effets proTh2, l'IL-4 induit également des réponses Th1 pendant la phase précoce de certaines infections parasitaires (Biedermann et al., 2001) et induit même, directement *via* STAT6, la production d'IFN-γ par les cellules NK (Morris et al., 2006).

3.9. Régulation par l'IL-21

Egalement membre des cytokines de la famille γc, l'IL-21 a une structure très proche de l'IL2, de l'IL-4 et de l'IL15. Son récepteur, l'IL-21R ressemble également à la chaîne β du récepteur de l'IL-2. Une fois fixé sur son récepteur, celui-ci se couple à la chaîne γc et active les voies de signalisation JAK1 et STAT5. L'IL-21 est exprimée essentiellement par les lymphocytes T CD4 activés, tandis que son récepteur est exprimé par les cellules lymphoïdes (Kasaian et al., 2002; Parrish-Novak et al., 2000). L'IL-21 a des effets contraires. Elle agit en inhibant la prolifération des cellules NK induite par l'IL-15, mais potentialise l'effet de l'IL-15 sur la différenciation terminale des cellules NK et dans l'acquisition des fonctions effectrices (Brady et al., 2004, 2010; Kasaian et al., 2002; Strengell et al., 2003). L'expression forcée d'IL-21, *in vivo*, entraîne une réduction, dépendante des cellules NK, du nombre de tumeurs pulmonaires obtenues après injection de lignées tumorales (Brady et al., 2010). Comme l'IL-21 renforce la prolifération des cellules T *in vitro*, il a été proposé que sa production par les lymphocytes T activés stoppe les réponses des cellules NK, une fois l'immunité adaptative mise en place. L'IL-21 produite par les lymphocytes T CD4 est essentielle pour prévenir l'épuisement des lymphocytes T CD8 lors d'infections virales chroniques (Elsaesser et al., 2009; Fröhlich et al., 2009; Yi et al., 2009). Le fait que l'IL-21 contrôle également la fonction des cellules NK lors d'infections virales chroniques doit également être testé, mais le traitement *in vitro* par l'IL-21 de cellules NK de patients atteints du virus du SIDA, augmente leurs fonctions effectrices (Iannello et al., 2010; Strbo et al., 2008)

3.10. Régulation par les IFN de type I

Les interférons de type I (IFN-I), en particulier les IFN-α/β ont d'abord été identifiés comme des protéines responsables de la résistance cellulaire aux infections virales. Ils sont produits par différents types cellulaires d'origine hématopoïétique, ou non. Les effets de ces molécules sur les cellules NK sont connus depuis longtemps. Ils induisent la prolifération et la cytotoxicité des cellules NK (Gidlund et al., 1978). L'effet des IFN-I, est, au moins partiellement, indirect sur les cellules NK puisque leur absence peut être compensée par l'injection d'IL-15 (Nguyen et al., 2002). Cependant, d'autres études ont montré des effets directs des IFN-I sur la cytotoxicité des cellules NK (Beuneu et al., 2011; Martinez et al., 2008). Les IFN-I modulent également positivement la production d'IFN-γ *via* STAT4 et négativement *via* STAT1 en fonction du contexte (Miyagi et al., 2007).

4. Cellules NK et Cancer

4.1. Concept d'immunosurveillance

L'immunosurveillance est un concept selon lequel le système immunitaire est capable de reconnaître et d'éliminer des cellules tumorales. D'abord évoquée par Ehrlich en 1909, cette théorie a été reprise par Burnet et Thomas dans les années 60 après la mise en évidence d'antigènes spécifiques de tumeurs (Old and Boyse, 1964). Si cette théorie est aujourd'hui admise, elle a été remise en cause dans les années 70 par une série d'articles montrant que des souris athymiques ne développaient pas plus de cancers spontanés ni de cancers induits par des carcinogènes chimiques que les souris sauvages (Stutman, 1974, 1979). Depuis, des observations faites chez l'homme comme l'augmentation de l'incidence des cancers chez les patients immunodéprimés (Boshoff and Weiss, 2002; Vajdic et al., 2006) ou la relation entre l'infiltration des tumeurs par les cellules du système immunitaire et le pronostic ont définitivement validé la théorie de l'immunosurveillance. Aujourd'hui, le concept d'immunosurveillance a évolué vers le concept d'immuno-editing, processus dynamique qui comporte 3 phases: l'élimination de la tumeur, l'équilibre et l'échappement (Dunn et al., 2002). Au cours de la phase d'élimination, les cellules du système innée et adaptatif agissent en synergie pour détecter une tumeur naissante et l'éliminer avant que les signes cliniques n'apparaissent. Les mécanismes par lesquels le système immunitaire est alerté du développement d'une tumeur ne sont vraisemblablement pas tous connus.

Les IFN de type I semblent jouer un rôle important dans ce processus puisqu'ils activent les DC et favorisent une réponse adaptative anti tumorale. Les signaux de dangers (DAMP) doivent également être pris en compte car ils sont libérés directement des cellules tumorales mourantes ainsi que des tissus endommagés (Sims et al., 2010). Un autre mécanisme de reconnaissance met en jeu l'intéraction entre les ligands de stress comme MICA/B avec NKG2D, conduisant à la sécrétion de cytokines pro-inflammatoires comme l'IFN-γ et favorisant un microenvironnement défavorable au développement tumoral. En effet, l'IFN-γ est bien connu pour protéger l'hôte contre la croissance de tumeurs transplantées, induites par des carcinogènes mais également de cancers spontanées (Dunn et al., 2004). Les protéines cytotoxiques comme la perforine et TRAIL jouent également un rôle clé dans la phase précoce d'élimination tumorale. Les souris déficientes pour perforine sont beaucoup plus sensibles que les animaux immunocompétents à des tumeurs induites ou transplantées (Brennan et al., 2010). Chez l'homme, des polymorphismes du gène codant la perforine comme l'allèle A91V semblent prédisposer l'apparition de différents types de cancers (Clementi et al., 2005; Santoro et al., 2005).

La seconde phase de l'immunoediting est la phase d'équilibre pendant laquelle le système immunitaire contient les cellules tumorales dans un état de dormance. Un des modèles utilisés chez la souris est l'injection de faibles doses d'un carcinogène, le methylcholanthrene, qui induit chez des souris immunocompétentes le développement de tumeurs contrôlées par le système immunitaire (Koebel et al., 2007). L'injection d'anticorps déplétant les lymphocytes T induit le redéveloppement rapide de ces tumeurs dormantes alors que la déplétion des cellules NK n'a pas d'effet. Ceci suggère que cette phase d'équilibre entre la tumeur et l'hôte semble dépendante des cellules de l'immunité adaptative mais pas des cellules NK.

Dans la phase d'échappement, les cellules tumorales ont acquis la capacité d'échapper à leur reconnaissance et à leur élimination par le système immunitaire. Différents mécanismes facilitent l'échappement tumoral. Au niveau des cellules tumorales, on peut citer par exemple la diminution de l'expression des molécules de classe I du CMH qui vont diminuer leur reconnaissance ou la surexpression de molécules anti-apoptotiques qui vont augmenter leur résistance. L'échappement est également facilité par la création d'un état immunosupresseur dans le microenvironnement via la sécrétion de cytokines comme le TGF-β et le recrutement de cellules immunosupressives : Treg et cellules myéloïdes suppressives.

La capacité d'échappement est une caractéristique importante des cellules cancéreuses et fait partie des 4 « nouvelles » capacités distinctives des cellules tumorales (et qui s'ajoutent aux 6 capacités déjà connues) décrites par Hanahan et Weinberg en 2011 (Hanahan and Weinberg, 2011).

4.2. Rôle des cellules NK dans le cancer

Depuis la découverte des capacités des cellules NK à éliminer spontanément des cellules cancéreuses, leur rôle dans la réponse anti-tumorale a largement été étudié chez la souris et chez l'homme. Elles sont, en effet, impliquées dans l'immunosurveillance des cancers hématologiques, mais également des cancers solides. Du fait de cette propriété, les cellules NK sont donc rapidement devenues des cibles thérapeutiques pour renforcer la réponse anti-tumorale chez des patients atteints de cancers. Même si la première génération de traitements ciblant les cellules NK, basé sur l'utilisation de cellules NK autologues activées par de l'IL-2, n'a pas eu les effets escomptés, de nombreux traitements visant à augmenter l'activité des cellules NK ont été développés ou sont en cours d'évaluation.

4.2.1. Cancers solides

Le rôle des cellules NK ne semble pas, comme il l'a longtemps été supposé, limité aux tumeurs d'origine hématopoïétique. L'absence du récepteur NKG2D, chez la souris, augmente la sensibilité au cancer de la prostate (Guerra et al., 2008) tandis que la déplétion des cellules NK augmente le nombre de métastases pulmonaires induites par l'injection de cellules de mélanomes de la lignée B16 (Gorelik et al., 1982). Chez l'homme, l'activité des cellules NK (mesurée par la capacité des cellules NK à éliminer des cellules tumorales *in vitro*) est corrélée au risque de développer un cancer (Imai et al., 2000). De plus, dans les cancers gastriques, colorectaux ou pulmonaires, une infiltration plus importante de cellules NK dans le tissu tumoral est associée à un meilleur pronostique (Coca et al., 1997; Ishigami et al., 2000; Villegas et al., 2002).

Enfin et quelle que soit leur origine, la majorité des cellules tumorales expriment des ligands des récepteurs activateurs des cellules NK (Tableau 3).

Récepteur Activateur	Ligand(s)	Expression des ligands par des cellules tumorales	Exemples
NKp46	Inconnu	Différentes lignées tumorales	(Elboim et al., 2010)
	HSPG	Surexprimé par les cellules tumorales	(Hecht et al., 2009)
NKp30	BAT3/BAG6	293T (cellules embryonnaires de rein transformées)	(Pogge von Strandmann et al., 2007)
	B7-H6	Carcinomes, mélanomes	(Brandt et al., 2009)
	HSPG	Surexprimé par les cellules tumorales	(Hecht et al., 2009)
NKp44	MLL5	Cancer du col de l'utérus, de la vessie, du rein, mélanome.	(Baychelier et al., 2013)
	HSPG	Surexprimé par les cellules tumorales	(Hecht et al., 2009)
NKG2D	MICA-B, ULBP1-6	Surexprimés par les tumeurs d'origine épithéliale et non épithéliale	(El-Gazzar et al., 2013)
DNAM-1	CD155, CD112	Mélanomes, neuroblastomes, carcinomes, glioblastomes	(Bottino et al., 2003)
NKp80	AICL	HeLaS3 et ME180 (carcinomes)	(Akatsuka et al., 2010)

Tableau 3: Expression des ligands des récepteurs activateurs des cellules NK par les cellules tumorales

Le rôle exact des cellules NK infiltrant les tumeurs reste, cependant, incertain. Dans la plupart des cas, au sein des cellules immunitaires infiltrant la tumeur, la fréquence des cellules NK est faible conduisant à un ratio Effecteur : Cible très en faveur des cellules tumorales. De plus, les cellules NK ne sont, en général, pas localisées en contact direct avec les cellules cancéreuses, mais à la proximité des vaisseaux sanguins. Les cellules NK rejoignent la tumeur par la circulation sanguine, traversent les vaisseaux et migrent jusqu'au tissu tumoral par des mécanismes qui mettent en jeu des interactions entre les chimiokines et leurs récepteurs comme CXCR3 et CX3CR1 (Grégoire et al., 2007). Malgré cela, plusieurs équipes ont étudié le nombre et la fonction des cellules NK infiltrantes. Dans la majorité des cas, des défauts de fonction sont observés et sont associés à une progression de la maladie. L'environnement tumoral contient, en effet, de nombreux facteurs inhibant la fonction des cellules NK, résumés dans la Figure 25.

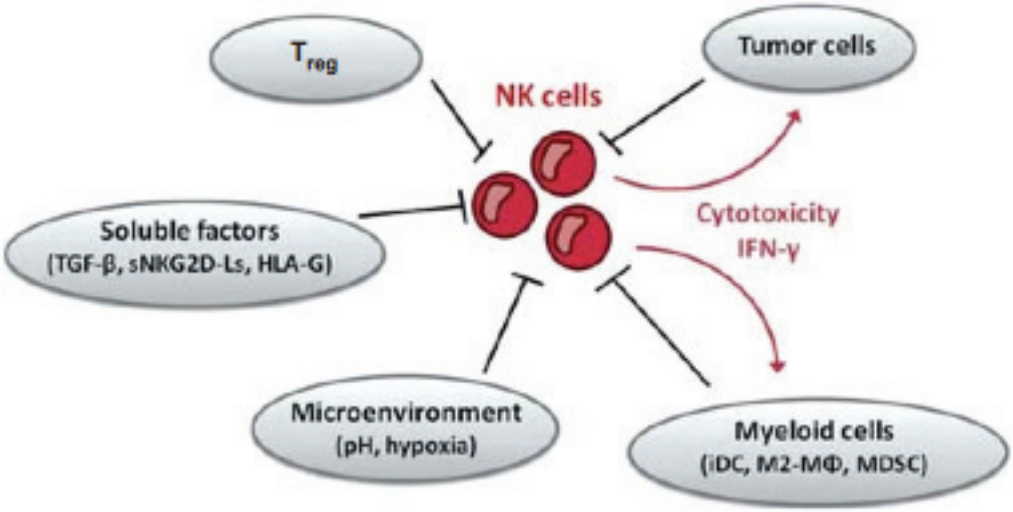

Figure 25: Facteurs du microenvironnement tumoral inhibant les cellules NK
Différents facteurs peuvent inhiber les réponses des cellules NK comme un pH acide, l'hypoxie, des molécules solubles comme les TGF-β, HLA-G ou des ligands solubles de NKG2D ainsi que les populations suppressives comme les Treg, les cellules myéloïdes suppressives ou les cellules tumorales elles-mêmes. (Stojanovic and Cerwenka, 2011)

Le premier mécanisme d'évasion tumoral est l'inhibition des cellules NK par les cellules de l'environnement. En effet, les cellules immunitaires associées aux tumeurs peuvent sécréter du TGF-β ou de l'IL-4 qui modulent l'activité des cellules NK. L'inhibition peut également être directe, par les Treg, qui sont capables d'inhiber les cellules NK *via* le TGF-β lié à leur membrane ou en entrant en compétition pour l'IL-2. Une réduction du nombre de Treg, chez des patients atteints de cancer, est d'ailleurs corrélée avec une meilleure activité des cellules NK (Ghiringhelli et al., 2006). D'autres cellules infiltrantes, comme les macrophages ou les fibroblastes, sont également capables d'inhiber la fonction des cellules NK(Balsamo et al., 2009; Sprinzl et al., 2013). Finalement, les cellules tumorales elles-mêmes sont également capables d'inhiber les cellules NK. C'est, par exemple, le cas des lignées dérivées de cellules primaires de mélanome qui inhibent l'expression des récepteurs NKp30, Nkp44 et NKG2D par les cellules NK (Pietra et al., 2012) ou des cellules de neuroblastome, qui, en modulant l'expression des récepteurs aux chimiokines des cellules NK, modifient leur capacité migratoire, *via* la production de TGF-β (Castriconi et al., 2013). Un dernier élément inhibiteur est représenté par le niveau d'oxygénation du microenvironnement tumoral. L'hypoxie altère à la fois le phénotype et la fonction des principaux récepteurs activateurs des cellules NK (Balsamo et al., 2013).

Le deuxième mécanisme d'échappement tumoral est le phénomène d'évasion *via* l' « immuno-editing » des cellules tumorales. L'exposition chronique des tumeurs à des cellules NK semble favoriser la sélection et le développement de cellules tumorales ayant une expression altérée des ligands des récepteurs activateurs et inhibiteurs. Il a été montré, que les cellules de mélanomes ne pouvant être rapidement éliminées par les cellules NK augmentaient l'expression des molécules de classe I du CMH et devenaient résistantes à la reconnaissance et à l'élimination médiée par les cellules NK(Balsamo et al., 2012). De plus, les tumeurs qui se développent dans des souris déficientes pour NKp46 expriment d'avantage de ligands de NKp46, en comparaison avec les tumeurs se développant dans des souris sauvages (Elboim et al., 2010). Enfin, les cellules tumorales peuvent également éviter la reconnaissance médiée par NKp44 ou NKG2D en diminuant, à leur surface, l'expression de ligands de NKG2D (El-Gazzar et al., 2013) ou en surexprimant les protéines PCNA (Proliferating cells nuclear Antigen), qui, en s'associant avec les molécules de classe I du CMH, engagent NKp44 et délivrent des signaux inhibiteurs paradoxaux conduisant à une réduction de la cytotoxicité des cellules NK (Horton et al., 2013).

4.2.2. Cancers hématologiques

Dans la plupart des cancers hématologiques, des défauts de l'activité des cellules NK ont été rapportés.

a. Mécanismes d'échappement dans les hémopathies malignes

L'échappement tumoral peut, selon la pathologie, impliquer des déficits qualitatifs ou quantitatifs des cellules NK. Ces défauts sont résumés dans le Tableau 4.

Mécanisme d'échappement	Récepteur	Ligand	Hémopathie
Baisse du nombre de cellules NK			MDS
Surexpression de signaux inhibiteurs		CMH de classe I	LAM, LAL, LMC MM, LNH
Défauts d'expression des récepteurs activateurs ou de leurs ligands	NKp30		LAM
	NKp46/NKG2D		LLC
	NKG2D		LLC, LAM, LMC
		Ligands des NCRs	LAM
		MICAs et MICBs	LAM, LMC, MM
	NKG2C, 2B4		LAM
	CD16		LAM, MM
Défaut de différenciation des cellules NK			LMC
Défaut de production de cytokines			MDS, SMP, LAM, LAL

Tableau 4: Récepteurs et ligands impliqués dans l'échappement immunitaire des hémopathies malignes
SMD : Syndrome Myélodysplasique, LAM : Leucémie Aigue Myéloïde, LAL : Leucémie Aigue Lymphoïde, LMC : Leucémie Myéloïde Chronique, MM : Myélome Multiple, LNH : Lymphome Non Hodgkinien, LGL : Large Granular Lymphocytes, SMP : Syndrome Myéloprolifératif (adapté de Farnault et al., 2012)

Dans les syndromes myélodysplasiques, il a été proposé que la réduction de l'activité des cellules NK était causée par une diminution du nombre de cellules NK circulantes (Kerndrup et al., 1984). Cette réduction étant associée à une élévation de la concentration plasmatique du récepteur soluble de l'IL-2 (RsIL-2), il est possible que la production de RsIL-2, par les cellules dysplasiques, puisse altérer le développement normal des cellules NK.

L'échappement tumoral peut être également lié à des déficits qualitatifs. Tout d'abord, certaines tumeurs hématologiques, comme les cellules de leucémie aigue myéloïde (LAM), expriment des niveaux normaux de molécules de classe I du CMH, inhibant ainsi la reconnaissance des cellules NK. D'autres, comme les leucémies aigues lymphoïdes (LAL), les leucémies lymphoïdes chroniques (LLC), les cellules de lymphomes et de myélomes sont même capables d'augmenter l'expression de molécules de classe I du CMH, pour échapper à la lyse médiée par les cellules NK. Ensuite, certaines hémopathies induisent des défauts d'expression des récepteurs activateurs. Dans les LAM, le défaut d'expression de NKp30 et NKp46 est associé à une diminution de la cytotoxicité des cellules NK (Costello et al., 2002). Ce

phénomène, qui conduit à un phénotype de cellules NK « NCR^{dull} » est réversible après traitement, et est donc probablement acquis par un contact direct des cellules NK avec les blastes. Des défauts d'expression de ligands des NCR, observés dans différentes hémopathies, participent également au défaut de reconnaissance des cellules NK *via* ce type de récepteurs activateurs. Une autre interaction ligand-récepteur activateur est altérée dans les hémopathies malignes, il s'agit de celle impliquant NKG2D/MICA. Des concentrations anormalement élevées de la forme soluble de MICA ont été mises en évidence dans le sang de patients atteints de LMC et de myélome multiple (MM) tandis que des défauts d'expression de NKG2D ont été rapportés dans les LMC, LLC et LAM (Farnault et al., 2012). D'autres anomalies comme des défauts d'expression de 2B4 ou CD16 ont également été mises en évidence chez des patients atteins de leucémie aigue ou de MM (Fauriat et al., 2006). Finalement, les autres mécanismes pouvant conduire à l'échappement de la réponse immunitaire des cellules NK par les cellules tumorales sont des défauts de différenciation ainsi qu'une inhibition médiée par des cytokines inhibitrices comme le TGF-β ou le PDGF.

b. Cas du myélome

Le MM est une hémopathie lymphoïde caractérisée par la prolifération d'un clone de plasmocyte dans la moelle osseuse. De nombreuses études, résumées dans le Tableau 5, se sont intéressées à la description des cellules NK dans le MM.

Nombre de cellules NK	Récepteurs activateurs	Cytotoxicité	Référence
Augmenté		Inchangée	(Famularo et al., 1992)
Inchangé			(King and Radicchi-Mastroianni, 1996)
Augmenté		Diminuée	(Frassanito et al., 1997)
Augmenté dans la moelle osseuse			(Sawanobori et al., 1997)
	Diminution de CD16, 2B4		(Fauriat et al., 2006)
	Diminution de NKG2D		(Jinushi et al., 2008)
		Diminuée	(Jurisic et al., 2007)
	Diminution de NKG2D		(Lilienfeld-Toal et al., 2009)
Augmenté dans le sang et la moelle osseuse			(Pessoa de Magalhaes et al., 2013)

Tableau 5: Phénotype et fonction des cellules NK chez des patients atteints de myélome multiple.

Un des travaux les plus anciens a d'ailleurs montré que les cellules NK étaient capables d'éliminer des cellules autologues de MM d'une manière dépendante de NKG2D et des NCR (Carbone, 2005). De plus, l'évolution de l'expression des molécules de classe I du CMH et des ligands de NKG2D, MICA, au cours de la maladie, suggère l'existence d'une pression de sélection médiée par les cellules NK. Si les défauts d'activation des cellules NK *via* NKG2D contribuent à l'échappement des cellules myélomateuses, d'autres mécanismes sont également impliqués. L'expression de certains récepteurs, comme 2B4 ou CD16 (Fauriat et al., 2006), est également altérée chez les patients atteints de MM tandis qu'à la différence de cellules NK « normales », les cellules NK de patients atteints expriment PD-1 (Programmed cell Death 1) (Benson et al., 2010). Le blocage, par un anticorps bloquant, de l'interaction de PD-1 avec son ligand (PD-L1), exprimé par les cellules myélomateuses, augmente l'activité des cellules NK vis-à-vis des cellules tumorales autologues. En plus des anomalies phénotypiques sus-décrites, les cellules NK de patients atteints de MM présentent également des défauts de cytotoxicité, dont la sévérité dépend du stade de la maladie (Jurisic et al., 2007). Dans un article récent utilisant un modèle murin de myélome, les auteurs ont mis en évidence le rôle de

DNAM-1 (Guillerey et al., 2015) dans le contrôle de la maladie. Ils ont observé que l'expression de DNAM-1 diminue au cours de la progression de la maladie et que son absence accélère cette progression. Ils ont également montré, que le contrôle de la maladie par DNAM-1 mettait en jeu son interaction avec son ligand, CD155, molécule surexprimée par les lignées de cellules myélomateuses (El-Sherbiny et al., 2007), faisant de DNAM-1 une cible potentielle des traitements anticancéreux.

c. Traitements visant à moduler l'activité des cellules NK

Depuis la découverte du potentiel inné des cellules NK à éliminer des cellules tumorales, l'intérêt de leur utilisation thérapeutique en cancérologie n'a cessé de grandir. Les premières stratégies développées consistèrent en l'administration systématique de cytokines activatrices comme l'IL-2, puis au transfert de cellules NK autologues activées, avec des résultats plutôt décevants et des effets indésirables importants. La découverte du phénomène de disparité entre les KIRs et leurs ligands, au cours duquel la reconnaissance du non-soi par des cellules NK allogéniques, greffées à des patients atteints de cancer, entraine un effet de GVL (Greffon Vs Leukemia) a relancé l'intérêt des cellules NK en thérapeutique. De nombreuses études, associant la greffe de cellules NK hétérologues à une chimiothérapie classique, sont en cours. L'évaluation de leur intérêt, en terme de bénéfice/risque, doit encore être déterminé. Une alternative, plus simple en terme pratique, à l'utilisation de cellules NK primaires est l'utilisation de lignées de cellules NK. La lignée NK-92 a d'ailleurs reçu des autorisations de la part de la FDA (Food and Drug Administration) pour être testée dans différents cancers, puisque son utilisation semble sûre et qu'elle présente des effets bénéfiques anticancéreux potentiels. En plus des avantages certains des cellules de lignées comme la facilité d'obtention et d'entretien et une plus grande homogénéité, ces cellules sont également plus facilement modifiables au niveau génétique que les cellules primaires du fait d'une plus grande permissivité. Là encore, des études sont en cours pour évaluer l'intérêt de la modulation génétique de différentes voies d'activation (IL-2, IL-15, SCF, TGF-β) ou d'induire l'expression de récepteurs chimériques spécifiques d'antigènes tumoraux couplés à des récepteurs à ITAM (Topfer et al., 2015).

Une autre stratégie thérapeutique partiellement dépendante des cellules NK est l'utilisation d'anticorps monoclonaux capables d'activer et d'induire la dégranulation des cellules NK par le phénomène d'ADCC. Les deux principaux exemples pour lesquels l'efficacité est démontrée sont l'anti-CD20 dans le traitement des LNH et

l'anti-Her2 dans les formes métastatiques des cancers du sein et du colon. Différentes modifications structurales, comme l'humanisation, permettent de réduire les effets indésirables liés à l'activation du complément, en maintenant, voire en augmentant la capacité d'induire l'ADCC.

Comme la plupart des molécules administrées au cours des chimiothérapies ont un impact sur le système immunitaire, la nécessité d'y associer une immunothérapie est maintenant admise. Parmi ces traitements immunomodulateurs ciblant les cellules NK et utilisés en cancérologie, on peut citer les inhibiteurs du protéasome comme le Bortézomib, les anti-KIRs ou certains agents déméthylants Il est important de noter qu'un grand nombre de molécules visant à inhiber la voie du TGF-β sont en phase d'essai clinique (Akhurst and Hata, 2012). Une autre grande famille de médicaments immunomodulateurs est celle des dérivés de la thalidomide qui composent la classe des « IMiDs ». Ces médicaments ont des effets antiangiogéniques (D'Amato et al., 1994; Dredge et al., 2002; Gupta et al., 2001), des effets antiprolifératifs directs (Mitsiades et al., 2002; Verhelle et al., 2007) en plus de leurs propriétés immuno-modulatrices (inhibition de la production de cytokines pro-inflammatoires, activation des lymphocytes T et des cellules NK) (Corral et al., 1999). Les premières études s'intéressant aux effets des IMiDs sur les cellules NK ont montré que ces médicaments augmentaient la cytotoxicité des cellules NK (à la fois vis-à-vis de cibles déficientes pour les molécules de classes I du CMH (Zhu et al., 2008) et de cibles recouvertes d'anticorps monoclonaux (Wu et al., 2008)). Les mécanismes d'action sur la potentialisation des fonctions des cellules NK ne sont, en revanche pas totalement compris. Certains auteurs proposent qu'ils soient plutôt indirects, *via* la sécrétion d'IL-2 par les lymphocytes T CD4 (Hayashi et al., 2005) et d'autres cytokines/chimiokines par les DC (Reddy et al., 2007). Des données obtenues récemment sur des cellules NK purifiées mettent en évidence un effet direct du lénalidomide, *in vitro*. Il augmenterait les fonctions des cellules NK en diminuant leur seuil d'activation et en favorisant le remodelage du cytosquelette d'actine(Lagrue et al., 2015). Cependant, ses effets sur les cellules NK *in vivo*, chez l'homme, n'ont pas clairement été démontrés. La principale limite à leur étude, *in vivo*, est le fait qu'ils soient souvent associés à des médicaments cytotoxiques et rarement utilisés en monothérapie.

OBJECTIFS DU TRAVAIL DE THESE

Comme nous l'avons évoqué dans l'introduction, la biologie des cellules NK est largement influencée par l'environnement cytokinique. Alors que certaines cytokines pro-inflammatoires comme l'IL-2/15, l'IL-12 ou l'IL-18 potentialisent l'activité des cellules NK, d'autres comme le TGF-β l'inhibent. La sécrétion de TGF-β par certaines cellules cancéreuses est d'ailleurs un mécanisme commun d'échappement tumoral.

Si les voies de signalisation activées par les cytokines ainsi que les mécanismes moléculaires impliqués sont souvent connus, ils sont parfois incomplets et ne permettent pas d'expliquer tous les phénomènes biologiques observés. En effet, le TGF-β est connu depuis longtemps pour contrer les effets de l'IL-2 et de l'IL-15, mais seule l'inhibition de T-bet *via* les protéines Smad est décrite pour expliquer ce phénomène. L'objectif de ce travail a donc été de revisiter l'effet du TGF-β ainsi que son mécanisme d'action sur les cellules NK.

Pour cela, nous avons créé deux modèles murins permettant d'étudier l'effet d'un défaut de signalisation ou d'une activation constitutive de la voie du TGF-β sélectivement dans les cellules NK. Tout d'abord, nous avons étudié les conséquences d'une absence de signalisation par le TGF-β. Une souche de souris transgénique exprimant une forme tronquée de TGFbRII dans les cellules $CD11c^{+}$ existait déjà, mais ce modèle n'est pas spécifique des cellules NK puisque les DC ainsi que d'autres types cellulaires expriment CD11c. Nous avons donc voulu créer un modèle plus spécifique des cellules NK. Pour cela, nous avons croisé des souris NKp46Cre avec des souris floxées (*tgfbr2*$^{lox/lox}$) pour supprimer l'expression de TGFbRII sélectivement dans les cellules NK (Figure 26).

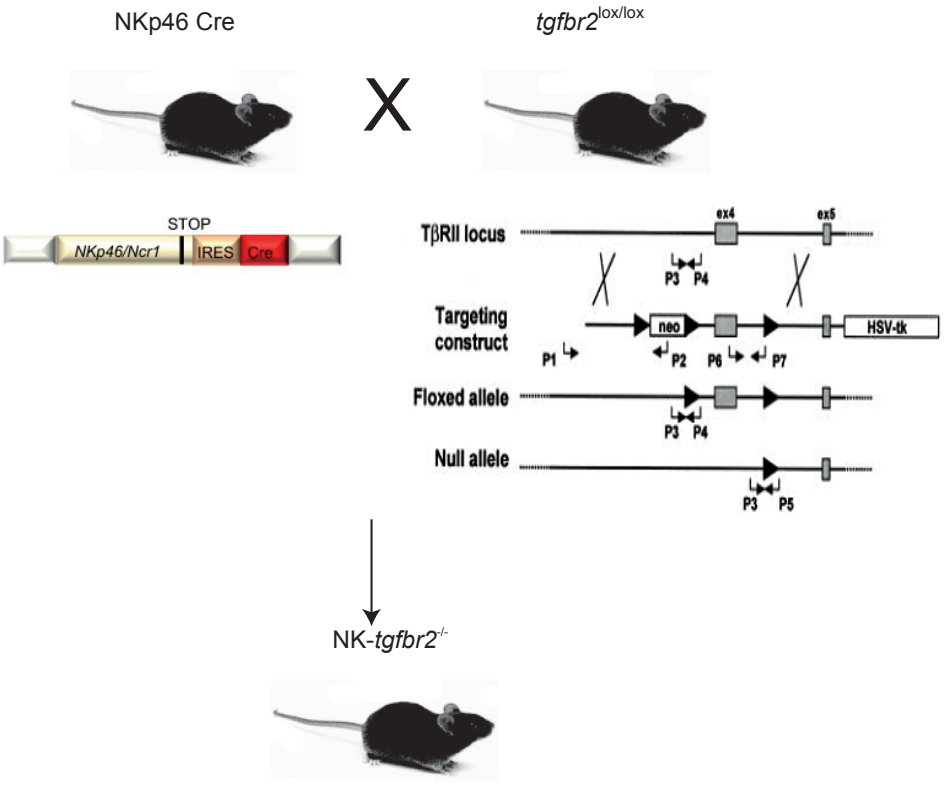

Figure 26: Création des lignées NK-*tgfbr2*$^{-/-}$ par croisement
Les souris NKp46 Cre ont été créées par l'insertion du gène *icre* dans la partie 3' du gène codant pour NKp46 par recombinaison homologue (Narni-Mancinelli et al., 2011). Les souris *tgfbr2*$^{lox/lox}$ ont été créées en insérant 2 sites « flox » avant et après l'exon 4 du gène codant pour *tgfbr2* (Leveen, 2002). Le croisement de ces deux lignées conduit à l'excision des sites floxés sous la dépendance de la Cre, c'est à dire à l'excision de l'exon 4 sélectivement dans les cellules NKp46^{+}.

Dans ces souris, nous avons d'abord étudié la maturation, la distribution et le phénotype des cellules NK en condition homéostatique *in vivo*, leur réponse à l'IL-15 *in vitro* ainsi que leurs capacités anti tumorales *in vivo*.

Inversement, nous avons voulu étudier les conséquences d'une activation constitutive des voies activées par le TGF-β, dans les cellules NK. Pour cela, nous avons croisé des souris NKp46 Cre avec des souris lox-stop-loxTβRI^{CA}. L'excision du codon stop par la Cre permet en fait l'expression d'une forme constitutivement activée de TGFbRI (Figure 27).

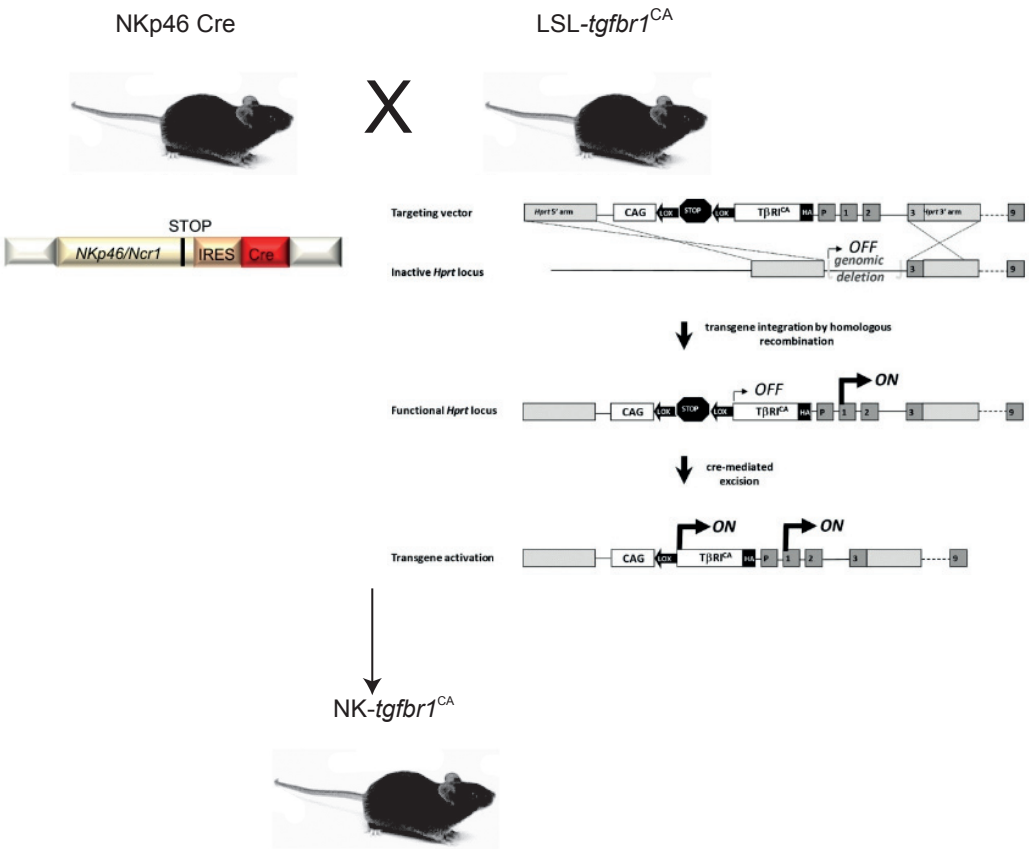

Figure 27: Création des lignées NK-*tgfbr1*^{CA} par croisement
Les souris NKp46 Cre ont été créées par l'insertion du gène *icre* dans la partie 3' du gène codant pour NKp46 par recombinaison homologue (Narni-Mancinelli et al., 2011). Les souris LSL-*tgfbr1*^{CA} ont été créées par intégration du transgène tgfbr1^{CA} précédé d'un codon stop « floxé ». Le croisement de ces deux lignées conduit à l'excision du codon stop par la Cre et à l'expression du transgène sélectivement dans les cellules NKp46^{+}.

Dans ces souris, nous avons étudié le phénotype des cellules NK, leur maturation, distribution et essayé de comprendre les mécanismes moléculaires qui pouvaient expliquer le phénotype obtenu.

Le deuxième objectif de cette thèse a consisté à étudier le compartiment cellulaire NK chez des patients atteints de différents cancers, afin de mieux comprendre l'interaction entre cellules NK et tumeurs. Pour cela, nous avons mis en place une plate-forme d'immuno monitorage axée sur l'analyse étendue du phénotype et de la fonctionnalité des cellules NK humaines. Cette plateforme, localisée dans un laboratoire d'immunologie hospitalier (Centre Hospitalier Lyon Sud), a permis la réalisation d'études cliniques dans un contexte tumoral (Article 6, Annexe) et permis aussi l'analyse étendue des cellules NK dans d'autres contextes (obésité, infections bactériennes). Cette plateforme favorise également le transfert technologique de la recherche fondamentale à l'Inserm à la recherche clinique à l'hôpital.

A partir d'un tube de sang frais, l'analyse phénotypique consiste en la mesure de l'expression des récepteurs activateurs et inhibiteurs ou de marqueurs associés à la cytotoxicité des cellules NK chez l'homme. Ce test, standardisé à l'aide de billes fluorescentes, permet un suivi longitudinal des patients ainsi qu'une comparaison des données obtenues dans le temps. La liste des marqueurs phénotypiques analysés est présentée dans le tableau 6.

Récepteurs activateurs	Récepteurs inhibiteurs	Marqueurs d'activation	Marqueurs de maturation	Marqueurs cytotoxiques
CD16	NKG2A	CD69	CD57	perforine
DNAM-1	KIR2DL1	granzyme B	NKG2C	granzyme B
2B4	KIR2DL2		CD94	NKG7
NKG2C	KIR3DL1		CD56	
NKG2D	CD161			
NKp46				
NKp30				

Tableau 6: Principaux marqueurs des cellules NK analysés sur la plate-forme d'immunomonitorage NK.

L'analyse phénotypique est complétée par une analyse fonctionnelle des cellules NK en ce qui concerne leurs deux fonctions principales : cytotoxicité et production de cytokines. Pour ce faire, les cellules mononuclées sont isolées par centrifugation en gradient de densité, puis mises en co-culture avec deux types de cibles des cellules NK, les cellules K562 et les cellules granta. Les cellules K562 dérivent d'une lignée érythroleucémique. Elles ont la particularité de diminuer l'expression des molécules de classe I du CMH et constituent ainsi des cibles « naturelles » des cellules NK. Les cellules granta dérivent d'un lymphome B. La pré-incubation de ces cellules avec un anti-CD20 (Rituximab) permet l'étude des fonctions des cellules NK dépendantes des anticorps.

Après 4 heures de culture avec leurs cibles, les cellules NK sont perméabilisées, puis nous déterminons le pourcentage de cellules qui ont dégranulé ($CD107a^+$) et qui ont produit de l'IFN-γ, du TNF-α ou du MIP1-β (CCL3) en réponse à la stimulation.

Ce test a été utilisé dans des contextes tumoraux comme le myélome ou dans des contextes infectieux comme les infections à Staphylocoques ou encore dans l'obésité. D'autres études basées sur ce test sont en cours, notamment pour tester

l’effet de médicaments immunosuppresseurs (évérolimus, étude RAPANK) sur les cellules NK ou l’effet de co-infection (VHC/HIV) sur ces cellules.

DEUXIEME PARTIE : RESULTATS

Article 1 : TGF-β inhibits NK cell activation and functions through repression of the mTOR pathway

TGF-β inhibits NK cell activation and functions through repression of the mTOR pathway

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One sentence summary: TGF-β signaling restricts mTOR activation and bioenergetic metabolism induced by IL-15 in Natural Killer cells.

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Abstract

TGF-β is a major immunosuppressive cytokine maintaining immune homeostasis and preventing autoimmunity. TGF-β has anti-proliferative and anti-inflammatory properties in various cell types that remain to be characterized at the molecular level. Here, we provide genetic, pharmacologic and biochemical evidences that a major target of TGF-β in mouse and human Natural Killer (NK) cells is the serine/threonine kinase mTOR. In vitro treatment with TGF-β leads to a rapid blockade of IL-15-induced mTOR activation in mouse or human NK cells. TGF-β and the mTOR-specific inhibitor rapamycin had identical effects on NK cell metabolic activity, proliferation, expression of various NK cell receptors and cytotoxic activity. In vivo, constitutive TGF-β signaling or mTOR deletion result in similar developmental arrests in NK cells, while reciprocally TGF-βRII deletion releases mTOR activity and cytotoxic potential upon IL-15 treatment. Suppression of TGF-β signaling in NK cells impacts neither NK cell development nor homeostasis. However, it endows NK cells with a better ability to control metastases in two different tumor models. Altogether, these results establish the mTOR kinase as a crucial signaling integrator of pro and anti-inflammatory cytokines in NK cells. Moreover, they suggest that improving metabolic activity of anti-tumor lymphocytes could be a valid strategy to promote tumor suppression.

Keywords: Natural Killer cells, cytokine signaling, IL-15, TGF-β, Metabolism

Introduction

Natural Killer (NK) cells are innate lymphoid cells with an important role in the defense against intracellular pathogens and against tumors. They have the ability to kill other cells recognized as targets through an arsenal of receptors recognizing MHC class I molecules or various surface ligands associated with cellular stress (*1*). They also secrete large amounts of IFN-γ and other cytokines in response to stimulation through NK cell or cytokine receptors. NK cells develop in the bone marrow (BM) and in other organs such as the liver (*2*, *3*), in response to IL-15. IL-15 is pivotal to instruct the NK cell lineage and also to maintain NK cell survival at the periphery by inducing STAT5 phosphorylation (*4*). After commitment to the NK cell lineage, NK cells undergo a phase of intense proliferation before reaching the blood circulation. This is followed by a process of maturation that includes at least three stages defined by the expression of CD11b and CD27 (*5*, *6*). $CD11b^{-}CD27^{+}$ (also called $CD11b^{-}$) are the most immature cells, found mostly in the BM and in the lymph nodes (LN). $CD11b^{+}CD27^{+}$ (or DP) represent the intermediate stage while $CD11b^{+}CD27^{-}$ (or $CD27^{-}$) are the most mature cells, expressing the full set of NK cell receptors and the highest amount of S1P5, a sphingosine-1 phosphate receptor allowing egress from the bone marrow and promoting circulation in the blood (*7*).

NK cell cytotoxic potential can be enhanced upon inflammation mediated by TLR ligands. Mechanistically, this phenomenon involves trans-presentation of IL-15/IL15Rα complexes by myeloid cells such as dendritic cells, and surface amounts of these complexes are increased during infections (*8*). We previously showed that

high IL-15 concentrations induce activation of the mTOR kinase in NK cells, boosting both their metabolism and their cytotoxic arsenal. Stimulating mTOR activity is essential for peripheral NK cell activation, demonstrating that it is a key checkpoint to control the effector potential of these innate effectors (*9*). Similarly, the control of mTOR and of the cellular metabolism has been shown to be central for the regulation of effector functions of other immune subtypes such as T cells or dendritic cells (*10*). Hence, pharmacological agents such as rapamycin or derivatives are powerful immunosuppressant molecules, used in clinic to inhibit graft rejection in solid organ transplantation (*11*).

TGF-β is a cytokine of the BMP/activin family that mediates a wide range of actions in the immune system (*12*). Many cell types produce TGF-β and virtually all cells of the immune system express TGF-β receptor. TGF-β is secreted as an inactive dimer that requires processing via different mechanisms to be active. Active TGF-β binds to a tetrameric receptor composed of two TGF-βRI and two TGF-βRII chains. On binding TGF-β, the type II receptors phosphorylate the type I receptors that then propagate the signal by phosphorylating the transcription factors Smad2 and Smad3. This complex then shuttles to the nucleus and binds to Smad4 and additional cofactors to repress or activate target genes. Besides this Smad-dependent pathway, often referred to as the canonical signaling pathway, TGF-β receptors also activate various other signaling events that involve p38, MAPK, PI3K-Akt, TRAF6 and others. The importance of this Smad-independent pathway is variable depending on cell types, and other parameters such as proliferation and environment (*13*).

TGF-β is commonly viewed as the most powerful immunosuppressive cytokine (*14*). Deletion of TGF-β or TGF-β receptor induces a massive T-cell mediated autoimmune syndrome that causes rapid death in mice. This is due to an essential role of TGF-β in the control of T cell tolerance in part by promoting the development of the natural regulatory T cell (T-reg) lineage and inducing the differentiation of peripheral induced T-regs and also by directly restraining CD4 and CD8 T cell activation and differentiation (*14*). Furthermore, TGF-β plays an important role in the suppression of innate immune cells, such as NK cells (*15*, *16*). Addition of TGF-β to NK cell cultures with IL-2 has long been shown to inhibit NK cell proliferation, cytotoxicity and IFN-γ secretion (*17*, *18*). In vivo, transgenic expression of a dominant negative form of TGF-β receptor in $CD11c^+$ cells (CD11c-dnTGFβRII mice, CD11c being expressed at least in dendritic cells and NK cells) induces massive NK cell proliferation and promotes their maturation, but does not induce adverse autoimmune reactions (*19*). TGF-β production by tumors or in the context of chronic infections has been shown to negatively impact on NK cell function, in particular via the decreased abundance of NK cell receptors (*20*, *21*). Reciprocally, deletion of TGF-β receptors in T cells leads to activation and NK cell-like differentiation, suggesting that TGF-β is a powerful repressor of NK cell receptor expression (*22*). The molecular mechanism by which TGF-β inhibits NK cell differentiation is unclear. SMADs have been shown to repress T-bet transcription by directly binding to T-bet promoter thereby limiting IFN-γ production by NK cells (*23*). Whether this is sufficient to explain the range of inhibitory activities of TGF-β on NK cell proliferation, cytotoxicity and expression of NK cell receptors is unknown.

Here, we created several mouse models allowing deletion of TGF-β receptor or constitutive TGF-β signaling in NK cells to revisit TGF-β mechanisms of action in NK cells. We found that TGF-β induced a very early inhibition of mTOR activity in NK cells stimulated with IL-15. The effect of TGF-β on mTOR was comparable to the effect of rapamycin, a specific mTORC1 inhibitor, both in intensity and kinetics. Moreover, TGF-β and rapamycin had very similar impacts on NK cell activation in vitro and mTOR deletion or constitutive TGF-β signaling in NK cells had comparable deleterious effects on NK cell development and differentiation in vivo. Altogether, our data establish that a major target of early TGF-β signaling in NK cells is the mTOR kinase.

7

Results

TGF-β is not essential for conventional NK cell development

To study the role of TGF-β signaling in NK cells, we measured the expression of TGF-βRII in NK cell subsets defined by CD11b/CD27 expression. As shown in Fig. 1A, TGF-βRII was expressed in all subsets but maximal in immature $CD11b^{-}CD27^{+}$ NK cells. Accordingly, upon ex vivo treatment with TGF-β, $CD11b^{-}$ NK cells expressed higher amounts of pSMAD2/3 than mature NK cells (Fig. 1B) while total SMAD2/3 expression was similar between subsets (Fig. 1B). As immature NK cells are known to actively proliferate to generate the pool of peripheral NK cells, these data suggested a possible role of TGF-β in the regulation of the size of this pool. Previous articles showing that expression of a dominant negative form of TGF-βRII in $CD11c^{+}$ cells resulted in an increased peripheral NK cell compartment further supported this hypothesis (*19*, *24*). To directly test it, we deleted *Tgfbr2* in NK cells by crossing $Ncr1^{Cre}$ mice (*25*) with $Tgfbr2^{fl/fl}$ mice (*26*) to obtain $Ncr1^{Cre/+}xTgfbr2^{fl/fl}$ mice (thereafter called NK-$Tgfbr2^{-/-}$). $Tgfbr2^{-/-}$ NK cells were totally unresponsive to TGF-β similar to NK cells from transgenic *CD11c-dnTGFβRII* mice (Fig. 1C). However, and in contradiction with results obtained in the *CD11c-dnTGFβRII* model, the distribution and the maturation of NK cells was normal in NK-$Tgfbr2^{-/-}$ mice (Fig. 1, D to F). The only evidence of an inhibitory role of TGF-β on NK cell development was the increased proliferation measured for BM NK cells in NK-$Tgfbr2^{-/-}$ mice compared to control mice (Fig. 1G). Thus, TGF-β has a very limited activity on conventional NK cell development and homeostasis under steady-state conditions. The difference in NK cell phenotypes between *CD11c-dnTGFβRII* and NK-$Tgfbr2^{-/-}$ mice could be due to the different approaches used to abrogate TGF-β signaling.

TGF-β inhibits anti-tumor NK cell function

To identify in vivo situations of NK cell exposure to TGF-β, we then measured SMAD2/3 phosphorylation in ex vivo-isolated cells from mice challenged or not with different agents. Very little pSMAD2/3 was measured in spleen NK cells from unchallenged mice or mice injected with the classical NK cell activator poly(I:C) or with IL-15/IL-15Rα complexes (Fig. 2A). Similar results were obtained when mice were infected with *Influenza* virus or *Listeria Monocytogenes*. However, strong SMAD2/3 phosphorylation was measured in NK cells extracted from solid tumors such as NEU15 mammary tumors (Fig. 2A), suggesting exposure of NK cells to TGF-β within the tumor environment. To examine the role of TGF-β in NK cells in a tumoral context, we challenged NK-*Tgfbr2*$^{-/-}$ mice and littermate controls with B16-F10 melanoma cells or RM1 cells *i.v.* and counted lung metastases 2 weeks after injection. Results in Fig. 2, B and C showed a better suppression of metastases by NK-*Tgfbr2*$^{-/-}$ mice. NK cell depletion in both mouse strains led to an equivalent high increase in the number of metastases. Thus, we concluded that TGF-β impairs NK cell anti-tumor function.

Loss of TGF-β signaling releases in vitro NK cell activation

To investigate how TGF-β inhibited NK cell activation, we compared the effect of stimulation with IL-15 on parameters of NK cell activation in vitro between NK-*Tgfbr2*$^{-/-}$ and control mice. As shown in Fig. 3A, *Tgfbr2*$^{-/-}$ NK cells expressed more GZMB, T-bet and KLRG1 than control NK cells upon IL-15 stimulation. Interestingly, IL-15 stimulated *Tgfbr2*$^{-/-}$ NK cells also had higher FSC, SSC and CD98/CD71 expression than control NK cells. The latter parameters strongly correlate with metabolic activity, as we previously showed (*9*). For this reason, we hypothesized that *Tgfbr2*$^{-/-}$ NK cells

may have higher mTOR activity upon IL-15 stimulation. To test this hypothesis, we measured phosphorylation of the ribosomal S6 protein, which is a substrate of S6K, a major target of mTORC1 upon IL-15 signaling (*9*). We detected significantly higher S6 phosphorylation in *Tgfbr2*$^{-/-}$ NK cells but no difference of STAT5 phosphorylation upon IL-15 stimulation (Fig. 3B), suggesting that endogenous TGF-β may inhibit NK cell activation through limiting mTOR activity.

TGF-β inhibits mTOR and metabolic activity induced by IL-15 in mouse and human NK cells

Next, we directly measured the influence of exogenous TGF-β on mTOR activity induced by IL-15 stimulation in spleen NK cells in vitro. TGF-β had a strong inhibitory effect on the induction of S6 phosphorylation (Fig. 4A). This effect occurred with the same kinetics as SMAD2/3 phosphorylation suggesting that mTOR inhibition is occurring just downstream of TGF-β receptor. We also observed SMAD2/3 phosphorylation in the absence of exogenous TGF-β, suggesting that spleen cells secreted active TGF-β in these conditions. Accordingly, the addition of a blocking TGF-β antibody increased mTOR activity induced by IL-15 in NK cells (Fig. 4A). Similar results were obtained using an inhibitor of TGF-βRI kinase activity (Fig. 4B). Importantly, neither TGF-β, nor anti TGF-β altered STAT5 phosphorylation induced by IL-15, thus showing a very specific inhibition of mTOR activity by TGF-β and no general impairment of IL-15 signaling (Fig. 4A). The inhibitory effect of TGF-β was not restricted to S6 phosphorylation but also observed on other mTOR substrates 4EBP1 and Akt (Fig. 4C). This effect was comparable to that of the specific mTORC1 inhibitor rapamycin, with a notable difference regarding pAkt, insensitive to rapamycin (*27*) but inhibited by TGF-β. Moreover, TGF-β also significantly inhibited

mTOR activity induced by a short treatment (1 hour) with IL-15 in human NK cells (Fig. 4D).

As we previously demonstrated that mTOR controls NK cell bioenergetic metabolism, we compared the effect of TGF-β and rapamycin on the following metabolic parameters: expression of amino-acid transporters CD71 and CD98, FSC FACS parameters proportional to cell size, 2NBDG glucose incorporation, glycolysis (glycolytic capacity, GC and acidification due to glycolysis, ADG) and oxidative phosphorylation (OxPhos). As shown in Fig. 4, E and F, TGF-β was as efficient as rapamycin to inhibit NK cell metabolic activity, irrespective of the parameter analyzed, and to inhibit the expression of associated markers CD71 and CD98. Thus, TGF-β inhibits mTOR-dependent metabolic activity induced by IL-15 in NK cells.

mTOR inhibition is not a consequence of IL-15 receptor decreased abundance or inhibition of cell cycle

Even though the effect of TGF-β on mTOR activity was extremely rapid in NK cells, we considered the possibility that it was a consequence of the well-described inhibitory effect of TGF-β on cell cycle activity rather than a contributing cause. To address this point, we first stimulated freshly isolated NK cells with IL-15 in the presence of TGF-β or of a wide range of chemotherapeutic compounds known to inhibit proliferation through various mechanisms. As shown in Fig. 5A, doxorubicin, methotrexate, cyclophosphamide or 5-FU all inhibited NK cell proliferation at various intensities. However, unlike TGF-β or rapamycin, none of them decreased early mTOR activity induced by IL-15 (Fig. 5A). Second, we measured the effect of TGF-β on in vitro BrdU incorporation by proliferating NK cells during in vitro stimulation with IL-15. As shown in Fig. 5B, a significant effect of TGF-β on NK cell proliferation was

not detected before 48 hours of culture, days after the observed inhibitory effect of this cytokine on mTOR activity. These experiments excluded that mTOR inhibition was the consequence of cell cycle inhibition in TGF-β treated NK cells.

Next, we considered the possibility that mTOR inhibition by TGF-β was indirectly due to the inhibition of T-bet expression. Indeed, T-bet is well known to regulate the expression of CD122, the beta chain of Il-15 receptor (*28*) and TGF-β has been previously shown to inhibit T-bet expression (*23*). However, CD122 (or CD132, the gamma chain of the IL-15 receptor) expression was not altered by TGF-β or anti TGF-β, at least in the first 4 hours after in vitro treatment (Fig. 5C). Moreover, TGF-β still had an inhibitory effect on NK cells isolated from *Tbx21*$^{-/-}$ mice that lack T-bet, regardless of the parameter analyzed *i.e.* mTOR activity or cell proliferation, as assessed by BrdU incorporation (Fig. 5D). Altogether, these results suggest that mTOR inhibition is a proximal signaling event downstream TGF-β receptor in NK cells and not a distal effect indirectly due to the inhibition of other biological processes.

TGF-β and rapamycin have similar impacts on NK cell activation in vitro

To evaluate the contribution of mTOR inhibition in the inhibitory effect of TGF-β on NK cells, we compared the effect of TGF-β and rapamycin on NK cell activation parameters in vitro, upon stimulation with IL-15 for 3 days. We measured NK cell proliferation, using BrdU incorporation, and expression of a large panel of cytotoxic or lymphocyte markers. TGF-β and rapamycin had equivalent negative effects on NK cell proliferation (Fig. 6A-B). The flow cytometry analysis, presented as a heatmap revealed that most markers analyzed were similarly regulated by TGF-β and rapamycin, which resulted in co-clustering of TGF-β and rapamycin conditions apart

from the “IL15 alone” and the “IL15 + anti-TGF-β” conditions, when performing a global clustering analysis (Fig. 6C). Many of the markers analyzed had a decreased expression in the presence of TGF-β or rapamycin. For some of them, TGF-β was a more potent inhibitor (see for example GZMB, CD24 or CD223) and for some others, it was the reciprocal (CD71, CD98, Fig. 6C). Of note, the abundance of CD122, the beta-chain of the IL-15 receptor was highly decreased by both rapamycin and TGF-β at this late culture time-point. Thus, overall, except for TNF related apoptosis inducing ligand (TRAIL) which abundance was increased by TGF-β but not changed by rapamycin and for CD62L that displayed the reciprocal pattern, there was a strong similarity in the effect of both compounds. A similar conclusion was reached when we compared the effects of TGF-β and rapamycin on NK cell cytotoxicity directed against YAC1 cells (Fig. 6D). Finally, TGF-β and rapamycin had analogous influences on human NK cell GZMB/perforin expression, and degranulation/IFN-γ and MIP-1β secretion in response to stimulation by the classical targets K562 cells, in the presence of IL-2 (Fig. 6 E and F). Thus, in vitro the impact of TGF-β on IL-2/15-mediated NK cell activation can be recapitulated by inhibiting mTOR with rapamycin.

Similar effects of mTOR deletion and constitutive TGF-β signaling on NK cell development

Next, we sought to compare the effects of TGF-β signaling and mTOR inhibition on NK cells in vivo. For this we took advantage of *mTor*$^{fl/fl}$ mice (*29*) and of *Tgf-βRI*^{CA} mice (*30*). The former allow mTOR deletion and the latter allow constitutive TGF-β signaling in Cre-expressing cells. These mice were crossed with *Ncr1*^{Cre} mice to generate NK-*mTor*$^{-/-}$ and NK-*TgfβRI*^{CA} mice. The percentage of peripheral NK cells was very low in both strains (Fig. 7A). Moreover, spleen NK cells in both mouse

strains had a strikingly similar phenotype, with a predominance of immature $CD27^+$ $CD11b^-$ NK cells (Fig. 7B). In the bone marrow, the frequency and number of NK cells were similar in all three strains, while NK cells tended to be more immature in NK-*mTor*$^{-/-}$ and NK-*TgfβRI*^{CA} mice compared to control mice. We performed an in-depth analysis of NK cell phenotype in both strains by flow cytometry, narrowing our analysis on spleen $CD27^+CD11b^-$ NK cells that are present in all strains to avoid a bias caused by the skewed maturation in NK-*mTor*$^{-/-}$ and NK-*TgfβRI*^{CA} strains. We then selected the FACS parameters that were statistically different between control and at least one of the mutant strains. Results are shown in Fig. 7C. Three groups of markers were identified: those that showed co-regulation in *mTor*$^{-/-}$ and *TgfβRI*^{CA} NK cells, significantly different to that of WT NK cells (first row on the Figure). This category was the most important one and notably included KLRG1, CD146, GZMB, Ly49H, T-bet, and CD122. The other categories correspond to genes which expression was not correlated between NK-*mTor*$^{-/-}$ and NK-*TgfβRI*^{CA} mice and included for example the NK cell receptors 2B4 and NKG2D, which abundance was decreased in *mTor*$^{-/-}$ but not *TgfβRI*^{CA} NK cells. Next, we compared homeostatic proliferation and early phosphorylation events in *TgfβRI*^{CA} vs control NK cells following IL-15 stimulation. Results in Fig. 7D show that similarly to *mTor*$^{-/-}$ NK cells[9], *TgfβRI*^{CA} NK cells had a defective proliferation in the BM but not in the spleen. This was associated with a decreased amount of pS6 but not pSTAT5 in response to stimulation, in comparison with control NK cells (Fig. 7E). Altogether, these data show a very close proximity between NK cells deleted of mTOR or expressing a constitutively active form of type 1 TGF-β receptor, which further demonstrates the contribution of mTOR inhibition to the overall effect of TGF-β on NK cells. This inhibition is relevant for the anti-tumor NK cell function as mTOR deficient NK cells

are poorly responsive to IL-15 in vivo and have reduced effector functions upon engagement of activating NK cell receptors (*9*). Moreover, both *mTor*$^{-/-}$ NK cells and *TgfβRI*^{CA} NK cells have reduced ability to degranulate in response to YAC1 tumor targets (Fig. 7F).

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Discussion

The molecular mechanisms of TGF-β inhibitory activity on NK cells have remained mostly unknown. Here, we provide compelling evidence that a major target of TGF-β in NK cells is the S/T kinase mTOR. As direct evidence, we showed that TGF-β signaling opposed phosphorylation of mTOR substrates S6, 4EBP1 and Akt induced by IL-15. This effect was very rapid, concomitant with SMAD phosphorylation, thereby consistent with a proximal event downstream TGF-β receptor. Indirect evidence included the observation that mTOR deletion and constitutive TGF-β signaling in NK cells had strikingly comparable impacts on NK cell development, NK cell maturation and cytotoxic potential. Moreover, TGF-β and rapamycin, a highly specific mTORC1 inhibitor had almost identical effects on IL-15-mediated NK cell activation in vitro, in terms of bioenergetics metabolism, proliferation, cytotoxic activity and expression of cytotoxicity-associated receptors and transcription factors. Similarly, previous reports described that TGF-β can be substituted with rapamycin to induce regulatory T cell (*31*) or follicular T helper cell differentiation (*32*) and that TGF-β and rapamycin have similar effects on anti-CD3/CD28 mediated T cell proliferation following in vitro culture for several days (*33*).

The control of metabolic activity emerges as a key event in immune cell regulation (*10*). Rapamycin and its derivatives are currently used for the prevention of kidney transplant rejection, for the treatment of graft-versus-host disease, and for chemotherapy of some cancers (*11*). The mechanisms by which rapamycin suppresses immune responses have been extended from inhibition of T cell proliferation to suppression of DC maturation and sustenance of regulatory T cells (*34*). Our own studies previously highlighted the crucial role of mTOR on NK cell activation. Treatment with rapamycin dampened in vivo NK cell cytotoxicity against

MHC-I negative target cells, clearly demonstrating the central role of mTOR and bioenergetics metabolism in the control of NK cell function. Mechanistically, we found that mTOR was essential to control the expression of several cytotoxic mediators such as granzyme B and perforin, but also to increase NK cell reactivity to target cells via a positive feedback on signaling through activating NK cell receptors (*9*). mTORC2-dependent regulation of actin cytoskeleton could also contribute to the establishment of immune synapses (*35*). The remarkable similarity of effects between rapamycin and TGF-β thus demonstrates that a major effect of TGF-β in cytotoxic lymphocytes is to restrain bioenergetic metabolism through mTOR inhibition, in order to antagonize the effect of pro-inflammatory cytokines IL-2 and IL-15.

Smad-induced inhibition of T-bet was proposed to explain the inhibition of IFN-γ secretion by NK and T cells exposed to TGF-β (*23*, *36*). However, as shown here, the amounts of T-bet and of its downstream target CD122 were not significantly altered by TGF-β before at least 4 hours of treatment. Moreover, we showed that the well-known anti-proliferative effect of TGF-β occurs days after the inhibition of mTOR activity. These data thus establish that mTOR inhibition is a very early event during TGF-β signaling in NK cells and contributes to the inhibition of proliferation rather than being a surrogate marker of this inhibition. How does TGF-β inhibit mTOR? A previous study in epithelial cells found many interactions between TGF-β signaling and other signaling pathways, which opens several possibilities (*37*). We found that TGF-β-mediated inhibition required TGF-βRI kinase activity. TGF-β receptor kinase activity controls Smad-dependent and Smad-independent pathways. Previous studies have established that TGF-β can activate Akt and mTOR in a Smad-independent way in epithelial cell types during the epithelial to mesenchymal transition (EMT), highlighting the context-dependent effects of TGF-β (*38*, *39*).

Interestingly, Type 1 TGF-β receptor constitutively binds to FKBP12 (*40*) and this signaling molecule is released upon TGF-β signaling. Rapamycin also binds with high affinity to FKBP12 (*41*) and the FKBP12-rapamycin complex is known to inhibit mTOR activity. Endogenous FKBP12 partners with similar activity as rapamycin are yet to be identified but FKBP12 represents a potential link between TGF-β and mTOR. Of note, Treg development, which strongly depends on both TGF-β and mTOR inhibition, is reduced in *Fkbp12*$^{-/-}$ mice (*42*). Moreover, in transplanted patients, treatment with tacrolimus or FK506, a calcineurin and FKBP12 inhibitor decreases the percentage of Tregs (*43*).

The early effect of TGF-β on mTOR activity is likely to be reinforced in an indirect way at later stages. Indeed, our results confirm previous findings that the amount of CD122, the IL-15 receptor, is decreased upon TGF-β signaling in vitro or in vivo, which may contribute to the overall decrease in metabolic activity observed in NK cells at late stages of TGF-β signaling. Overall mTOR inhibition by TGF-β is therefore likely to be due to direct and indirect effects operating with different kinetics.

Our results thus identify mTOR as a crucial integrator of cytokine signaling, capable of translating antagonistic signals into adapted cellular responses. As mTOR activity is also modulated by antigen receptors, it is tempting to speculate that mTOR activity is a key molecular switch in immune cells such as NK cells, integrating signals from surface receptors for various metabolic, immunologic or hormonal signals. In this context, it will be interesting to study the impact of inhibitory cues such as adenosine or corticosteroids on mTOR activity in NK cells.

We noted a few differences between TGF-β and rapamycin effects on in vitro NK cell activation. For example TGF-β induced TRAIL expression in NK cells while rapamycin inhibited their expression. Similarly, constitutive TGF-β signaling in NK

cells led to the induction of CD24, CD29 and BTLA in vivo while mTOR deletion did not influence these markers. Reciprocally mTOR deletion had a profound inhibitory effect on a series of markers like 2B4, NKG2D, IL-12 receptor (CD212) and CD11c while constitutive TGF-β signaling was ineffective on the expression of these molecules. Therefore, TGF-β cannot be seen only as an "endogenous rapamycin" but probably has activities beyond the control of mTOR that could be linked to the transcriptional activity of Smads on target genes. Future studies will have to identify all Smad target genes in NK cells to precisely address this point.

We found very little consequences of *Tgfbr2* deficiency in NK cells at steady state. This result is in clear contrast with previous findings that transgenic expression of human TGF-βRII truncated from its kinase domain in $CD11c^+$ cells induced massive NK cell expansion (*19*). The reason for this discrepancy is unclear as we found that both *Tgfbr2*$^{-/-}$ and *CD11c-dnTGFβRII* NK cells are unresponsive to TGF-β in terms of Smad phosphorylation. A similar discrepancy has been reported in CD8 T cells when comparing the effect of both genetic approaches (*44*). One can speculate that the CD11c promoter may be expressed at earlier stages of NK cell development than NKp46 that drives *Tgfbr2* deletion in our system. Another possibility could be an effect of the dominant negative receptor both in cis and in trans on myeloid cells interacting with NK cells and trans-presenting IL-15. Our work demonstrates a lack of a role of TGF-β on the homeostasis of $NKp46^+$ NK cells that includes the vast majority of conventional NK cells, suggesting that under physiological conditions, NK cells are not exposed to active TGF-β. Similarly, we found very little Smad2/3 phosphorylation in NK cells exposed to various inflammatory stimuli such as TLR ligands, IL-15, intracellular bacteria or viruses, suggesting a limited role of TGF-β in controlling NK cell activation in these contexts. By contrast, very strong Smad2/3

phosphorylation was observed in NK cells extracted from tumors, confirming the relevance of the TGF-β pathway in immunosuppression induced by the tumor microenvironment. This may be especially true in the context of breast cancer as several studies have highlighted a role for TGF-β in suppressing NK cell activity in models of breast cancer in vitro (*45*) or in vivo (*46*, *47*).

In conclusion, we demonstrated the existence of an evolutionarily conserved molecular pathway whereby TGF-β inhibits NK cell metabolic activity via opposing the induction of mTOR activity mediated by IL-2 or IL-15. These data specify a molecular mechanism for the immunosuppressive effects of TGF-β and point to mTOR as a key integrator of cytokine signals in NK and T cells. Our data provide a rationale for developing novel therapeutics aiming at increasing mTOR activity in tumor infiltrating lymphocytes such as NK cells to restore their cytolytic activity by countering the effect of TGF-β.

Material and Methods

Mice

This study was carried out in strict accordance with the French recommendations for the ethical evaluation of experiments using laboratory animals, the European guidelines 86/609/CEE, and the QIMR Berghofer Medical Research Institute animal ethics committee. C57BL/6 mice were purchased from Charles River Laboratories (L'Arbresle). *Ncr1*^{iCre} mice were crossed with *mTOR*$^{fl/fl}$ mice or LSL-*Tgf-βRI*^{CA} or *TGF-βRII*$^{fl/fl}$ mice. *Tbx21*$^{-/-}$ mice were previously described (*48*). All strains were bred at the Plateau de Biologie Experimentale de la souris (PBES) or the QIMR Berghofer Medical Research Institute. Litters with mice 8 to 24 weeks old were used for NK cell analysis. In some experiments, mice were treated i.p. with 5 µg IL-15-IL15Rα complex (eBioscience), twice, at 24h intervals and sacrificed 24h later or with 150 µg Polyinosinic:polycytidylic acid (poly(I:C), Invivogen) and sacrificed 18 hours later. For in vivo BrdU incorporation, mice were injected twice at 24 hour interval with 2 mg Brdu in saline. BrdU incorporation was measured the next day. In some experiments, mice were infected with 2 x 10^5 TCID50 Influenza virus intranasally or 2 x 10^3 Listeria Monocytogenes intravenously. Mice were sacrificed at day 6 after infection.

Flow cytometry

Single cell suspension of BM, blood, spleen and liver from mice were obtained and stained. Human whole-blood samples from healthy donors were collected by venous puncture in heparin-containing vials. PBMCs were isolated by Ficoll gradient centrifugation. Intracellular stainings for transcription factors or intracellular cytotoxic mediators were performed using Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBiosciences). Intracellular stainings for phosphorylated proteins were done

using Lyse/Fix and PermIII buffers (BD Bioscience). BrdU incorporation was measured using a kit (BD Bioscience), Flow cytometry was carried out on a FACS Canto, a FACS LSR II, a FACS Fortessa (Becton-Dickinson) or a Navios (Beckman Coulter). Data were analysed using FlowJo (Treestar). The antibodies we used are listed in Table S1.

Cell culture and stimulation

Splenic lymphocytes were prepared and cultured with cytokines (rmIL-15 100 ng/ml; rhTGFβ1 10 ng/ml from R&D Systems), In some culture experiments, we used anti TGF-β blocking antibody (1 μg/ml, 1D11, BioXcell), rapamycin (25 nM, Sigma-Aldrich), TGF-βRI kinase inhibitor SB431542 (1-10 nM, Sigma-Aldrich), Doxorubicin (5 μM, Healthcare), methotrexate (50 μM, Mylan), 5-FU (50 μM, Pfizer), Cyclophosphamide (50 μM, Baxter) or BrdU (10 μM, Sigma-Aldrich). Surface and intracellular stainings were then performed. PBMCs were isolated by Ficoll gradient centrifugation and stimulated using 1.000 UI/mL rhIL-2 (corresponding approximately to 15ng/ml) or hIL-15 (Peprotech) in the presence or absence of TGF-β or rapamycin.

Assessment of glucose uptake.

Glucose uptake was measured with 2- NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose; Invitrogen). Freshly isolated cells were resuspended in RPMI-1640 medium (Life Technologies) in the presence of 100 μM 2-NBDG and were cultured for 10 min at 37 °C, then surface markers were stained.

Killing assay

Spleen cells from Rag2-/- mice were stimulated overnight with IL-15 alone or IL-15 +

anti TGF-β, TGF-β or rapamycin then co-cultured for 4 hours at different effector target (E/T) ratios with YAC1 cells previously labeled with CFSE (Invitrogen). The percentage of dead cells within CFSE positive YAC1 cells was measured by flow cytometry after staining with propidium iodide.

Degranulation assay

Human PBMCs were stimulated overnight with IL-2 alone or IL-2 plus anti TGF-β, TGF-β or rapamycin then co-cultured for 4 hours with K562 cells (ratio 1:1). The percentage of NK cells positive for CD107a, IFN-γ or MIP1-β was measured by flow cytometry.

Tumor models

B16-F10 melanoma (2×10^5) or RM-1 prostate adenocarcinoma cells (2×10^5) cells were re-suspended in PBS and injected into the tail veins of the mice. The numbers of lung nodules were counted under a dissecting microscope 14 days after injection, as previously described (*49*). The NEU15 cell line was established from a spontaneous mammary tumor harvested from an MMTV-neu transgenic female mouse (*50*). FVB/N mice were injected with 5×10^6 NEU15 cells into the fourth mammary fat pad and tumors were harvested 7 weeks after injection. Tumor volume was between 124 and 628 mm^3.

Seahorse analysis:

O_2 Consumption Rate (OCR) and ExtraCellular Acidification Rate (ECAR) were measured in XF media (non-buffered DMEM containing 2 mM Glutamine, 10mM Glucose at pH7.4) with the XF-24 Extracellular Flux Analyzer (Seahorse Bioscience).

Rates ECAR and OCR were measured under basal condition and following the sequential addition of Oligomycin 2 μM, Antimycin A 4 μM + Rotenone 0,1 μM, and 2-deoxyglucose (30mM) (all from Sigma-Aldrich) allowing for the accurate quantitation of oxygen consumption due to oxidative phosphorylation and acidification due to glycolysis. Cultured NK cells were purified, activated with IL-15 (100 ng/ml) for 20 hours and plated (750,000 cells per well) in a CellTak (BD Biosciences) coated Seahorse plate.

Statistical analyzes

Error bars represent the standard deviation. Statistical analyses were performed using two-tailed t-tests or non-parametric tests when appropriate. These tests were run on the Prism software (GraphPad). Levels of significance are expressed as p-values (*p<0.05, **p<0.01, ***p<0.001).

Supplementary material

Table S1 lists the antibodies used in this study

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Figure legends

Figure 1: TGF-β receptor is functional on NK cells but does not regulate their homeostasis at steady-state

(A) Flow cytometry analysis of TGF-βRII expression in gated NK cell subsets from the spleen. left panel: representative histogram plot of one out of 3 experiments. Right panel: averaged MFI of the TGF-βRII staining calculated from a total of 3 mice in 2 experiments. (B-C) Spleen cells from the indicated mouse strains were cultured for one hour with TGF-β. Smad2/3 phosphorylation was then measured by flow cytometry. (B) Left panel: representative histogram plot of one out of three experiments. Middle panel: averaged MFI of the pSMAD2/3 staining calculated from total of 6 WT mice in 2 experiments. Right panel: averaged MFI of the total SMAD2/3 staining calculated from total of 7 WT mice in 2 experiments. (C) Averaged MFI of the pSMAD2/3 staining calculated from N=3 WT, 3 NK-*Tgfbr2*$^{-/-}$ and 3 CD11c-dnTGFβRII mice. (D-F) Flow cytometry analysis of NK cells in the indicated organs of WT and NK-*Tgfbr2*$^{-/-}$ mice. (D) Frequency and (E) number of NK cells calculated from N=8 mice for each group. (F) Density plot showing expression of CD27 and CD11b in NK cells, representative of N=3 experiments in each group. (G) Flow cytometric analyzes of BrdU incorporation by BM NK cells from WT and NK-*Tgfbr2*$^{-/-}$ mice. The bar graph shows average percentages of BrdU positive cells (total of 6 mice in 3 independent experiments). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. (*t*-test).

Figure 2: TGF-β inhibits NK cell anti-tumor function

(A) Flow cytometry analysis of Smad2/3 phosphorylation in gated NK cells. NK cells were analyzed in the spleen of mice either left unchallenged, or injected with Poly(I:C) 18 hours before, or injected twice with IL-15/IL-15Ra complex, or infected with Influenza or Listeria 7 days before. NK cells were also analyzed in tumors from NEU15 cell-injected mice. Results are averaged from at least N=4 mice. MFI is normalized to the pSMAD2/3 amount in control NK cells. (B) Control and NK-*Tgfbr2*$^{-/-}$ mice were injected with B16-F10 or RM-1 cells (2 x 10^5) i.v. Groups of mice were treated with 50 μg asialo-GM1 antibody or control Ig on day -1,0 and 7 relative to tumor inoculation (day 0). Mouse lungs were harvested and fixed on day 14 and the number of metastases were counted under a dissecting microscope. Each symbol represents an individual mouse. (* P < 0.05; ** P < 0.01, Mann Whitney test).

Figure 3: Loss of TGF-β signaling releases NK cell activation induced by IL-15

Spleen cells from WT and NK-*Tgfbr2*$^{-/-}$ mice were cultured with IL-15 (100 ng/ml) for 3 days (A) or 1 hour (B). The expression of various intracellular or surface proteins was measured by flow cytometry (A). Bar graphs show averaged MFI ratios calculated from a total of 5 mice in 3 experiments, relative to control NK cells. Histograms show forward side scatter (FSC) and side scatter (SSC) in a representative experiment. In (B) the expression of pS6 was measured in spleen cells from CD45.1 WT and NK-*Tgfbr2*$^{-/-}$ mice mixed at a 1:1 ratio at the beginning of the culture. This procedure was used to minimize experimental variations within experiments. Bar graphs show averaged MFI calculated from a total of 4 mice in 2 experiments. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. (*t*-test).

Figure 4: TGF-β counters activation of mTOR and bioenergetic metabolism induced by IL-15

(A-C) Spleen cells from WT mice were stimulated in the indicated conditions. (A) pS6, pSTAT5 and pSMAD2/3 expression were measured over time. Graphs show averaged MFI ratios calculated from a total of 3 mice in 3 experiments, relative to the control, unstimulated condition. (B) S6 and SMAD2/3 phosphorylation was measured in the presence of different concentrations of TGF-βRI kinase inhibitor (SB-431542). N=3 in 3 experiments. (C) Phosphorylation of Akt, 4EBP1 and S6. Histograms represent phosphorylation of different proteins in NK cells in the indicated condition, one representative experiment out of 3 is shown. The bar graphs show averaged phosphoprotein MFI in NK cells (n=4 mice in 2 independent experiments). (D) Phosphorylation of S6 in human NK ($CD56^{bright}$ $CD3^{-}$) cells. PBMC were cultured in the indicated conditions for 1 hour. The bar graph shows averaged pS6 MFI in NK cells (n=7 in 3 independent experiments). (E) Spleen cells from WT mice were stimulated in the indicated conditions for 24 hours. The expression of various intracellular or surface proteins or 2-NBDG incorporation was measured by flow cytometry. Bar graphs show averaged MFI calculated from N=3 mice in 2 experiments. (F) Primary NK cells were cultured in low dose IL-15 for 5 days, as described in (*51*), then stimulated with IL-15 (100 ng/ml) for 20 hours and NK cell metabolism was analyzed in the indicated conditions. ADG (Acidification Due to Glycolysis), OXPHOS (OCR for O_2 Consumption Rate), GC (Glycolytic Capacity) were analyzed in real time. Bar graphs show the average of 3 independent mice performed in triplicate (3 independent experiments). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. (*t*-test).

Figure 5: Early inhibition of mTOR activity by IL-15 is not a consequence of cell cycle inhibition and is independent of T-bet and IL-15R quantities.

(A-C) WT spleen cells were cultured in vitro in the indicated conditions. (A) S6 phosphorylation was measured after 1 hour (right panel) and cell proliferation was measured after 3 days (left panel). N=4 in 2 experiments. (B) WT spleen cells were cultured in the presence of BrdU. BrdU incorporation by NK cells was measured at the indicated time points using flow cytometry. N=3 in 2 experiments. (C) CD122 and CD132 amounts were measured at the indicated time points. N=3. (D) *Tbx21*$^{-/-}$ spleen cells were cultured with IL-15, in the presence of BrdU. S6 phosphorylation was measured at 1 hour GzmB and BrdU incorporation was measured at 3 days. For pS6, results are shown as MFI ratios, relative to the condition with IL-15 alone. N=5 in 2 experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (*t*-test).

Figure 6: TGF-β and rapamycin have similar effects on IL-15 induced NK cell activation and cytotoxicity in vitro.

(A-B) IL-15 expanded WT NK cells were restimulated in the indicated conditions for 12 hours in the presence of BrdU. BrdU incorporation was measured by flow cytometry. (A) shows representative flow cytometry density plots of NK1.1 and BrdU in gated NK cells. (B) shows the mean +/-SD frequency of BrdU positive NK cells calculated from a total of 4 mice in 2 experiments. (C) Flow cytometry analysis of the indicated parameters measured in spleen NK cells after 3 day-cultures in the indicated conditions. Results are presented as a hierarchical cluster heatmap; each row denotes a parameter and each column a cell culture condition, as indicated; the color scale indicates protein expression intensities. Dendrograms denote the Euclidean distance between clustered conditions. Results are representative of 4

independent experiments. (D) Cytotoxic assay of overnight-stimulated WT NK cells vs YAC1 cells, cultured at different effector to target ratio for 4 hours. Results show the mean +/-SD cytotoxicity calculated from 4 mice in two independent experiments. (E) Perforin and granzyme B in gated NK cells among human peripheral blood mononuclear cells cultured for 36 hours in medium alone or with IL-2 alone or IL-2 plus TGF-β or rapamycin, analyzed by flow cytometry. The bar graph shows averaged MFI ratio in NK cells (Total of 9 healthy donors in 3 independent experiments) relative to the "medium" condition. (F) CD107a, IFN-γ and MIP1-β in stimulated NK cells (by IL-2 + TGF-β or IL-2 alone or IL-2 + TGF-β or IL-2 + rapamycin) after a 4 hour culture with K562 cells, ratio 1:1. N=6 donors. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (*t*-test).

Figure 7: Constitutive TGF-β signaling and mTOR deletion have comparable impacts on NK cell development and maturation

(A-B) Ex vivo analysis of NK cell frequency and number (A) and NK cell maturation determined by CD11b/CD27 (B) in the spleen and bone marrow of WT vs NK-*Tgfbr1*^{CA} and NK-*mtor*$^{-/-}$ mice. N=10. (C) Flow cytometry analysis of the expression of various surface or intracellular proteins in gated spleen CD11b^{-}CD27^{+} NK cells from WT vs NK-*Tgfbr1*^{CA} and NK-*mtor*$^{-/-}$ mice ex vivo. Results show the averaged MFI +/-SD of the staining, calculated from a total of 6 mice in 3 experiments in each group. Results are also normalized to the control condition. Histograms show forward scatter (FSC) and side scatter (SSC) in a representative experiment. (D) Homeostatic NK cell proliferation, as measured by BrdU incorporation in the spleen and BM of WT vs NK-*Tgfbr1*^{CA} mice. Bar graphs show the average frequency of BrdU positive cells calculated from N=4 mice in 2 experiments. (E) S6 and STAT5

phosphorylation in gated spleen CD11b^{-}CD27^{+} NK cells from the indicated mice, as measured after one-hour culture of spleen cells in the presence or absence of IL-15. N=3 mice in each group. (F) Spleen cells from WT, NK-*Tgfbr1*^{CA} and NK-*mtor*$^{-/-}$ mice were co-cultured for 4 hours with YAC1 cells at a 1:1 ratio. NK cell degranulation was then measured by flow cytometry. Results show the average (N=3 experiments) frequency of CD107a+ cells among gated CD11b- NK cells for the different mouse strains. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001. (*t*-test).

Figure 1

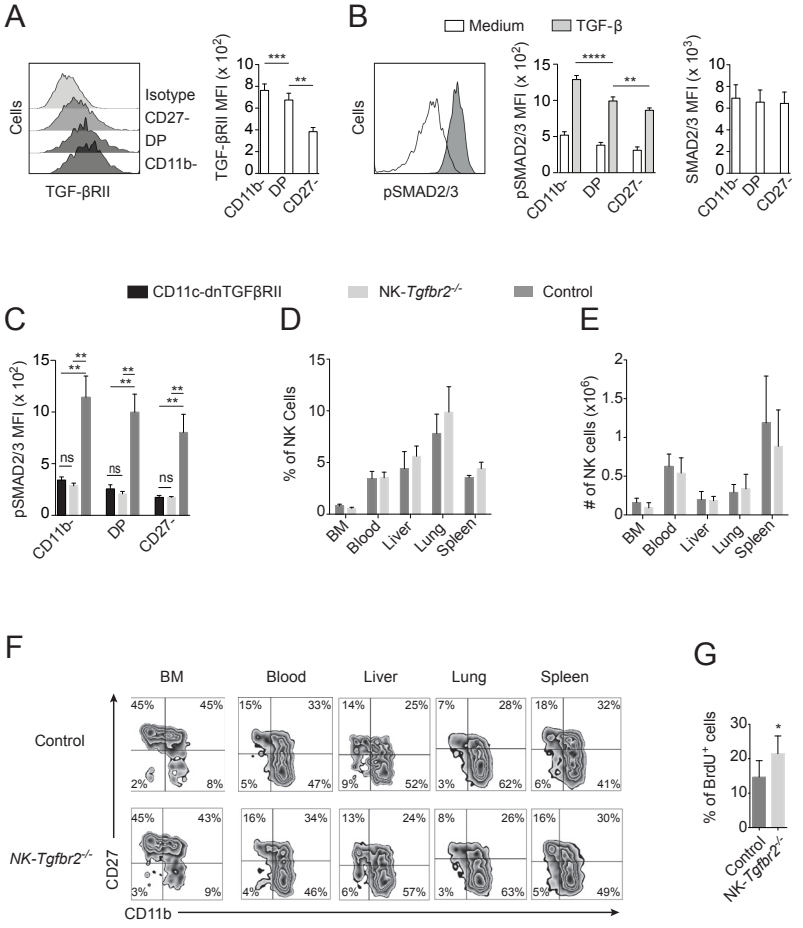

A
Cells
Isotype
CD27-
DP
CD11b-
TGF-βRII
TGF-βRII MFI (x 10²)
CD11b-
DP
CD27-
B
Medium
TGF-β
Cells
pSMAD2/3
pSMAD2/3 MFI (x 10²)
SMAD2/3 MFI (x 10³)
CD11c-dnTGFβRII
NK-Tgfbr2-/-
Control
C
pSMAD2/3 MFI (x 10²)
ns
D
% of NK Cells
BM
Blood
Liver
Lung
Spleen
E
# of NK cells (x10⁶)
F
BM
Blood
Liver
Lung
Spleen
Control
NK-Tgfbr2-/-
CD27
CD11b
45%
45%
2%
8%
15%
33%
5%
47%
14%
25%
9%
52%
7%
28%
3%
62%
18%
32%
6%
41%
45%
43%
3%
9%
16%
34%
4%
46%
13%
24%
6%
57%
8%
26%
3%
63%
16%
30%
5%
49%
G
% of BrdU+ cells
Control
NK-Tgfbr2-/-

Figure 2

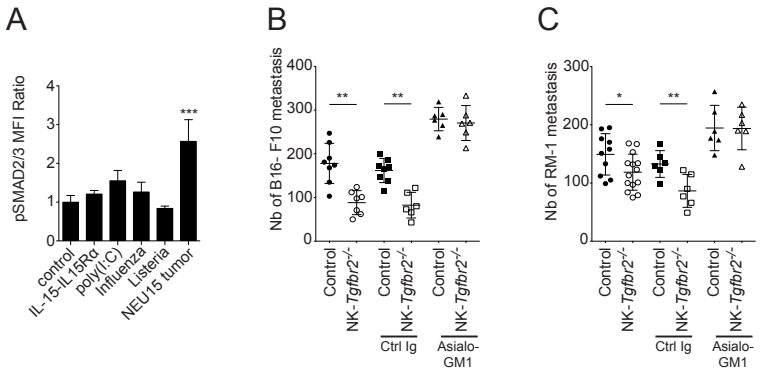

A
pSMAD2/3 MFI Ratio
0
1
2
3
4
***
control
IL-15-IL15Rα
poly(I:C)
Influenza
Listeria
NEU15 tumor
B
Nb of B16- F10 metastasis
0
100
200
300
400
**
**
Control
NK-Tgfbr2−/−
Control
NK-Tgfbr2−/−
Ctrl Ig
Control
NK-Tgfbr2−/−
Asialo-GM1
C
Nb of RM-1 metastasis
0
100
200
300
*
**
Control
NK-Tgfbr2−/−
Control
NK-Tgfbr2−/−
Ctrl Ig
Control
NK-Tgfbr2−/−
Asialo-GM1

Figure 3

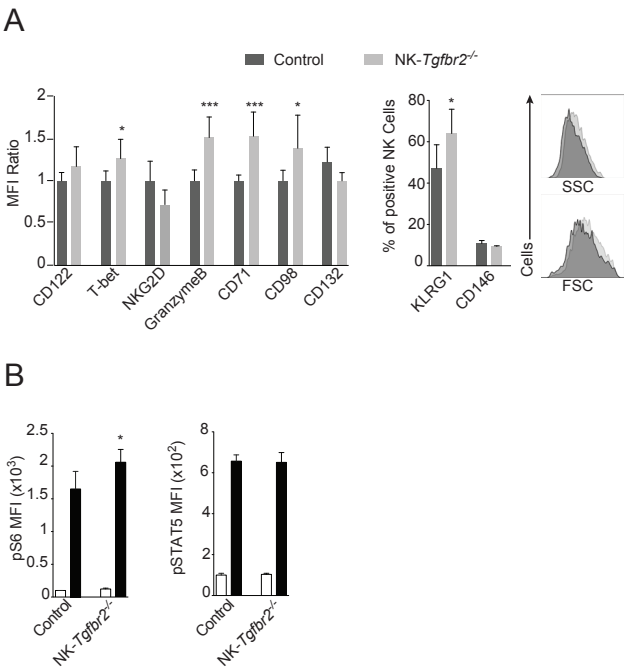

A
Control
NK-Tgfbr2-/-
MFI Ratio
CD122
T-bet
NKG2D
GranzymeB
CD71
CD98
CD132
% of positive NK Cells
KLRG1
CD146
Cells
SSC
FSC
B
pS6 MFI (x10-3)
pSTAT5 MFI (x10-2)
Control
NK-Tgfbr2-/-

Figure 4

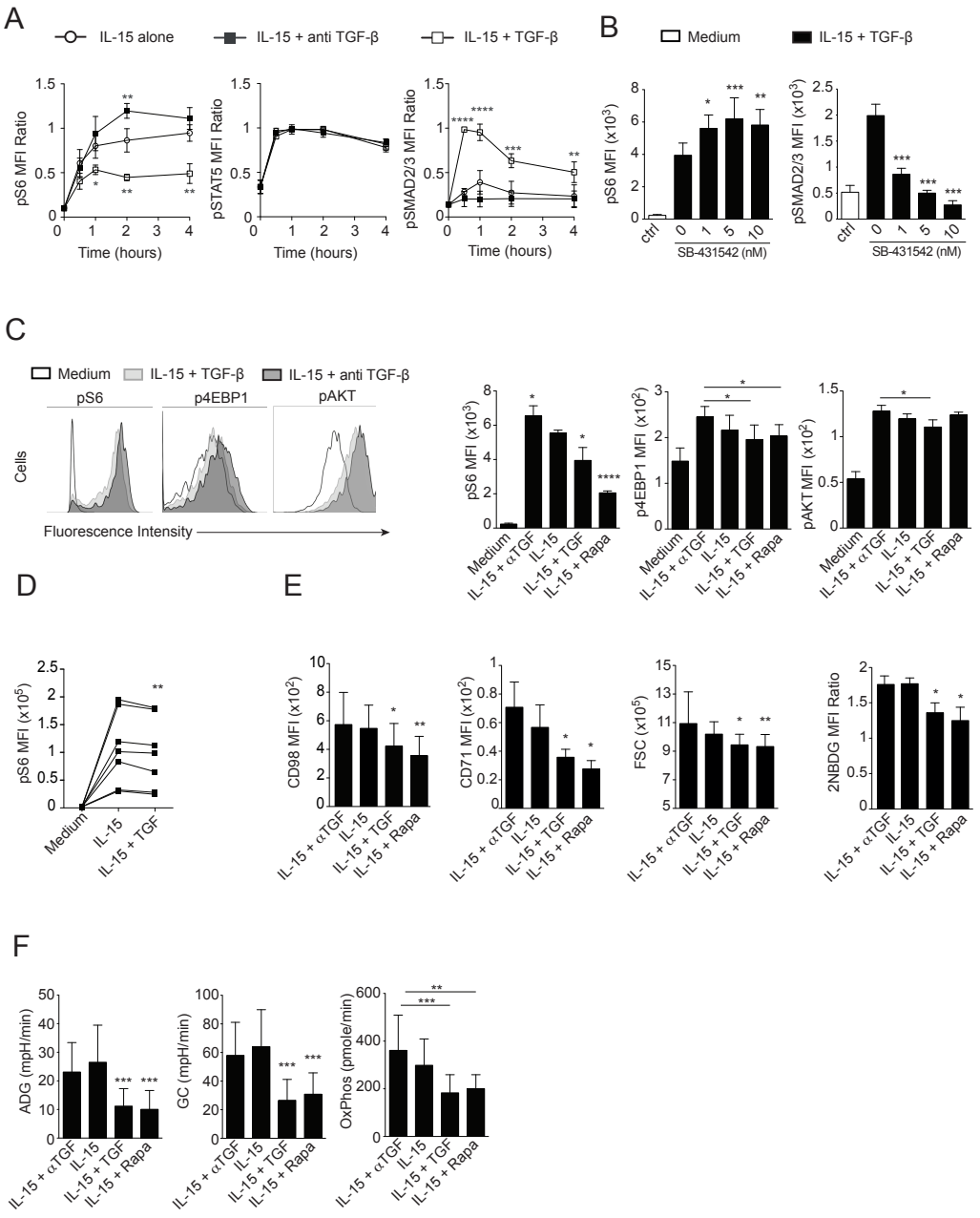

A
IL-15 alone
IL-15 + anti TGF-β
IL-15 + TGF-β
pS6 MFI Ratio
pSTAT5 MFI Ratio
pSMAD2/3 MFI Ratio
Time (hours)
B
Medium
IL-15 + TGF-β
pS6 MFI (x10³)
pSMAD2/3 MFI (x10³)
ctrl
SB-431542 (nM)
C
Medium
IL-15 + TGF-β
IL-15 + anti TGF-β
pS6
p4EBP1
pAKT
Cells
Fluorescence Intensity
pS6 MFI (x10³)
p4EBP1 MFI (x10²)
pAKT MFI (x10²)
Medium
IL-15 + αTGF
IL-15
IL-15 + TGF
IL-15 + Rapa
D
pS6 MFI (x10⁵)
Medium
IL-15
IL-15 + TGF
E
CD98 MFI (x10²)
CD71 MFI (x10²)
FSC (x10⁵)
2NBDG MFI Ratio
IL-15 + αTGF
IL-15
IL-15 + TGF
IL-15 + Rapa
F
ADG (mpH/min)
GC (mpH/min)
OxPhos (pmole/min)
IL-15 + αTGF
IL-15
IL-15 + TGF
IL-15 + Rapa

Figure 5

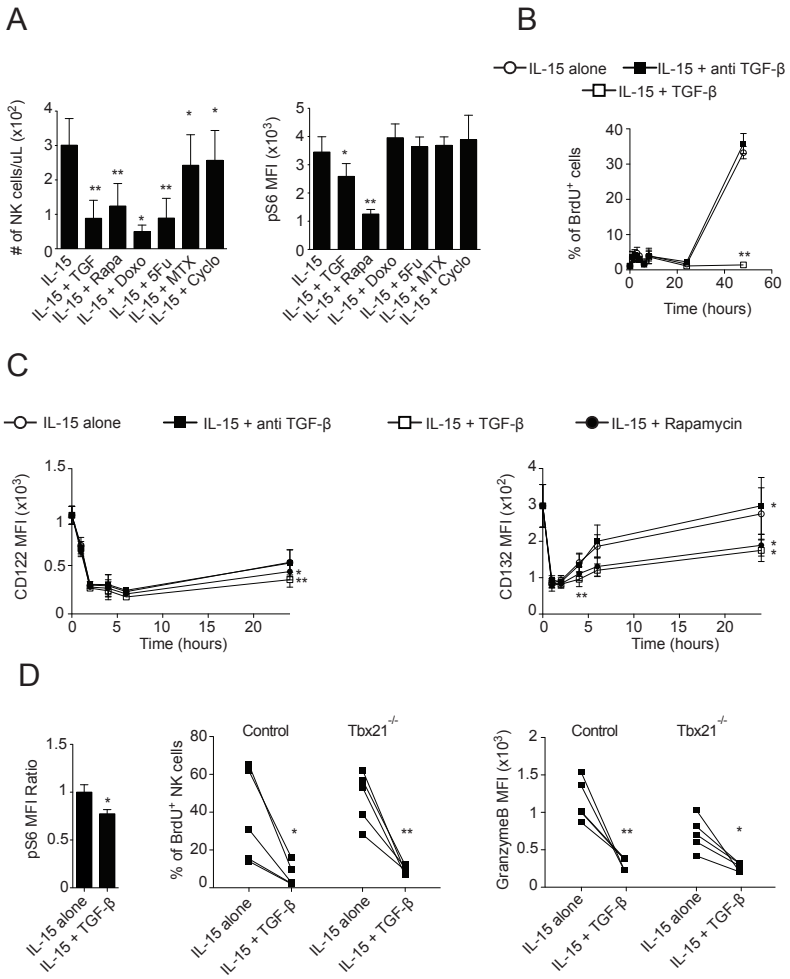

A
B
C
D
IL-15 alone
IL-15 + anti TGF-β
IL-15 + TGF-β
IL-15 + Rapamycin
# of NK cells/uL (x10²)
pS6 MFI (x10³)
% of BrdU⁺ cells
Time (hours)
CD122 MFI (x10³)
CD132 MFI (x10²)
pS6 MFI Ratio
% of BrdU⁺ NK cells
GranzymeB MFI (x10³)
Control
Tbx21-/-
IL-15
IL-15 + TGF
IL-15 + Rapa
IL-15 + Doxo
IL-15 + 5Fu
IL-15 + MTX
IL-15 + Cyclo

Figure 6

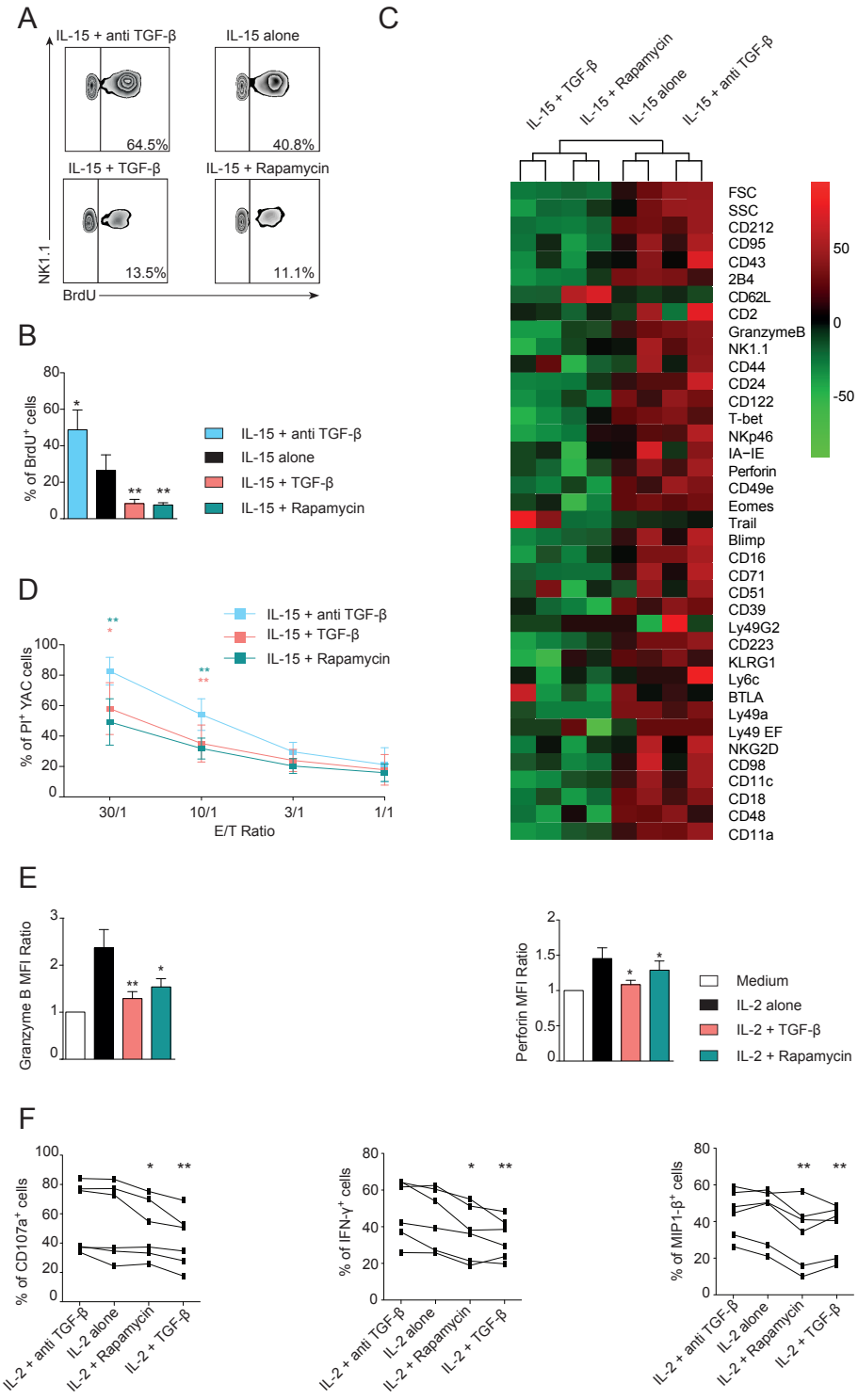
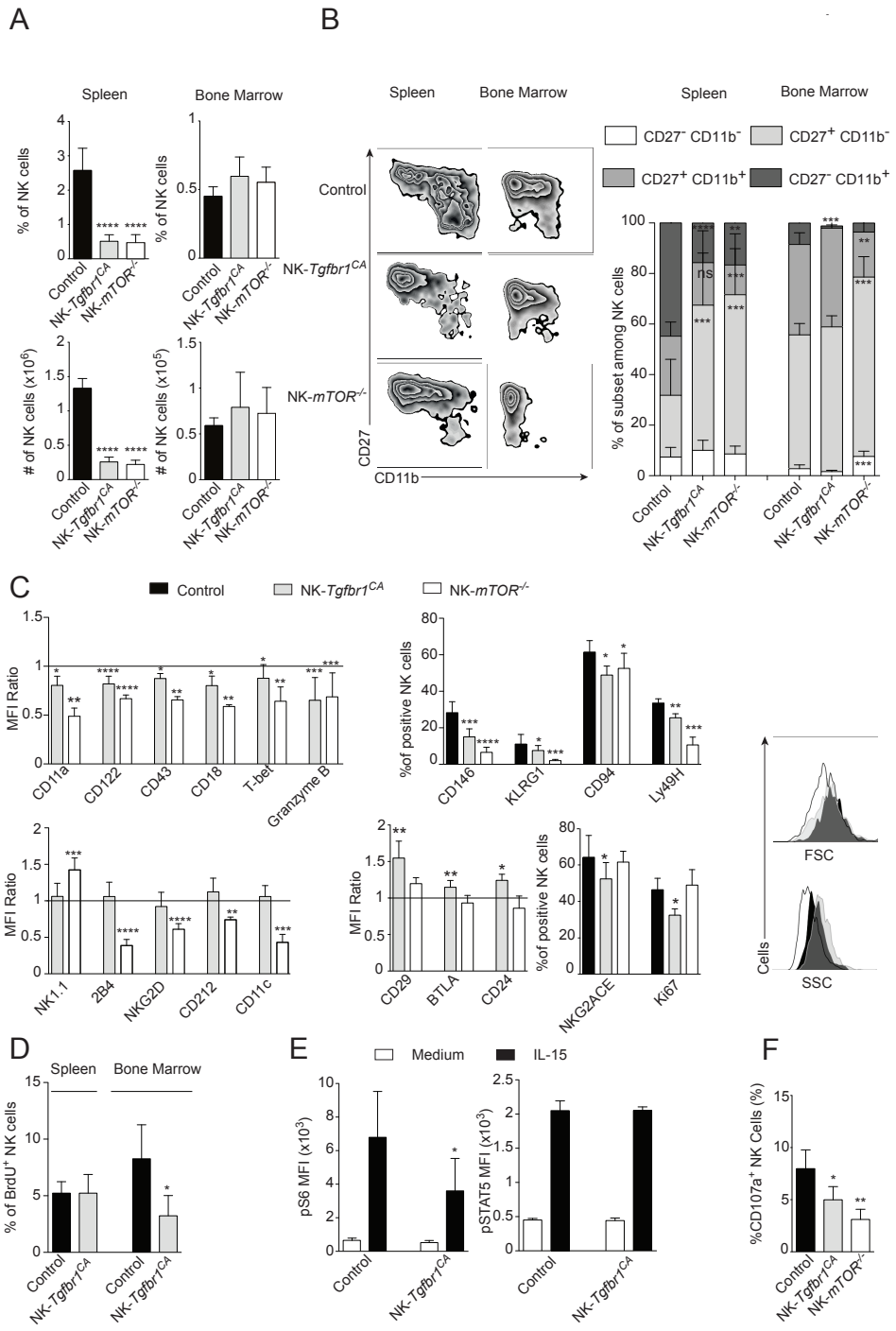

A
IL-15 + anti TGF-β
IL-15 alone
64.5%
40.8%
IL-15 + TGF-β
IL-15 + Rapamycin
13.5%
11.1%
NK1.1
BrdU
B
% of BrdU+ cells
IL-15 + anti TGF-β
IL-15 alone
IL-15 + TGF-β
IL-15 + Rapamycin
C
IL-15 + TGF-β
IL-15 + Rapamycin
IL-15 alone
IL-15 + anti TGF-β
FSC
SSC
CD212
CD95
CD43
2B4
CD62L
CD2
GranzymeB
NK1.1
CD44
CD24
CD122
T-bet
NKp46
IA−IE
Perforin
CD49e
Eomes
Trail
Blimp
CD16
CD71
CD51
CD39
Ly49G2
CD223
KLRG1
Ly6c
BTLA
Ly49a
Ly49 EF
NKG2D
CD98
CD11c
CD18
CD48
CD11a
50
0
-50
D
% of PI+ YAC cells
E/T Ratio
30/1
10/1
3/1
1/1
IL-15 + anti TGF-β
IL-15 + TGF-β
IL-15 + Rapamycin
E
Granzyme B MFI Ratio
Perforin MFI Ratio
Medium
IL-2 alone
IL-2 + TGF-β
IL-2 + Rapamycin
F
% of CD107a+ cells
% of IFN-γ+ cells
% of MIP1-β+ cells
IL-2 + anti TGF-β
IL-2 alone
IL-2 + Rapamycin
IL-2 + TGF-β

Figure 7


A
Spleen
Bone Marrow
% of NK cells
# of NK cells (x10^5)
Control
NK-Tgfbr1^CA
NK-mTOR^-/-
B
Spleen
Bone Marrow
Control
NK-Tgfbr1^CA
NK-mTOR^-/-
CD27
CD11b
CD27- CD11b-
CD27+ CD11b-
CD27+ CD11b+
CD27- CD11b+
% of subset among NK cells
ns
C
Control
NK-Tgfbr1^CA
NK-mTOR^-/-
MFI Ratio
CD11a
CD122
CD43
CD18
T-bet
Granzyme B
%of positive NK cells
CD146
KLRG1
CD94
Ly49H
NK1.1
2B4
NKG2D
CD212
CD11c
CD29
BTLA
CD24
NKG2ACE
Ki67
FSC
SSC
Cells
D
Spleen
Bone Marrow
% of BrdU+ NK cells
E
Medium
IL-15
pS6 MFI (x10^3)
pSTAT5 MFI (x10^3)
F
%CD107a+ NK Cells (%)

Résultats complémentaires

1. L'effet du TGF-β sur les cellules NK est, en partie, médié par FKBP12

En introduction, nous avons déjà évoqué le lien intéressant entre la rapamycine et le TGF-β au regard des protéines de la famille des FKBP. En effet, la protéine FKBP12 maintient TGFbRI sous sa forme inactive et est libérée lors de l'engagement de ce récepteur. La rapamycine, en s'associant à FKBP12, agit quant à elle en inhibant l'activité kinase de mTOR. D'autres médicaments, comme le FK506, peuvent s'associer à FKBP12, mais n'ont pas d'effet sur mTOR puisque le complexe FK506-FKBP12 cible la calcineurine. Comme un prétraitement par FK506 est connu pour inhiber les effets de la rapamycine en entrant en compétition pour leur fixation à FKBP12, nous nous sommes demandés si un tel prétraitement pouvait également avoir un effet sur l'inhibition de mTOR par le TGF-β. Pour le tester, nous avons cultivé des splénocytes avec de l'IL-15 en présence de TGF-β ou de rapamycine en combinaison ou non avec du FK506. Nous avons ensuite mesuré par cytométrie en flux l'expression de pS6 après une heure de stimulation ainsi que différents paramètres connus pour être régulés par mTOR (Granzyme B, Taille/structure, CD71, CD98) après 12h de stimulation. Nous avons ensuite calculé le pourcentage d'inhibition de chacun de ces paramètres par la rapamycine ou le TGF-β, en présence ou absence de FK506.

Comme illustré dans la Figure 28, le FK506 inhibe partiellement l'effet de la rapamycine sur la phosphorylation de S6 et inhibe totalement son effet sur d'autres paramètres comme la taille/structure ainsi que l'expression de CD122, Granzyme B, CD18 et NK1.1. De façon intéressante, le FK506 bloque également significativement l'effet du TGF-β sur la phosphorylation de S6 et sur les autres paramètres testés. Ces données suggèrent que les protéines FKBP et notamment FKBP12 jouent un rôle dans l'inhibition de mTOR induite par le TGF-β.

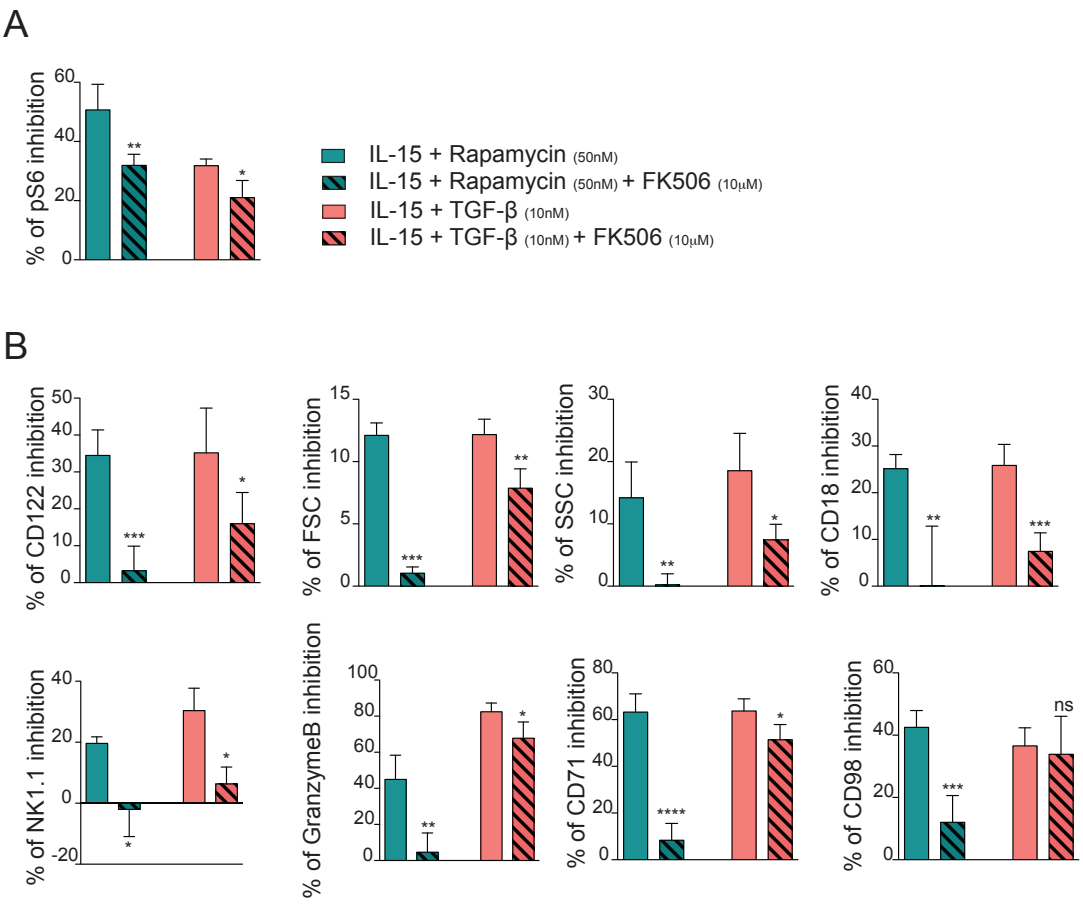


Figure 28: Effets du FK506 sur l'inhibition de mTOR induite par la rapamycine et le TGF-β.

Pourcentage d'inhibition du TGF-β ou de la rapamycine sur différents paramètres contrôlés par mTOR après 1 (A) ou 12 heures (B) de stimulation par de l'IL-15 en présence ou en absence de FK506.

2. La sur-activation de mTOR compense partiellement l'activation constitutive des voies du TGF-β dans les cellules NK

Afin d'étudier si le phénotype observé dans les cellules NK qui expriment la forme constitutivement activée du récepteur du TGF-β (NK-*tgfbr1*^{CA}) pouvait être compensé par une sur-activation de mTOR, nous avons créé des souris NK-*tgfbr1*^{CA}-TSC$^{-/-}$. TSC (Tuberous Sclerosis Complex) est un complexe à activité GTPase. mTORC1 est connu pour être activé par la protéine Rheb-GTP. TSC, dont l'activité GTPase induit la conversion de Rheb-GTP en Rheb-GDP, est donc un régulateur négatif de mTOR et son absence conduit à une sur-activation de mTORC1. Nous avons donc étudié le phénotype des cellules NK dans les souris NK-*tgfbr1*^{CA}-TSC$^{-/-}$ et nous l'avons comparé à celui des NK-*tgfbr1*^{CA} et des NK-*mtor*$^{-/-}$.

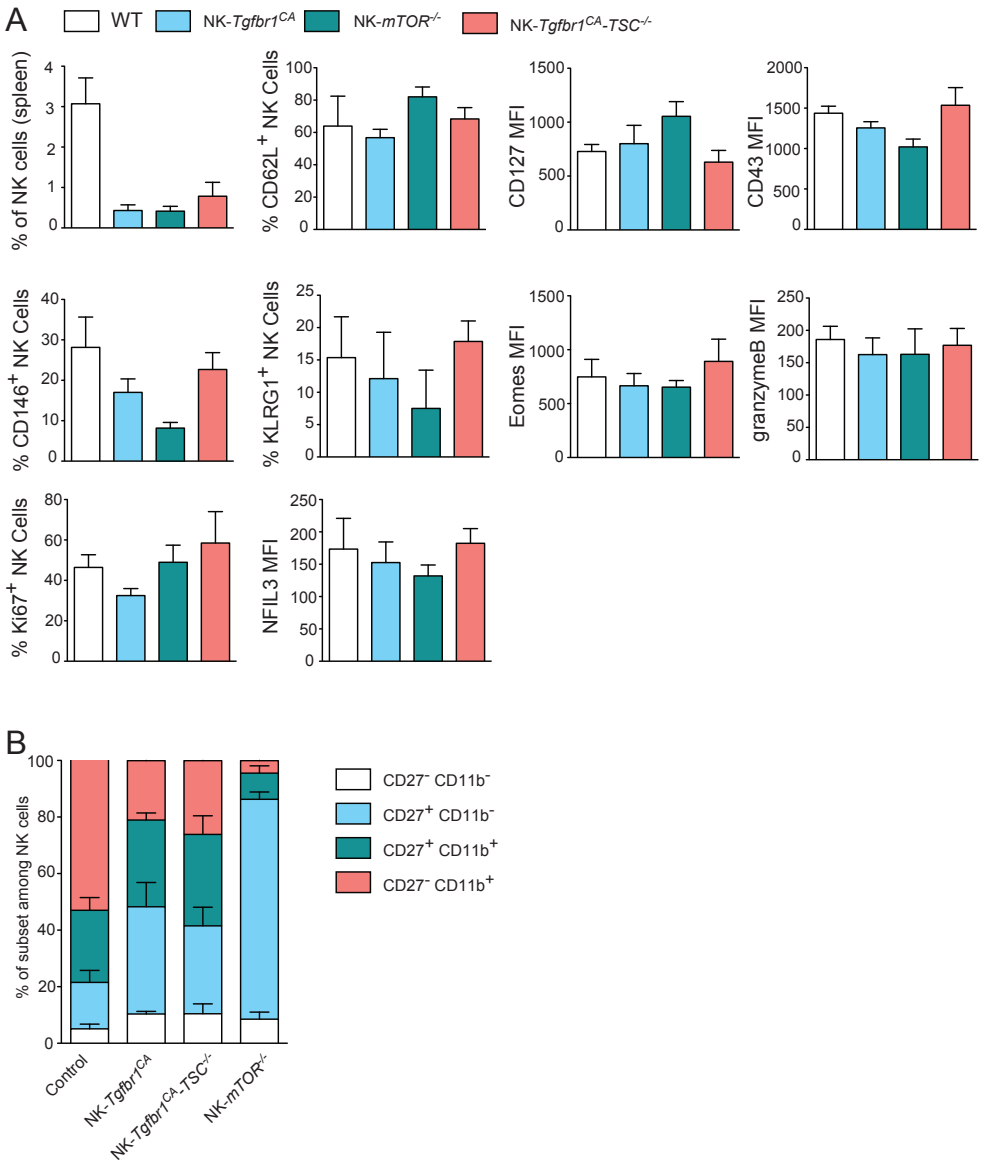


Figure 29: La sur-activation de mTOR compense partiellement l'activation constitutive des voies du TGF-β.
Comparaison de la distribution, du phénotype (A) et du profil de maturation (B) des cellules NK-*tgfbr1*^{CA}-TSC$^{-/-}$ avec les cellules NK-*tgfbr1*^{CA}.

Comme illustré dans la Figure 29, nous observons une tendance à la normalisation de certains paramètres comme le pourcentage de cellules NK dans la moelle, ainsi que d'un certain nombre de paramètres phénotypiques décrits dans la Figure 29-A (CD62L, CD127, CD43, CD146, KLRG1, Eomes, granzyme B, Ki67 et NFIL3). Même si elle est faible, cette tendance persiste toujours sur la maturation des cellules NK (Figure 29-B). En revanche, pour les autres paramètres mesurés (CD16, CD51, CD29, CD11a, FSC/SSC, CD44, CD48, CD39, 2B4, NKG2D, NKG2ACE, CD98, CD49a,d,e,f, CD218a, CD122, CD132, CD226, CD212, CD160, CD223, CMHII, Trail,

Ly6C, CD69 CD2, BTLA Ly49A,D,E,F,G2, T-bet, Perforine) aucun effet n'a été mis en évidence.

Ces données, qui doivent cependant être confirmées, suggèrent qu'au moins en partie, l'effet du TGF-β peut être compensé *in vivo* par une sur-activation de mTOR.

3. L'activation des cellules NK par l'IL-15 induit un rétrocontrôle négatif médié par le TGF-β *in vitro*.

Au cours des expériences réalisées pendant ce travail, nous avons observé que l'activation de cellules NK murines par de l'IL-15 seule *in vitro* induisait la phosphorylation de smad2/3 de façon concomitante à la phosphorylation de S6 (Article 1, Figure 4A). Ces résultats suggèrent l'existence d'une boucle de rétrocontrôle négatif de la voie de l'IL-15 par le TGF-β activé. Pour savoir si les cellules NK elles-mêmes produisaient du TGF-β actif, nous avons purifié des cellules NK murines à l'aide de billes magnétiques pour éliminer d'autres sources cellulaires potentielles de TGF-β actif. Nous avons ensuite activé ces cellules avec différentes doses d'IL-15 en présence ou non d'anti-TGF-β et mesuré l'expression de pS6 et psmad2/3 après 1 heure de stimulation.

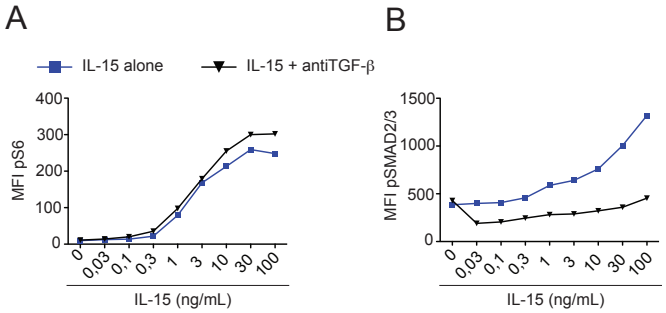


Figure 30: Activation des voies smad par l'IL-15
Mesure de l'expression de pS6 et psmad2/3 dans des cellules NK purifiées à différentes doses d'IL-15 en présence ou non d'anti-TGF-β

Comme attendu, plus la dose d'IL-15 utilisée est importante, plus l'expression de pS6 augmente (Figure 30-A). Comme nous l'avions déjà observé (Article 1, Figure 4A), l'addition d'anti-TGF-β augmente l'intensité de pS6, possiblement, en bloquant le TGF-β endogène (Figure 30-A). De façon intéressante, lorsque nous avons mesuré l'expression de psmad2/3, nous avons observé le même phénomène : plus la concentration d'IL-15 est forte, plus l'expression de psmad2/3 augmente (Figure 30-B). De plus, l'ajout d'anti-TGF-β dans la culture inhibe presque totalement ce

phénomène. Ces données suggèrent donc bien que l'IL-15 induit la sécrétion de TGF-β actif par les cellules NK, qui en retour contribue à limiter les effets de l'IL-15.

Si ces données ne sont pas décrites dans notre article, c'est qu'elles n'ont malheureusement pas été confirmées *in vivo*. En effet, l'injection de poly(I:C) induit une forte induction de pS6 dans les cellules NK chez la souris (Marçais et al., 2014), mais son effet sur l'expression de psmad2/3 reste très faible (Article 1, Figure 2A). Par ailleurs, l'induction de pS6 dans les cellules NK de souris NK-*tgfbr2*$^{-/-}$ suite à l'injection de poly(I:C) n'est pas supérieure à celle observée dans les souris contrôles. La voie d' « autorégulation » des cellules NK que nous avons mise en évidence *in vitro*, s'applique donc peut-être dans d'autres contextes qui restent à découvrir.

DISCUSSION

Au cours de ce travail, nous nous sommes intéressés aux mécanismes d'action du TGF-β sur les cellules NK. Si les effets antagonistes entre le TGF-β et l'IL-2/15 sont connus et décrits depuis une trentaine d'années (Malygin et al., 1993; Ortaldo et al., 1991; Rook et al., 1986), les mécanismes moléculaires qui expliquent ces effets restent inconnus.

Nous avons tout d'abord vérifié que les cellules NK murines exprimaient TGFbRII et que ce récepteur était fonctionnel. En effet, exposées à du TGF-β exogène, les cellules NK phosphorylent smad2/3. La réponse au TGF-β en terme d'intensité d'expression de psmad2/3 diminue avec la maturation des cellules NK. Ces résultats peuvent s'expliquer, au moins en partie, par le fait qu'au cours de la maturation, l'expression de TGFbRII diminue tandis que l'expression de smad2/3 total n'est pas modifiée. Nous avons ensuite testé la réponse au TGF-β des cellules NK issues de deux modèles de souris génétiquement modifiées pour le récepteur de type 2 du TGF-β dans les cellules NK : le modèle CD11c-dnTGFbRII, généré par l'équipe de Flavell et le modèle NK-*tgfbr2*$^{-/-}$, généré au laboratoire. Si l'approche génétique utilisée pour la génération de ces 2 modèles est différente, l'effet sur l'expression de psmad2/3 après stimulation est le même puisque psmad2/3 n'est pas détectée après ajout de TGF-β exogène dans les deux cas. En revanche, le phénotype obtenu est très différent. Dans le modèle CD11c-dnTGFbRII, l'expression transgénique d'une forme tronquée de ce récepteur par les cellules CD11c^{+} induit une forte expansion des cellules NK et augmente leur capacité de production d'IFN-γ (Laouar et al., 2005). En revanche, dans le modèle NK-*tgfbr2*$^{-/-}$, nous n'observons pas de conséquence majeure de la délétion de TGFbRII dans les cellules NK en condition homéostatique. En effet, ni la distribution des cellules NK, ni leur état de maturation ou leur phénotype ne sont altérés. La seule différence significative obtenue par rapport aux souris contrôles est une plus importante prolifération des cellules NK dans la moelle osseuse. Les mêmes différences ont été observés dans les lymphocytes T CD8 mémoires en utilisant les mêmes approches (Ishigame et al., 2013). Une des raisons pouvant expliquer ces résultats est le fait que d'autres populations leucocytaires, exprimant également CD11c, participent au développement des cellules NK *via* la transprésentation d'IL-15. En agissant indirectement sur ces cellules, il est alors possible que l'expansion des cellules NK

observée dans le modèle dominant négatif soit les conséquences d'un mécanisme indirect. Une autre possibilité est le fait que l'expression de CD11c soit plus tardive que celle de NKp46 (qui contrôle la délétion de *tgfbr2* dans notre modèle), dans le développement des cellules NK. Dans des lignées tumorales, il a été montré que la surexpression du transgène dnTGFbRII altérait à la fois les voies activées par TGF-β1, mais également par BMP2 (Dumont and Arteaga, 2003). Il est donc possible que dnTGFbRII interagisse avec d'autres récepteurs que TGFbRI et bloque d'autres voies de signalisation de la superfamille du TGF-β ce qui pourrait potentialiser les effets observés sur la prolifération et les capacités effectrices des cellules NK. Les cellules NK sont d'ailleurs sensibles à d'autres ligands de la famille du TGF-β comme l'activine A, qui est également décrite pour inhiber les fonctions des cellules NK chez l'homme (Robson et al., 2009) *in vitro*. L'absence de phénotype dans le modèle NK-*tgfbr2*$^{-/-}$, plus spécifique des cellules NK, semble néanmoins indiquer que l'expression du récepteur du TGF-β n'est pas essentielle dans la biologie des cellules NK en condition homéostatique.

Nous avons ensuite cherché à mettre en évidence quelles situations pouvaient induire l'activation des voies du TGF-β *in vivo*. Des infections virales, bactériennes ou des stimulations par des ligands de TLR ou par le complexe IL-15-IL-15Rα n'induisent que faiblement la phosphorylation de smad2/3 dans les cellules NK. En revanche, des cellules NK de l'environnement tumoral de cancers mammaires, chez la souris, présentent une expression élevée de psmad2/3 en accord avec le rôle important du TGF-β dans l'échappement tumoral (Flavell et al., 2010). Du fait des propriétés pro- et anti-tumorales du TGF-β, les gènes impliqués dans sa signalisation sont fréquemment la cible de mutations par de nombreuses lignées cancéreuses chez l'homme (Akhurst and Derynck, 2001). En plus de muter les gènes impliqués dans les voies du TGF-β, les cellules tumorales sont également capables de produire elles-mêmes du TGF-β dans le but de favoriser leur dissémination et le phénomène d'échappement du système immunitaire. Si de nombreuses études décrivent que l'élévation de la concentration plasmatique de TGF-β1 observée dans différents types de cancers est associée à la survie, les sources cellulaires du TGF-β ne sont souvent pas souvent définies (Teicher, 2007). D'autres études ont cherché à mettre en évidence l'origine de ce TGF-β produit. Dans le cancer de la prostate, il a été observé que cette origine était à la fois

tumorale et stromale (Gerdes et al., 1998). Dans cet article, les auteurs n'ont cependant pas mis en évidence de différence d'expression entre les cellules stromales du tissu sain et du tissu carcinomateux ce qui suggère que la surexpression de TGF-β mise en évidence est bien d'origine tumorale. De la même façon, dans le mélanome, il a été montré que seules les formes sévères présentaient une augmentation de l'expression de TGF-β qui n'était pas observée dans les formes bénignes ou dans les lésions *in situ* (Reed et al., 1994). Cette surexpression de TGF-β est associée à une augmentation du nombre de métastases ainsi qu'à la gravité des lésions dans cette maladie. Une augmentation de la concentration sérique du TGF-β est également décrite dans le myélome. Elle est corrélée à la concentration de b2-microglobuline qui sert de marqueur de sévérité de la maladie. Le tri de cellules myélomateuses a montré qu'elles produisaient significativement plus de TGF-β que les autres types cellulaires mononucléés. De plus, la sécrétion de TGF-β par les cellules stromales est supérieure chez les patients myélomateux que chez les donneurs sains ce qui suggère également que les cellules stromales participent à la surexpression du TGF-β1 dans le myélome (Urashima et al., 1996). Dans le cancer du sein, seules les formes sévères du cancer sont capables de produire du TGF-β1 actif *in vitro*. Cette propriété est partagée avec les cellules avoisinantes ce qui favorise la dissémination (Baillie et al., 1996). Si les cellules tumorales et stromales sont donc capables de sécréter du TGF-β, les cellules immunitaires du microenvironnement participent également à ce phénomène. C'est par exemple le cas des cellules NKT de type II qui, dans des modèles murins de tumeurs fibroblastiques, induisent la production de TGF-β par les cellules myéloïdes suppressives *via* l'IL-13 (Terabe et al., 2000). C'est également le cas des lymphocytes Treg, dont une population particulière n'exprimant ni CD25, ni Foxp3 est capable de produire fortement du TGF-β après l'engagement de CD69 (Han et al., 2009). Ces cellules semblent d'ailleurs jouer un rôle dans la croissance de tumeurs déjà installées. L'environnement tumoral est donc riche en TGF-β, quelle que soit sa source. Une fois sécrété, il participe au mécanisme d'échappement du système immunitaire en inhibant les fonctions de nombreux types cellulaires dont les cellules NK.

Nos résultats corroborent ces données puisque, dans des modèles de cancers contrôlés par les cellules NK (B16-F10 et RM-1), le nombre de métastases est significativement plus faible dans les souris NK-*tgfbr2*$^{-/-}$ que dans les souris

sauvages. Nous confirmons donc ici des résultats déjà décrits dans le cancer du sein (Krneta et al., 2015) à propos des effets inhibiteurs du TGF-β sur l'activité des cellules NK dans la réponse anti tumorale *in vivo.*

Afin d'essayer de comprendre les mécanismes par lesquels le défaut d'expression de TGFbRII par les cellules NK avantage leur réponse anti tumorale, nous avons activé ces cellules NK *in vitro* par de l'IL-15 et suivi l'activité de mTOR. La majorité des paramètres dépendants de mTOR comme la taille, la granularité ou l'expression de marqueurs métaboliques (CD71, CD98) ou cytotoxiques (granzyme B) augmente dans les souris NK-*tgfbr2*$^{-/-}$. Ces données suggèrent que le TGF-β endogène a un effet inhibiteur sur la kinase mTOR, que nous avons confirmé par une augmentation de l'expression de pS6 après une courte stimulation par IL-15 dans ces souris.

Concernant les effets du TGF-β *in vitro*, nous avons observé que le TGF-β inhibe la phosphorylation des substrats de mTOR S6, 4EBP1 et Akt chez la souris. Inversement, le blocage du TGF-β endogène par un anticorps bloquant augmente l'activité de mTOR. L'utilisation d'un autre antagoniste du TGF-β, le SB-431542, confirme ces résultats ce qui suggère que l'activité kinase de TGFbRI est requise pour l'induction de l'inhibition de mTOR par le TGF-β.

Cet effet inhibiteur est rapide puisque sa cinétique d'apparition se superpose à l'apparition de la phosphorylation de Smad2/3 et apparaît en moins d'une heure. Chez l'homme, nous avons également détecté une diminution de l'expression de pS6 induite rapidement par le TGF-β pour tous les patients testés. Ces données suggèrent que le mécanisme d'inhibition du TGF-β sur mTOR est conservé au cours de l'évolution. L'effet mesuré est cependant moins marqué que chez la souris. Ceci est peut-être dû à de réelles différences entre la souris et l'homme, ou alors au fait que chez l'homme, les cellules NK utilisées proviennent du sang alors qu'elles proviennent de la rate chez la souris. Une dernière explication possible est le fait que chez l'homme, la majorité des cellules NK qui répondent à l'IL-15 à des temps courts sont les cellules NK CD56^{bright} et que leur proportion est très variable selon les donneurs.

Nous avons également mis en évidence des effets très proches du TGF-β et de la rapamycine, un inhibiteur spécifique de mTOR sur le métabolisme des cellules NK activées par l'IL-15 *in vitro*. En effet, le TGF-β inhibe l'expression de marqueurs métaboliques comme CD98 ou CD71, la taille cellulaire ainsi que l'incorporation de

glucose dans des proportions similaires à la rapamycine. Les effets inhibiteurs du TGF-β et de la rapamycine sur l'activité glycolytique et la phosphorylation oxydative de cellules NK activées sont également très comparables. Des données de la littérature avaient d'ailleurs souligné les effets similaires du TGF-β et de la rapamycine sur l'induction de la différenciation des lymphocytes Treg (Sauer et al., 2008) ainsi que sur la prolifération des lymphocytes T activés par des anticorps anti-CD3/CD28 *in vitro* (Delisle et al., 2013). Le contrôle de l'activité métabolique apparaît comme un évènement clé de la régulation des cellules immunitaires. La rapamycine et ses dérivés sont essentiellement utilisés pour la prévention du rejet de greffe rénale, pour la prévention et le traitement des réactions de type GVH (Graft Versus Host). Parmi les mécanismes d'actions immunosuppresseurs de la rapamycine, on peut citer l'inhibition de la prolifération des lymphocytes T, l'inhibition de la maturation des DC et l'induction de l'expression de Foxp3 favorisant le développement des lymphocytes Treg (Zeng and Chi, 2013). Nos travaux précédents ont souligné le rôle central de la kinase mTOR dans l'activation des cellules NK. Le traitement par de la rapamycine *in vivo* réduit la cytotoxicité des cellules NK vis à vis de cibles déficientes pour le CMH de classe I objectivant clairement le rôle clé de mTOR et du métabolisme cellulaire dans le contrôle de la fonction des cellules NK. Nous avons montré que mTOR était essentiel dans l'induction de l'expression des protéines cytotoxiques comme perforine et granzyme B, mais également pour augmenter l'activité des cellules NK *via* un rétrocontrôle positif sur les voies de signalisation en aval des récepteurs activateurs (Marçais et al., 2014). La régulation du cytosquelette d'actine dépendante de mTORC2 favorise également l'établissement de la synapse immunologique entre les cellules NK et leurs cibles.

Les similarités d'effets observés entre le TGF-β et la rapamycine ne se limitent pas qu'au métabolisme. Chez l'homme, le TGF-β et la rapamycine inhibent également la dégranulation et la sécrétion de cytokines ainsi que l'expression de granzyme B et de perforine induites par l'IL-15 *in vitro*. De la même façon, chez la souris, ces deux molécules inhibent la prolifération des cellules NK, la cytotoxicité ainsi que l'expression d'un nombre important de marqueurs phénotypiques. Nous avons cependant observé quelques discordances concernant l'expression de ces marqueurs. Par exemple, le TGF-β induit l'expression de TRAIL alors que la rapamycine l'inhibe. A ce sujet, il serait ainsi intéressant d'étudier plus profondément le phénotype des ILC1 du foie, qui expriment TRAIL, dans les souris NK-*tgfbr2*$^{-/-}$.

Inversement, la rapamycine induit l'expression de CD62L, mais pas le TGF-β. Des travaux récents ont d'ailleurs mis en évidence le même type d'induction de CD62L par la rapamycine dans les lymphocytes T CD8 (Hukelmann et al., 2015). Si le TGF-β et la rapamycine ont globalement des effets proches, on ne peut visiblement pas limiter les effets du TGF-β à ceux d'une « rapamycine endogène ».

Dans notre travail, l'inhibition de la kinase mTOR par le TGF-β est également soutenue par des résultats indirects. Les cellules NK de souris exprimant une forme constitutivement activée du récepteur du TGF-β ont un phénotype très semblable à celui des cellules NK déficientes pour mTOR. Dans les deux cas, nous avons observé une diminution très importante du nombre de cellules NK (à la fois en pourcentage et en valeur absolue) dans la rate. Ces résultats s'expliquent au moins par le fait que ces 2 modèles induisent un biais important de la maturation des cellules NK. En effet, l'activation constitutive des voies du TGF-β ou la déficience de mTOR provoque un blocage de la maturation des cellules NK caractérisé par la quasi absence de cellules NK matures $CD27^{-}CD11b^{+}$ en périphérie, mais également dans la moelle. Concernant le phénotype de ces cellules NK, nous avons mis en évidence des résultats similaires entre les deux modèles pour des molécules dont l'expression dépend de mTOR comme T-bet, granzyme B, CD122 ou KLRG1. Comme *in vitro*, nous avons également observé des discordances concernant l'expression d'autres marqueurs (2B4, NKG2D, CD212, CD11c…) qui renforcent l'idée que les effets du TGF-β ne se limitent pas à mimer parfaitement ceux de la rapamycine. Cependant, d'autres similarités entre les 2 modèles de souris ont été mises en évidence. L'activation constitutive des voies du TGF-β, dans les cellules NK, induit une diminution de leur prolifération dans la moelle, comme nous l'avions observé pour les cellules NK déficientes pour mTOR (Marçais et al., 2014). Concernant la réponse précoce à l'IL-15, les cellules NK-*tgfbr1*^{CA} présentent une baisse significative de l'expression de pS6 alors que l'expression de pSTAT5 n'est pas touchée, ce qui confirme la spécificité de l'effet du TGF-β sur mTOR et non sur l'ensemble des voies activées par l'IL-15. Enfin, le dernier point commun mis en évidence dans ces 2 modèles est un défaut de la dégranulation des cellules NK après co-culture avec des cellules déficientes pour le CMH de classe I. Afin de déterminer si l'effet du TGF-β, mis en évidence dans les souris NK-*tgfbr1*^{CA}, était réversible et compensé par une sur-activation de mTOR, nous avons généré des

souris NK-*tgfbr1*^{CA}-TSC$^{-/-}$, TSC étant un régulateur négatif de mTORC1. Comme présenté dans les résultats complémentaires, nous avons observé qu'une partie des anomalies phénotypiques présentes dans les NK-*tgfbr1*^{CA} était corrigée par une sur-activation de mTOR. En plus de l'étude de l'impact sur le phénotype, il paraît également indispensable de confirmer que l'effet de la sur-activation de mTOR corrige les autres paramètres régulés par mTOR dans les souris NK-*tgfbr1*^{CA} (phosphorylation de S6, prolifération des cellules NK dans la moelle et fonctions effectrices). Deux difficultés se sont opposées à l'obtention de tels résultats dans notre laboratoire. La première a été de générer en nombre suffisant des souris ayant le génotype adéquat (double homozygote) dans le temps imparti. La deuxième difficulté a été le fait que les souris TSC$^{lox/lox}$ utilisées pour générer les croisements n'étaient pas sur un fond génétique C57BL/6 pur. En plus d'influencer potentiellement les résultats, cela a eu pour conséquence de rendre plus difficile l'identification des cellules NK dans ces souris.

Les effets rapides du TGF-β mis en évidence au cours de ce travail sur la signalisation IL-15 sont renforcés par d'autres effets indirects, plus tardifs. Nos résultats montrent clairement que le TGF-β inhibe l'expression de T-bet et une de ses cibles, l'IL-15Rα (CD122) à des temps longs, ce qui contribue à la diminution globale de l'activité de mTOR induite par le TGF-β.

Si nos travaux mettent en évidence des preuves *in vitro* et *in vivo* de l'inhibition de mTOR par le TGF-β, nous n'avons pas été en mesure de déterminer précisément le mécanisme moléculaire à la base de cette inhibition.

L'inhibition de T-bet par Smad3 a été proposée pour expliquer l'inhibition de la production d'interféron induite par le TGF-β dans les cellules NK chez la souris et chez l'homme (Trotta et al., 2008; Yu et al., 2006). Nous avons cependant montré qu'en l'absence de T-bet, le TGF-β est toujours capable d'inhiber la voie mTOR à des temps courts sur l'expression de pS6 ou à des temps plus longs sur la prolifération et l'expression de granzyme B.

Les effets du TGF-β mis en évidence sur mTOR ne semblent pas non plus être une simple conséquence des propriétés antiprolifératives du TGF-β. Si de nombreux médicaments sont capables de bloquer le cycle cellulaire et d'inhiber la prolifération de cellules NK activées par l'IL-15, seuls la rapamycine et le TGF-β diminuent significativement l'expression de pS6 à des temps courts. L'effet du TGF-β sur la

prolifération n'est d'ailleurs détecté qu'après 48 heures de culture suggérant que cette baisse de la prolifération n'est pas la cause de l'inhibition de mTOR par le TGF-β, mais plutôt sa conséquence. De la même façon, l'inhibition de l'expression de CD122 par le TGF-β qui participe à la diminution globale de la signalisation par l'IL-15 n'apparaît qu'après 24 heures et ne peut expliquer les effets observés après 1 heure de traitement par le TGF-β.

L'effet du TGF-β décrit ici dépend-il des voies canoniques ou non canoniques ? Nous avons observé que l'effet du TGF-β nécessitait l'activité kinase de TGFbRI. Mais, ce récepteur contrôle à la fois les voies de signalisation dépendantes et indépendantes des protéines Smad. Des données de la littérature suggèrent l'existence d'un lien entre le TGF-β et mTOR. D'une part, dans les macrophages, la kinase TAK1, dont l'activation nécessite l'engagement de TGFbRI, inhibe la phosphorylation de la protéine S6 (Shin et al., 2013). D'autre part, la délétion du gène codant pour TGFbRII, dans les Treg, augmente l'activité basale de mTOR (Liu et al., 2014) et leur confère un phénotype Th1. Nous n'avons pas pu déterminer si les effets du TGF-β étaient dépendants ou non des protéines Smad. Nous avons, cependant, voulu tenter d'inhiber les voies dépendantes de Smad3 à l'aide d'un inhibiteur, le SIS3 (Jinnin et al., 2006) pour étudier si l'effet du TGF-β sur mTOR persistait ou non après inhibition de smad3. Malheureusement, la toxicité du composé n'a pas permis une interprétation correcte des résultats. Une autre approche envisagée a été la génération de la délétion conditionnelle de Smad4 dans les cellules NK en croisant des souris NKp46Cre avec des souris $Smad4^{lox/lox}$. Cette fois, ce sont des incompatibilités sanitaires entre animaleries qui nous ont empêché d'avancer sur ce point. La génération de telles souris reste primordiale pour évaluer la dépendance des voies smad sur l'inhibition de mTOR par le TGF-β. Inversement, pour tester si les effets du TGF-β sont indépendantes des Smad, il paraît intéressant de vérifier quelles voies non canoniques sont fonctionnelles dans les cellules NK et d'étudier si certains inhibiteurs de ces voies comme le 5Z-7-oxozeaenol, un inhibiteur spécifique de TAK1 (Fan et al., 2013), annihile l'effet du TGF-β sur mTOR.

Dans les autres types cellulaires, l'importance des voies non canoniques par rapport aux voies impliquant les protéines Smad n'est pas non plus très claire. En effet, la délétion de l'expression de TGFbRII dans les lymphocytes T induit un syndrome auto-inflammatoire massif et létal après 2 ou 3 semaines de vie (Marie et al., 2006).

A l'inverse, le phénotype obtenu grâce à la délétion sélective de smad4 dans les lymphocytes T est beaucoup moins sévère et se limite à des lésions prolifératives de l'intestin. Ces souris développent, en effet, des tumeurs épithéliales à de multiples endroits du tractus gastro-intestinal, sans doute en lien avec un défaut de la réponse Th17 (Hahn et al., 2011). Même si d'autres co-Smad, comme TIF1-γ, peuvent se substituer à smad4 pour activer certaines voies du TGF-β, ces données supposent que les voies non canoniques ont peut être une place importante dans les effets induits par le TGF-β dans les cellules T, et en particulier dans le contrôle de mTOR.

Un autre lien entre la voie TGF-β et la voie mTOR pourrait dépendre de FKBP12. TGFbRI fixe FKBP12 de façon constitutive (Wang and Donahoe, 2004), qui est libérée après l'activation de la voie du TGF-β. La rapamycine, qui se lie avec haute affinité à FKBP12 (Sehgal, 2003), est un inhibiteur spécifique de mTOR, mais les ligands endogènes de FKBP12, s'ils existent, ne sont, en revanche, pas identifiés. Le développement des Treg, qui dépend à la fois du TGF-β et de l'inhibition de mTOR, est fortement perturbé dans les souris déficientes pour FKBP12 (Chiasson et al., 2011). De plus, chez des patients transplantés, le traitement par le FK506, qui se fixe également à FKBP12, diminue le pourcentage de Treg dans le sang (Segundo et al., 2006). Les expériences présentées en résultats complémentaires supportent cette hypothèse. Comme attendu, le prétraitement des cellules NK par le FK506 bloque significativement et parfois totalement l'effet de la rapamycine sur de nombreux marqueurs. Le fait que le FK506 bloque également significativement l'effet du TGF-β sur mTOR suppose qu'au moins en partie, les effets du TGF-β sont médiés par les FKBP dont FKBP12. La confirmation de cette hypothèse impose de vérifier que le TGF-β induise bien une interaction entre mTOR et les FKBP. Un moyen de l'analyser serait de co-immunoprécipiter mTOR et FKBP12 après traitement par le TGF-β. Si le processus d'immuno-précipitation de mTOR est délicat, il peut être facilité par l'utilisation de tags comme la biotine. En ce sens, nous avons démarré la génération de lignées murine dont CD122 est « tagué ». Elles permettront d'immunoprécipiter efficacement les complexes de signalisation dépendants de l'IL-15 et d'analyser plus finement l'effet du TGF-β sur ces complexes.

Si les effets de l'IL-15 et du TGF-β s'opposent dans les cellules NK, nous avons également mis en évidence que l'activation de cellules NK purifiées, en l'absence de TGF-β exogène, induisait la phosphorylation de smad2/3, *in vitro*.

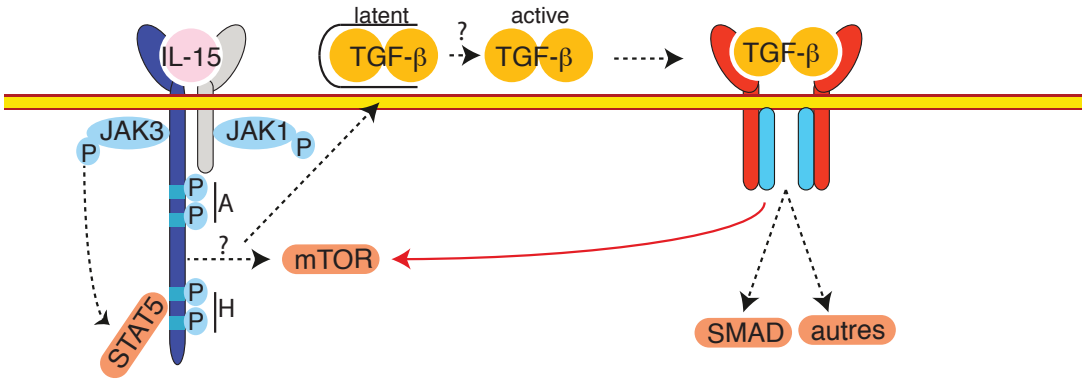


Figure 31: Rétrocontrôle de la voie IL-15 sur la voie du TGF-β *in vitro*
L'activation des cellules NK par l'IL-15 induit la production et l'activation de TGF-β qui, en retour, inhibe l'activation de mTOR induite par l'IL-15.

Ce phénomène, bloqué par l'addition d'anti-TGF-β, suggère que les cellules NK sont capables de produire/libérer du TGF-β actif après une stimulation par de l'IL-15 pour s'autoréguler (Figure 31). Ce mécanisme, qui n'est pas décrit à l'heure actuelle n'a, en revanche, pas été confirmé *in vivo* et plusieurs explications peuvent être avancées. Il est tout d'abord possible que ce phénomène existe *in vivo*, mais que nous ne l'ayons pas détecté du fait d'une cinétique d'apparition différente de celle de la phosphorylation de S6 induite par du poly(I:C). Il est aussi possible que le TGF-β produit par les cellules NK *in vivo* soit capté par d'autres types cellulaires qui expriment plus fortement le récepteur du TGF-β comme les lymphocytes T CD4.
Au cours de ce travail, nous nous sommes concentrés sur l'impact de la modulation de la voie du TGF-β dans les cellules NK. Mais, d'autres populations lymphoïdes innées comme les ILC1 du foie ou les ILC3 NCR^+ de l'intestin sont également impactées par ces modifications génétiques puisqu'elles expriment NKp46. Or, le TGF-β à un rôle majeur dans l'homéostasie de l'intestin. En effet, la délétion de Smad3 ou 4 dans les lymphocytes T favorise l'inflammation intestinale (Kim et al., 2006; Yang et al., 1999). Le TGF-β, qui est riche dans l'intestin, induit fortement la synthèse d'IgA (Cerutti and Rescigno, 2008) qui protègent la muqueuse intestinale en neutralisant les toxines et les pathogènes et en inhibant l'adhésion des bactéries commensales à l'épithélium. L'intestin est également un organe privilégié pour le développement des Treg puisqu'il est riche en TGF-β et en acide rétinoïque qui agissent en synergie pour induire l'expression de Foxp3 et favoriser la maturation des Treg (Coombes et al., 2007; Sun et al., 2007). Le TGF-β favorise également la

différenciation des lymphocytes T CD4 naïfs en cellules Th17. Le TGF-β agit indirectement en inhibant les réponses Th1 et Th2 mais, également de façon directe en induisant l'expression de RORγt (Mangan et al., 2006). Les LTi ainsi que les ILC3 NCR^+ dépendent également de RORγt pour leur développement. De plus, aucune donnée n'existe, à l'heure actuelle, sur l'effet d'une activation constitutive de la voie du TGF-β ou d'une extinction de cette voie dans ces cellules. Comme les ILC3 NCR^+ expriment NKp46, il apparaît intéressant d'étudier leur phénotype dans les souris NK-*tgfbr2*$^{-/-}$ ainsi que dans les souris NK-*tgfbr1*^{CA}. En ce sens, une collaboration avec le laboratoire d'Eric Vivier s'est engagée. Les résultats préliminaires ont montré que ces deux modèles induisent des modifications de la distribution des ILC3 NCR^+ dans ces souris.

Chez l'homme, de nombreuses maladies sont associées à des mutations germinales des composants de la voie de la superfamille du TGF-β. Elles se caractérisent essentiellement par des anomalies anatomiques majeures et font partie des troubles des tissus conjonctifs. Du fait du pléiotropisme du TGF-β, ces maladies associent des malformations cardiovasculaires, des anomalies du tissus conjonctif, des dysplasies musculo-squelettiques, des défauts du système génital, des troubles du développement, des désordres métaboliques ainsi que des prédispositions à certains cancers (Gordon and Blobe, 2008). En revanche, les conséquences sur le système immunitaire sont beaucoup moins connues. Parmi les maladies qui affectent les récepteurs du TGF-β figure le syndrome de Loeys-Dietz. Il associe des défauts du développement des structures crano-faciales, cardiovasculaires et osseuses (Loeys et al., 2005). Récemment, il a été mis en évidence que cette maladie s'accompagnait également de désordres immunologiques de type Th2 (Frischmeyer-Guerrerio et al., 2013). En effet, les patients présentent un nombre élevé de polynucléaires éosinophiles ainsi qu'une augmentation de la concentration des IgE sériques qui favorisent le développement de maladies atopiques comme l'asthme, l'eczéma, la rhinite allergique et les allergies alimentaires. De façon intéressante, si la majorité des mutations décrites chez ces patients sont des mutations faux-sens, les conséquences immunologiques supportent plutôt l'idée d'un excès de signal médié par le TGF-β dans cette maladie. En effet, il est observé une augmentation du nombre de Treg dans le sang, une diminution de l'expression de CCL5 et une augmentation de l'expression de CTLA4 par les Treg, molécules dont l'expression est respectivement diminuée et augmentée par le TGF-β (Dai et al., 2011; Huang et

al., 2010). De plus, les auteurs ont montré que l'expression de psmad2/3 dans les lymphocytes T du sang et dans le thymus était plus importante chez les patients que chez les donneurs sains. Si dans cet article l'impact des mutations des gènes codants les récepteurs du TGF-β n'a pas été évalué sur le phénotype des cellules NK, il paraît intéressant de l'étudier. Au cours de notre travail, nous avons eu accès à du sang d'un patient atteint du syndrome de Loeys-Dietz. Si des anomalies phénotypiques, comme une proportion importante de cellules NK $CD3^{-}CD56^{-}CD16^{+}$, et fonctionnelles ont été observées, nous n'avons pas pu définir le niveau d'expression de psmad2/3 dans les cellules NK. Il nous a donc été impossible d'essayer de comprendre les mécanismes impliqués dans l'apparition de ces défauts. La mise en place technique du marquage psmad2/3, chez l'homme, permettrait de mieux comprendre les mécanismes impliqués dans cette maladie mais également dans d'autres contextes pathologiques comme le cancer, dans lequel le TGF-β joue un rôle clé dans l'échappement du système immunitaire.

En conclusion, nous avons donc mis en évidence un nouveau mécanisme par lequel le TGF-β inhibe les fonctions des cellules NK en s'opposant à l'activation de mTOR induite par l'IL-2/15. Ces effets ont été observés chez la souris, mais également chez l'homme, suggérant que ce mécanisme est conservé au cours de l'évolution. Ces données décrivent un mécanisme moléculaire pour expliquer les effets immunosuppresseurs du TGF-β et font de mTOR un intégrateur clé des signaux cytokiniques dans les cellules NK. Nos données justifient de développer de nouvelles thérapeutiques visant à augmenter l'activité de mTOR dans les lymphocytes infiltrant les tumeurs comme les cellules NK pour restaurer leurs fonctions en contrant les effets du TGF-β. Si des ébauches de mécanisme *via* les protéines FKBP ont été avancées, le mécanisme d'action moléculaire à la base de cette inhibition n'a pas été clairement élucidé et nécessite des études supplémentaires.

ANNEXE

1. **Article 2 : Monitoring NK cell activity in patients with hematological malignancies**

REVIEW

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Monitoring NK cell activity in patients with hematological malignancies

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Natural killer (NK) cells are lymphocytes of the innate immune system that can recognize and kill various types of malignant cells. Monitoring the activity of peripheral NK cells in patients affected by hematological malignancies may provide prognostic information or unveil ongoing tumor-specific immune responses. Moreover, further insights into the biology of NK cells might also promote the development of novel strategies for stimulating their anticancer activity. Here, we review the main methods to monitor phenotypic and functional NK cell properties in cancer patients, focusing on individuals affected by multiple myeloma, a hematological malignancy currently treated with immunomodulatory drugs.

Introduction

The classification of cancers deriving from the hematopoietic system has become increasingly complex with the advent of novel techniques of molecular and cellular biology that can be used to precisely characterize malignant cell clones.[1] Nonetheless, hematological neoplasms can be roughly classified into lymphomas and leukemias. The former are lymphoid tumors initially confined to peripheral lymphoid organs and extranodal tissues, while the latter include both lymphoid and myeloid malignancies that originate in the bone marrow but generally invade the peripheral blood. All hematological cancers are therefore exposed very early during oncogenese and throughout tumor progression to effectors of the immune system. Thus, the immunological microenvironment should be taken into particular consideration to fully understand and treat hematological malignancies.

The term "cancer immunosurveillance" is generally employed to describe the process whereby the immune system eliminates newly formed malignant cells. After an initial debate on the physiological relevance of this progress, it is now widely accepted that the interaction between malignant cells and immune cells is one of the most prominent parameters determining disease outcome in cancer patients. In line with this notion, Hanahan and Weinberg have recently added two novel features that highlight the complex interplay between developing tumors and the immune system to the six hallmarks of malignancy that they had originally proposed in 2000.[2] These novel hallmarks are the ability of neoplastic cells to avoid immune destruction, and the ability of chronic inflammation to promote tumor progression.[3] As a result, pharmaceutical companies are now developing several anticancer drugs that operate via the immune system, both in its innate and adaptive components.

Natural killer (NK) cells are innate lymphocytes recently reclassified as members of the group 1 of innate lymphoid cells (ILC1).[4] NK cells are defined by their capacity to kill target cells upon recognition through a set of activating and inhibitory receptors. In the course of immune responses, NK cells are rapidly activated by monocytes[5] and dendritic cells[6] trans-presenting the immunostimulatory cytokine interleukin-15 (IL-15). This rapid (6–12 h) process primes NK cells to kill their targets mainly through the polarized release of cytotoxic granules that contain the pore-forming factor perforin, granzymes, and several other proteins. NK cells also secrete interferon γ (IFNγ) and other cytokines upon stimulation, in particular when this is mediated by the combination of IL-12 and IL-18.

NK cells play an important role in the early defense against intracellular pathogens.[7] Within lymphoid organs, they are strategically positioned in the proximity of sentinel macrophages that line the lymphatic sinus, where they can efficiently respond to cytokine signals emanated from pathogen-sensing phagocytes by secreting IFNγ.[8] NK cells have been shown to kill not only infected cells, but also malignant cells of various origin, in vitro and in vivo. This latter property underpinned their discovery in the 1970s and drew considerable interest from tumor immunologists. Subsequently, it was found that NK cells are capable to sense the absence of MHC class I molecules on the surface of target cells through inhibitory receptors of the killer cell immunoglobulin-like receptor (KIR) family in humans and Ly49 in mice.[9] Such an absence of MHC class I molecules, which is often referred to as "missing-self," characterize many cancers, in particular of hematological origin, and is thought to originate from a step of T cell-dependent selection. NK cells are also equipped

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with a variety of activating receptors that altogether contribute to their ability to recognize and kill neoplastic cells. The prototypical NK-cell activating receptor is killer cell lectin-like receptor subfamily K, member 1 (KLRK1, best known as NKG2D), which recognizes various proteins expressed on the surface of target cells in response to several forms of cellular stress, including DNA damage, infection and oncogenic stress.[10] In humans, many malignancies of hematopoietic or non-hematopoietic origin (but not healthy tissues) also express natural killer cell cytotoxicity receptor 3 ligand 1 (NCR3LG1, best known as B7-H6) on their surface, which can be recognized by the NK-cell activating receptor natural cytotoxicity receptor 3 (NCR3, also known as NKp30).[11]

Taken together, these observations suggest that NK cells are an important component of the endogenous arsenal of anticancer defenses, especially at early stages of oncogenesis and tumor progression. In this context, NK cells might indeed detect and kill transformed cells, in turn favoring the activation of tumor-associated antigen (TAA)-specific T and B lymphocytes. Later on, NK cells might also play an important effector functions, in particular in the presence of TAA-targeting antibodies. In fact, human NK cells are believed to be among the most prominent executor of antibody-dependent cellular cytotoxicity (ADCC), owing to a robust expression of Fc fragment of IgG, low affinity IIIa, receptor (FCGR3A, also known as CD16a).[12] Nonetheless, NK cells are often insufficient to mediated tumor regression, and a general decrease of NK-cell functions is frequently observed in cancer patients.[13–15] Presumably, this originates from tumor-derived mediators that negatively regulate NK-cell activity, such as transforming growth factor β (TGFβ), or to the intense chemotherapeutic regimens that are often employed in cancer patients.

Here, we review the rationale and the methods for monitoring the function of circulating NK cells in individuals affected by hematopoietic malignancies. In addition, we summarize the results of a series of studies on NK-cell function in multiple myeloma (MM) patients, to illustrate what principles can be learned from such a monitoring and what questions should be addressed in the near future.

Why Monitoring the Activity of NK Cells in Cancer Patients?

NK-cell activity as surrogate marker of general immunological functions

Jérôme Galon's group has defined the immune contexture as the type, functional orientation, density and location of adaptive immune cells within distinct regions of solid neoplasms.[16] They then quantified all these parameters in patients with colon cancer to delineate an "immunoscore" that turned out to have a better prognostic value than the classical scoring system relying on histological and anatomical features of neoplastic lesions.[17] This study highlighted not only the critical importance of immunological functions in tumor rejection but also the value of monitoring the immunological status of individuals affected by cancer. Of note, the immunoscore in its present form is not particularly adapted to tumors of hematological origin, as these grow within lymphoid organs that are naturally infiltrated with immune cells. Moreover, as T cells infiltrating solid tumors are generally believed to be TAA-specific, their level is supposed to reflect the intensity of antitumor immune responses. In the circulation, the situation is completely different, as TAA-specific T cells are diluted within a huge amount of T cells with unrelated specificities. Thus, the assessment of immune responses against hematological cancers must proceed by alternative techniques.

Circulating immune cells have been monitored for a long time for different purposes, as the peripheral blood is rapidly accessible without the need for invasive procedures. Often, the status of circulating immune cells is argued not to reflect that of their tumor-infiltrating counterparts. In the case of hematological malignancies, however, the blood de facto constitutes the tumor site or a site of intense cancer cell transit. Evaluating circulating immune cells is therefore highly relevant for many types of hematological tumors. But, what should be evaluated? How? Why? We believe that monitoring the activity of circulating NK cells is useful for the following reasons. First, this can be performed with simple assays (see below), as most NK cell subsets (in spite of their notable repertoire) can respond to malignant cells (be them nude or coated with antibodies) in a few hours. Second, it may provide indirect insights into the general proficiency of the immune system, as often the two parameters are correlated with each other. For example, in the course of chronic infection[18] or during metastatic tumor dissemination,[19] the activity of NK and T cells often decreases concomitantly, a phenomenon that may reflect a significant degree of interdependence between innate and adaptive components of the immune system. Third, it could give indications on tumor immunogenicity. In fact, the activation of NK cells may represent a hint of ongoing antitumor immune response. By analogy to the immunoscore approach, it would therefore be interesting to consider the prognostic value of the activity of circulating NK cells in patients with hematological malignancies. This parameter has already been shown to influence disease outcome in patients affected by other types of cancer.[20]

Predicting or monitoring the effect of anticancer (immune) therapy

Monitoring the function of circulating NK cells is obviously essential when patients are treated with drugs that are supposed to directly target NK cells. IPH2101 is a human monoclonal antibody specific that blocks the interaction between common KIRs (including KIR2DL1, KIR2DL2, and KIR2DL3) and their ligands, thus potentiating the cytotoxic activity of NK cells against autologous cancer cells in vitro.[21] At least theoretically, IPH2101 might be beneficial for the treatment of various cancers, provided that it really increases the cytotoxic activity of NK cells in patients. Phase I clinical trials involving IPH2101 have already been conducted in patients affected by refractory MM, a setting in which IPH2101 significantly increased the cytotoxic activity of NK against autologous myeloma cells ex vivo.[22]

The efficacy of several new anticancer agents relies (as a whole or in part) on the elicitation of anticancer immune responses. These therapeutic agents include an increasing large panel of tumor-targeting monoclonal antibodies. Among them, rituximab as well as second-generation anti-CD20 antibodies are

now commonly used for the treatment of B-cell malignancies, based on their ability to stimulate ADCC. However, a subset of patients does not respond well to anti-CD20 antibodies, some of them because of a polymorphism in the FCGR3A-coding gene that lowers the affinity of the receptor on NK cells for IgG_1.[23] Monitoring the activity of NK cells against autologous target cells coated with anti-CD20 antibodies prior to use would hence be helpful to identify those patients that have the highest chances to respond to therapy (an example of personalized medicine). A number of strategies are also under investigation to stimulate ADCC in patients receiving tumor-targeting therapeutic antibodies. In this context, monoclonal antibodies are often combined with immunostimulatory cytokines such as IL-2 and granulocyte macrophage colony-stimulating factor (GM-CSF) or immunomodulatory drugs (IMiDs). It will therefore be important to correlate the ability of peripheral NK cells to mediated ADCC with the therapeutic efficacy of these combinatorial regimens.[24]

Along similar lines, many conventional chemotherapeutic agents exert anticancer effects that depend on the immune system, especially when given at "metronomic," non-toxic doses.[25] Monitoring the activity of circulating NK cells in this setting may lead to potentially important discoveries. Vice versa, it will be important to understand which of the immunosuppressants that are currently employed in the clinics inhibit NK-cell function.[26] Indeed, as immunosuppressants are generally given to inhibit adaptive autoimmune responses, they should ideally spare the innate immune system, leaving patients with an important barrier against infections.

Understanding the impact of cancer cells on immunological functions

Why can NK cells function normally in cancer patients or, more often, be significantly impaired in their activity? Obtaining precise insights into the cell-intrinsic and cell-extrinsic circuitries that regulate the function of NK cells in physiological and pathological settings may lead to the development of novel strategies to stimulate for therapeutic purposes. It is therefore essential to monitor the activity of NK cells with appropriate protocols prior to the initiation of in-depth analyses.

Techniques to Monitor the Activity of Circulating NK Cells

Effector functions

For several decades, the activity of NK cells has been measured using the ^{51}Cr release assay. In this assay, target cells (most often the erythroblastic leukemia K562 cells) were loaded with ^{51}Cr and then co-cultured for 4 h with peripheral blood mononuclear cells (PBMCs). Eventually, the release of ^{51}Cr in culture supernatants was quantified as a marker of target cell death. This simple assay has several drawbacks. First, its results are highly dependent on the ratio between effector and target cells. Thus, limited extents of target cell death may originate from a low percentage of NK cells in the PBMC sample as well as from a poor cytotoxic activity of individual NK cells. Second, it does not provide insights into the activity of individual NK cells relative to other parameters, such as cytokine secretion. For these reasons, the activity of NK cells nowadays is preferentially measured by means of the so-called lysosomal-associated membrane protein 1 (LAMP1, best known as CD107a) assay.

The LAMP1 assay measures the release of secretory lysosomes (cytotoxic granules) by NK cells that enter in contact with target cells, a phenomenon commonly referred to as degranulation. The membrane of cytotoxic granules contains proteins, such as LAMP1, that are transiently exposed on the surface of NK cells upon fusion of granules with the plasma membrane. Thus, the staining of NK cells maintained in non-permeabilizing conditions with fluorochrome-labeled LAMP1-specific antibodies coupled to flow cytometry identifies cells that have recently undergone degranulation. Importantly, degranulation has been shown to correlate with the cytotoxic activity of NK cells,[27] and occurs rapidly after their contact with target cells (usually within an hour). The frequency of degranulating NK cells also turned out to be less dependent on the effector to target cell ratio than lysis of target cells (TW et al., unpublished observations). Moreover, the measurement of degranulation by flow cytometry can be combined with the evaluation of cytokine/chemokine expression levels through an intracellular staining with specific antibodies.[28,29] Upon ex vivo stimulation, NK cells mainly secrete IFNγ, tumor necrosis factor α (TNFα), chemokine (C-C motif) ligand 3 (CCL3), CCL4, and CCL5.[30] A low fraction of activated NK cells may also secrete GM-CSF, IL-10, and IL-13.[31] Flow cytometry allows for the precise identification of NK cells expressing one or more cytokine/chemokine(s). Usually, all cytokine-expressing cells also show signs of degranulation. Of note, a wide range of stimuli can be used to stimulate NK cells to degranulate and secrete cytokines/chemokines. For example, transformed cells including K562 cells, which do not express MHC class I molecules on their surface, and lymphoma B cells coated with anti-CD20 antibodies induce robust NK cell responses.

Expression of cell surface receptors and intracellular proteins

Multiparametric flow cytometry now allows for the measurement of up to 12 fluorescent signals in a single tube. Hence, even with limited blood samples, multiple phenotypic and functional parameters of circulating NK cells can be analyzed (**Table 1**). Mean fluorescence intensity is generally used to quantify the level of expression of each receptor. If both the cytofluorometer and the staining procedure are well calibrated, data from patients analyzed on different occasions can be compared with accuracy. This information can relate to:

NK-cell activation

Upon stimulation with cytokines such as IL-2 or IL-15 or interaction with target cells, NK cells express increased levels of CD25, CD69, CD25, NCR2 (also known as NKp44), and granzyme B. Recent reports have also demonstrated that in the course of various infectious diseases, NK cells upregulate expression the expression of KLRC2 killer cell lectin-like receptor subfamily C, member 2 (KLRC2, also known as NKG2C).[32,33]

NK-cell receptors and maturation

NK cells are equipped with a complex set of activating and inhibitory receptors that critically regulate their function in

Table 1. Phenotypic and functional parameters of NK cells that can be assessed by flow cytometry

Parameter	Flow cytometry-compatible marker
Cytotoxicity	CD107a, GZMA, GZMB, PRF1
Cytokine secretion	GM-CSF, IFNγ, IL-10, IL-13, TNFα
Chemokine secretion	CCL3, CCL4, CCL5, XCL1
Activation	CD25, CD69, GZMB, KLRC2, NCR2
Maturation	CD56, CD57, KIR, KLRC1, SELL
Activating receptors	CD16, CD226, CD244, KLRK1, NCRs, KIR-S
Inhibitory receptors	KIR-L, LILRB1

Abbreviations: CCL, chemokine (C-C motif) ligand; GM-CSF, granulocyte macrophage colony-stimulating factor; GZM, granzyme; IFNγ, interferon γ; IL, interleukin; KIR, killer cell immunoglobulin-like receptor; KLRC, killer cell lectin-like receptor subfamily C; KLRK1, killer cell lectin-like receptor subfamily K, member 1; LILRB1, leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1; NCR, natural cytotoxicity triggering receptor; PRF1, perforin 1; SELL, selectin L; TNFα, tumor necrosis factor α; XCL1, chemokine (C motif) ligand 1.

response to putative target cells, including malignant cells. In addition, NK cells express various cytokine and chemokine receptors, which also profoundly influence their activation and effector functions. Tumors often alter the expression of these receptors on the surface of NK cells, a process that may favor the escape of malignant cells from immunosurveillance.[34] Upon development in the bone marrow, NK cells operate a maturation process during which they stop proliferating, acquire effector functions, and express a peculiar profile of receptors (including activatory and inhibitory, as well as cytokine/chemokine receptors) on their surface. Also the maturation of NK cells is often inhibited by neoplasms.[35] The monitoring of this process by flow cytometry has been recently refined on the basis of several markers including selectin L (SELL, also known as CD62L)[36] and killer cell lectin-like receptor subfamily C, member 1 (KLRC1, also known as NKG2A).[37]

Repertoire of NK cells

Multiparametric flow cytometry and epitope-specific antibodies also allow for the study of the NK-cell repertoire. Using a panel of 5 antibodies against inhibitory receptors of the KIR and NKG2D family and a boolean gating strategy, Björkström et al. have recently demonstrated that 27 different NK-cell populations can be identified that express different combinations of the 5 receptors analyzed.[38] How the repertoire of NK cells evolves in the course of tumor progression is currently unknown.

Intracellular mediators

Intracellular staining procedures can be implemented to measure not only the expression level of cytokines and chemokines, but also that of proteins involved in the cytotoxic functions of NK cells, such as granzyme A, granzyme B, and perforin. Recently, several antibodies specific for transcription factors that regulate the activity of NK cells have also been generated. In particular, this is the case of T-box 21 (TBX21, best known as T-bet) and eomesodermin (EOMES), 2 transcription factors that are essential for the development and maturation of murine NK cells. The expression of these transcription factors by cytotoxic lymphocytes is altered in the course of chronic infection,[39] and could therefore be deregulated also in the context of oncogenesis and tumor progression.

Gene expression

Microarrays are valuable tools to measure global gene expression profiles and identify molecular pathways that are altered by tumors. So far, only a few studies have investigated global changes of gene expression in NK cells isolated from cancer patients. One study reported no difference in the gene expression profile of NK cells obtained from healthy individuals and melanoma patients.[40] Conversely, signs of activation were detected in NK cells infiltrating non-small cell lung carcinoma lesions as compared with NK cells located in the healthy lung parenchima of the same patient.[41] Clearly, this type of approach, which is not extensively used yet, will help deciphering the role of NK cells in cancer immunosurveillance and will open avenues to manipulate the antineoplastic functions of these cells.

Monitoring NK Cells in Multiple Myeloma Patients

Phenotype of NK cells in MM patients

MM is a malignant disease of plasma cells that, despite major therapeutic progresses, is incurable. Like many neoplasms, MM is associated with a marked deficiency in general immunological function, which presumably contributes to the escape of cancer cells from immunosurveillance.[42] The role of NK cells in the control of MM has been intensively investigated. A seminal study by Carbone et al. demonstrate that NK cells could kill autologous MM cells in a NKG2D- and NCR-dependent manner.[43] Moreover, MM cell lines derived from early-stage patients were found to express low levels of MHC class I molecules and high levels of the NKG2D ligand MHC class I polypeptide-related sequence A (MICA), while cell lines obtained from late-stage patients displayed the reverse phenotype, suggesting the existence of an NK cell-mediated pressure in the course of MM progression.[43]

Table 2 summarizes the results of several studies[44–52] that monitored the number, phenotype and functional activity of circulating NK cells in patients affected by MM at different stages of the disease, as compared with NK cells from the blood of healthy individuals. These studies suggest that patients with active MM have an increased number of circulating NK cells.[44,46,52] Of note, such an increase is also detectable in patients affected by monoclonal gammopathy of undetermined significance (MGUS), an asymptomatic pre-malignant disorder characterized by the proliferation of a plasma cell clone in the bone marrow that may precede overt myeloma. The expansion of NK cells in MM patients is not associated with their activation, as no increase in CD25, CD69, and HLA-DR levels has been observed in this setting.[45] In line with this notion, two studies reported a decrease in NKG2D expression on the surface of NK cells from MM patients.[49,51] MM progression has been shown to correlate with increased levels of soluble MICA in the circulation, an event that may promote the downregulation of NKG2D on cytotoxic lymphocytes.[49] However, no correlation between the levels of NKG2D on the surface of NK cells from MM patients and the concentration of circulating soluble MICA has been detected thus far.[51]

Table 2. Variations in phenotypic and functional NK-cell parameters as detected in the circulation of multiple myeloma patients

Ref.	NK cell number	Activation status	Activating receptors	Cytotoxicity	Notes
44	Increased			Unchanged	Increased NK also in MGUS
45	Unchanged	Unchanged			
46	Increased			Decreased	Increased NK also in MGUS
47	Increased in BM				Increased NK also in MGUS
48			Decreased CD16 Decreased CD244 Unchanged KLRK1 Unchanged NCR		Trend toward reduced KLRK1 levels in MM
49			Decreased KLRK1		Bortezomib increased MICA levels on MM cells
50				Decreased	Stage-dependent decrease
51			Decreased KLRK1		Not linked with soluble MICA levels
52	Increased also in BM				

Abbreviations: BM, bone marrow; KLRK1, killer cell lectin-like receptor subfamily K, member 1; MICA, HC class I polypeptide-related sequence A; MGUS, monoclonal gammopathy of undetermined significance; MM; multiple myeloma; NCR, natural cytotoxicity triggering receptor; NK, natural killer.

Irrespective of its cause, the downregulation of NKG2D may contribute to the escape of MM from immunosurveillance as well as to downregulation of MICA levels on the surface of MM cells. Presumably, this is not the only strategy whereby MM cells evade recognition by NK cells. Indeed, Fauriat et al. have observed a downregulation of the activating receptors CD244 (also known as 2B4) and CD16a on NK cells from MM patients.[48] Moreover, Benson et al. have reported that NK cells from MM patients express the inhibitory receptor programmed cell death 1 (PDCD1), while normal NK cells do not.[53] A therapeutic antibody that blocks the interaction between PDCD1 and its ligand CD274 (best known as PD-L1), which is expressed by MM cells, has been shown to enhance the activity of NK cells against autologous MM cells, through effects on both NK-cell trafficking and cytotoxicity. Alterations in the phenotype of NK cells commonly observed in MM patients might also be associated with a decrease in cytotoxic activity. This has been demonstrated in 2 studies based on the ^{51}Cr assay,[46,50] one of which even reported a tumor stage-dependent decrease in NK-cell functions.[50] The capacity of NK cells from MM patients to secrete cytokines and chemokines has not yet been addressed.

In summary, while the number of circulating NK cells increases in MM patients, these cells express an altered pattern of activating and inhibitory receptors and exhibit functional defects that impair their ability to control oncogenesis and tumor progression.

Effect of diverse treatments for MM on the phenotype of NK cells

A wide range of anticancer agents have positive effects on the immune system that de facto contribute to their therapeutic efficacy.[25] In particular, several novel treatments against hematological malignancies have an impact on NK cells.[54] In the case of MM, the therapeutic efficacy of IMiDs (such as lenalidomide) is believed to originate, at least in part, from the activation of antitumor immune responses. Thus, the administration of lenalidomide to PBMCs ex vivo activated the cytotoxic activity of NK cells (against both MHC class I-deficient cell lines and antibody-coated targets).[55] Such an activation is rather indirect, as it involves the secretion of IL-2 by $CD4^+$ T cells[56] as well as the production of specific cytokines by dendritic cells.[57] However, a clear effect of lenalidomide on NK cells in vivo remains to be formally demonstrated. In a murine model of severe immunodeficiency (SCID mice), the combination of rituximab and lenalidomide inhibited the growth of mantle cell lymphoma cells,[58] but the nature of the specific immune cell subset controlling tumor growth was not addressed. In fact, in the mouse, myeloid cells preferentially mediate ADCC, not NK cells.[12] The status of NK cells has been monitored in MM patients that underwent allogenic stem cell transplantation followed by several cycles of lenalidomide-based chemotherapy, both before and after each cycle of treatment. A transient slight increase in $NCR2^+$ NK cells[59] as well as a prolonged increase in their cytotoxic activity was observed,[60] suggesting a positive effect of lenalidomide on NK-cell activity. However, these studies fail to clarify whether the increase in NK-cell activity is due the administration of lenalidomide or to the suspension of prophylactic immunosuppressive treatments. As a matter of fact, many immunosuppressants impair the activity of NK cells. For example, dexamethasone has been shown to profoundly inhibit NK-cell function, even when administered in combination with lenalidomide.[61,62]

Other treatments against MM could also indirectly stimulate the activity of MK cells. For example, bortezomib (an inhibitor of the proteasome) has been shown to increase the levels of expression of MICA on the surface of MM cells, thus promoting NK-cell activation.[49]

Conclusions and Future Directions

It has now become clear that immunological functions must be taken into attentive consideration in the context of anticancer therapy. Usually, tumor-specific immune responses are indeed an indicator of good prognosis, and a wide range of conventional chemotherapeutics and novel immunomodulatory drugs can boost such responses. Many different aspects of the immunobiology of NK cells can now be monitored by flow cytometry, including their cytotoxic potential, their ability to secrete cytokines and chemokines, their activation status and their expression levels of activatory and inhibitory receptors. Monitoring phenotypic and functional parameters in circulating NK cells suggested that the activity of these cells is often reduced in patients affected by various neoplasms, including MM. Additional studies are now needed to characterize, with increasing precision, individual NK-cell responses. Notably, it will be interesting to see which cellular responses are mostly affected in the course of tumor progression, how the activity of circulating NK cells correlate with relapse or complications, how such an activity might be improved, and how various anticancer agents currently employed in the clinical routine impact on NK-cell function. The challenge of harnessing immunological monitoring to improve our understanding of antitumor NK cell responses (be them spontaneous or elicited by therapy) includes (1) to select appropriate cohorts of patients and (2) to take into account all the clinical parameters that may influence the correct interpretation of results. Moreover, high-content studies such as those based on microarrays, next-generation sequencing, and mass spectrometry should be performed to elucidate how hematological cancers impair the activity of NK cells and translate these findings into novel anticancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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2. Article 3 : Regulation of mouse NK cell development and function by cytokines

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Regulation of mouse NK cell development and function by cytokines

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Natural Killer (NK) cells are innate lymphocytes with an important role in the early defense against intracellular pathogens and against tumors. Like other immune cells, almost every aspects of their biology are regulated by cytokines. Interleukin (IL)-15 is pivotal for their development, homeostasis, and activation. Moreover, numerous other activating or inhibitory cytokines such as IL-2, IL-4, IL-7, IL-10, IL-12, IL-18, IL-21, Transforming growth factor-β (TGFβ) and type I interferons regulate their activation and their effector functions at different stages of the immune response. In this review we summarize the current understanding on the effect of these different cytokines on NK cell development, homeostasis, and functions during steady-state or upon infection by different pathogens. We try to delineate the cellular sources of these cytokines, the intracellular pathways they trigger and the transcription factors they regulate. We describe the known synergies or antagonisms between different cytokines and highlight outstanding questions in this field of investigation. Finally, we discuss how a better knowledge of cytokine action on NK cells could help improve strategies to manipulate NK cells in different clinical situations.

Keywords: natural killer cells, cytotoxicity, interferons, signal transduction, interleukin-15, interleukin-12, interleukin-18, TGF-beta

Natural killer (NK) cells are Innate Lymphoid Cells (ILC) involved in the immuno-surveillance of cancers and in the early control of infections by intracellular pathogens (1). They can kill cells recognized as targets through a battery of surface receptors (2) and produce large amounts of IFN-γ upon activation (1). Recently, the growing ILC family has been reclassified into three groups according to the pattern of cytokine they secrete. In this classification, NK cells are part of the group 1 ILC subset (3). In mice, NK cells mainly develop in the bone marrow (BM) (4, 5). If the earliest committed NK cell progenitor (pre-pro NK) does not express CD122 (6) which is the β subunit of the IL-2/IL-15 receptor, expression of this molecule is acquired soon after at the NK precursor (NKP) stage (7). The expression of this receptor is thereafter conserved and is a hallmark of the NK cell population. This underlines the fact that the various aspects of NK cell development, homeostasis, and function are conditioned by IL-15. If this cytokine is fundamental, a variety of other cytokines have been shown to influence the behavior of NK cells, alone or in synergy. In this review, we aim to describe the complex interplay between the molecular pathways triggered by these cytokines. We restricted our field of investigation to the direct effects of cytokines on NK cells. We believe that a good understanding of these pathways is essential to the rational design of drugs targeting the various aspects of NK cell functions.

THE γc FAMILY OF CYTOKINES

The central role of cytokines sharing the γc subunit as a co-receptor (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) in the constitution of the NK cell pool was appreciated some 20 years ago. Indeed, X-linked severe combined immunodeficiency patients presenting mutations leading to loss of γc function (8) and mice with targeted γc deletion (9) both presented a quasi absence of NK cells indicating that these cells relied on one or more of these cytokines. Absence of mature NK cells in mice genetically deficient in IL-2/15Rβ chain restricted the list of candidates to IL-2 and IL-15 (10). Finally, genetic ablation of IL-15 (11) or IL-15Rα (12), leading to a very similar phenotype with a near complete absence of mature NK cells, formally demonstrated the paramount importance of this cytokine for the generation of NK cells. In contrast, mice deficient for IL-2, IL-4, and IL-7 had normal NK cell numbers under homeostatic conditions (13). Importantly and despite expression of IL-2/15Rβ, NKPs appeared to be independent of γc signaling since they were present in normal numbers in γc deficient animals, suggesting that IL-15 only becomes important for subsequent maturation steps (13). Other γc cytokines are also involved in NK cell homeostasis and activation as summarized in **Figure 1**.

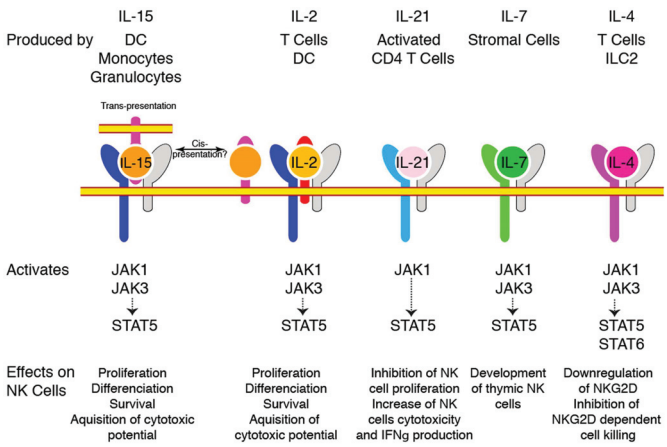

FIGURE 1 | γc Cytokines regulate NK cell homeostasis and function. Different cytokines use γc as a co-receptor to regulate NK cell biology. The cellular source of these cytokines is indicated on the top. The different receptor chains and the major signaling pathways they trigger are shown. The effect of each cytokine on NK cells is also mentioned at the bottom.

IL-15

DISCOVERY/RECEPTORS/TRANS-PRESENTATION

IL-15 was discovered as a result of its "IL-2-like" stimulatory activity since it was able to support proliferation of IL-2 dependent cell lines (14, 15). Structurally, IL-15 is a 14–15 kDa protein presenting sparse sequence similarity (19%) but extensive 3-dimensional analogy with IL-2 and belonging to the same four α-helix bundle cytokine family (15). IL-15 and IL-2 both interact with receptor complexes containing the common gamma chain (γc) (16) and IL-2/15Rβ chain (15, 17). The γc subunit also takes part in the formation of the receptors for IL-4, IL-7, IL-9, and IL-21 (18) whereas IL-2/15Rβ is only used for signaling by IL-2 and IL-15. IL-2 and IL-15 receptors only differ by their α chain, IL-2Rα (CD25) being dedicated to IL-2 and IL-15Rα to IL-15 (19). In contrast to IL-2Rα, IL-15Rα alone displays a high affinity of binding for IL-15 (1.4×10^{-11}M), equivalent to that of the heterotrimeric IL-2R for IL-2 (19). This last property is fundamental to understand the physiology of IL-15. Indeed, this high affinity, coupled to the fact that IL-15 and IL-15Rα are co-expressed by the same cells, allows intracellular binding of IL-15 to IL-15Rα in the endoplasmic reticulum. The complex is then shuttled to the cell membrane and presented to activate neighboring cells expressing IL-2/15Rβ/γc. This mechanism was called trans-presentation (20, 21) and proposed to explain the counter-intuitive fact that expression of IL-15Rα was not needed on responding NK cells to maintain their homeostasis, as would have been expected for a classical cytokine response scheme, but on neighboring cells (22–26). We can hypothesize that such a mechanism allows a very precise delivery of the cytokine stimulus perhaps coupled to other stimulating molecules. As NK cells rely on IL-15 for different purposes, trans-presentation would confine IL-15 signal to selective niches where it would sustain needs of specific NK cell sub-populations. Moreover, systemic availability of this cytokine could be detrimental as exemplified by the development of fatal leukemia in IL-15 transgenic animals (27, 28). The debate whether cis-presentation (i.e., autocrine presentation of IL-15) also occurs was recently revived by a paper showing that following bacterial challenge, NK cells produce and present IL-15 in a time frame and quantities matching DCs and that this cis-presentation was as important as DC trans-presentation to elicit NK cell IFN-γ production (29).

ROLE

The key role of IL-15 in NK cell biology is underlined by the complete absence of these cells in mice deficient in components of the IL-15 signaling axis (11, 12). However, the role of IL-15 in NK cell physiology is not limited to development. Indeed, this cytokine controls as well survival of mature NK cells in the periphery (24, 25, 30, 31), an effect that is probably mediated by up-regulation of anti-apoptotic Bcl2 family members and down regulation of apoptotic ones (31, 32). Moreover, resting NK cells are poor effectors and need to be primed beforehand to express their full effector capacity. This priming step is also controlled by IL-15, presented by dendritic cells, or monocytes (33–38). Mechanistically, IL-15 signals NK cells to constitute stocks of the effector proteins GzmB and Perforin, absent from unprimed NK cells (33). IL-15, synergizing with IL-12, is also mandatory for IFN-γ expression by NK cells (29, 34, 35). Finally, IL-15 controls NK cell homeostatic proliferation (25, 36, 39) as well as proliferation induced following bacterial, viral, or fungal infections (21, 35, 40, 41). How IL-15 can mediate such a wide range of effects, some homeostatic (differentiation, survival), and some context-dependent (priming, IFN-γ secretion) is still unresolved. One possibility would be that

varying IL-15 concentration triggers different responses on NK cells. In line with this idea, decreasing γc expression levels results in reduction of the peripheral NK cell pool (42, 43). This suggests that maximal expression of this receptor and hence maximal signal transduction is necessary for optimal transduction of the IL-15 signal. A recent study tested this model *in vivo* using mouse strains deficient for IL-15Rα or bearing chimeric IL-15Rα either as transgene or knocked in the IL-15Rα locus (44). This strategy allowed the authors to study NK cell populations exposed to five different levels of IL-15 trans-presentation (from null to normal levels). This disclosed the fact that on one hand, constituting a normal peripheral NK cell pool, relying on high proliferation rate in the BM, requires a high level of IL-15 trans-presentation. On the other hand, maturation is much less demanding. The impact of these different levels of IL-15 on the different signaling pathways downstream of the IL-15R has not been analyzed.

REGULATION

How is IL-15 regulated at the basal state remains largely unknown. IRF1, a transcription factor involved in type I IFN (IFN I)-induced IL-15 production, probably plays a role in this process. Indeed, expression of this factor is necessary on hematopoietic as well as non-hematopoietic cells for NK cell generation (45). IL-15 mRNA is expressed *in vivo* by a number of tissues and cell types, from hematopoietic (radiosensitive in chimera experiments) and non-hematopoietic origin (radio-resistant) (24, 46, 47). Chimera experiments have suggested that IL-15 trans-presentation by cells of the hematopoietic system is the most efficient since limiting IL-15Rα expression to the hematopoietic system is sufficient to generate normal NK cell numbers in the BM and only slightly decreased numbers in the periphery (26, 39). In line with its dual function in NK cell homeostasis and activation, IL-15 is expressed at low level under homeostatic conditions in monocytes/macrophages but this expression can be considerably enhanced by several pro-inflammatory agents like LPS (48), poly(I:C), or IFN I (49). More recently, using a transgenic mouse line in which emerald GFP (EmGFP) is expressed under the control of endogenous *Il15* regulatory elements, Lefrançois and collaborators have tracked the cell subsets expressing IL-15 mRNA under homeostatic or inflammatory conditions (50, 51). They confirmed the expression of this cytokine mRNA by a broad distribution of myeloid cells including monocytes, neutrophils, eosinophils, mast cells, and dendritic cells, the strongest expression being observed in basophils. More surprisingly, they described high transcription of IL-15 by Hematopoietic Stem Cells (HSC) and its progressive down regulation during T cell differentiation (51). The significance of this last result awaits further confirmation and functional tests. In addition, IL-15 expression is regulated at several steps including the post-transcriptional level. How much of this regulation is conserved in this reporter remains to be tested. It is however worth noting that these results perfectly correlate with the transcriptomic data available at the Immgen Consortium website (www.immgen.org) for the cell types analyzed (52).

SIGNALING

In terms of signaling, most of our knowledge was generated by studies focused on the IL-2-IL-2 receptor interaction (**Figure 2**).

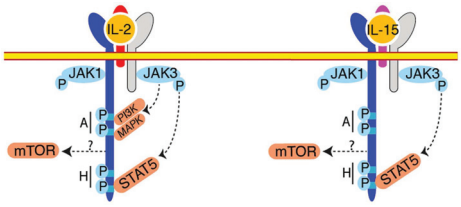

FIGURE 2 | Signaling downstream IL-2/IL-15Rβ. Upon engagement of IL-2 or IL-15 different tyrosine residues are phosphorylated in the IL-2/15Rβ intracytoplasmic regions, across two main regions ("A" and "H"). These phosphorylations lead to the recruitment and downstream phosphorylation of STAT5 by JAK1 and JAK3. The mTOR pathway takes also part to IL-15 signaling but the mode of mTOR activation is currently unknown. Activation of mTOR is essential to increase NK cell metabolism. PI3K and MAPK are also activated upon IL-2 engagement and probably also upon IL-15 engagement but this requires formal demonstration.

Given the shared receptor and the similarity of effect of IL-2 and IL-15 on cultured cells, it was inferred that IL-15 stimulation would lead to activation of the same pathways. And indeed, most of the experiments conducted so far suggested a remarkable conservation. However, these two cytokines are not functionally redundant as exemplified by the divergent immunological outcomes of IL-2 or IL-15 treatment (53). A recent *in vitro* study aiming at understanding these differences evidenced subtle changes in the gene transcription induced in CD8 T cells stimulated with IL-2 or IL-15 (54). This observation opens up the possibility that some differences exist in the signaling pathways downstream of the IL-2 or IL-15 receptors. In this context, the exact contribution of the different signaling pathways during NK cell development and activation is poorly understood. Upon IL-2 binding to its receptor, signaling is triggered by Janus Kinases (Jak) 1 and 3, bound to IL-15Rβ and γc (55–58). These kinases phosphorylate tyrosine residues of IL-15Rβ, which serve as docking sites for phosphotyrosine binding proteins such as the Shc adapter protein, Insulin Receptor Substrate (IRS) proteins, and STAT5a and b transcription factors and lead to the activation of three main transduction pathways: the Jak-STAT pathway, the phosphoinositide 3-kinase (PI3K)/Akt pathway, and the Mitogen Activated Protein Kinase (MAPK) pathway. Given its very proximal role in signal transduction, deficiency in Jak3 results in the absence of NK cells (59). In an attempt to dissect the importance of the different pathways stemming from the IL-2/15Rβ subunit, complementation of *Il2/15rβ*$^{-/-}$ mice with IL-2/15Rβ transgenes deleted for different cytoplasmic domains was undertaken (60). This approach demonstrated the necessity of the membrane distal H-region (**Figure 2**) containing tyrosine 392 and 510 and known to recruit STAT3 and 5 and potentially the p85 subunit of PI3K for the generation of a normal NK cell pool (60, 61). In contrast, the truncated protein generated after deletion of the membrane proximal A-region, known to interact with Lck, the p85 subunit of PI3K and Shc, was able to perfectly complement the IL-2/15Rβ deficient mice and to restore NK cell homeostasis and functional response to IL-15 and

seems thus dispensable. The importance of the STAT5 pathway was confirmed by several studies (62, 63). Indeed, NK cell number was reduced in STAT5b and to a lesser extend in STAT5a deficient animals and this was associated with a decreased response to IL-2 and IL-15 (63). NK cell specific deletion of both STAT5 factors lead to the complete disappearance of this population resulting from survival defects, probably associated with a block of differentiation at the NKP stage (62). In accordance with the putative role of the H-region of IL-2/15Rβ to recruit p85 via phosphorylated Y392 is a series of studies dissecting the role of the PI3K pathway in NK cell development and functions (64–66). Class IA PI3Ks comprise a p110 catalytic subunit associated with a p85, p55, or p50 regulatory subunit. The p110 catalytic subunits are encoded by three genes *Pik3ca*, *Pik3cb*, and *Pik3cd*. Class IB PI3Ks consist of the catalytic subunit p110γ (encoded by the *Pik3cg*) associated with the regulatory subunits p101 or p84. NK cells express all catalytic p110 (66), however only the role of p110γ and δ were examined using mice deficient for these proteins (65, 66) or bearing a catalytically inactive form of p110δ (64). Defect in p110γ or δ signaling lead to a decrease in peripheral NK cell number (64–66), combined defect in p110γ/δ prevented terminal NK cell maturation (66). Moreover, defect in PI3K signaling lead to impaired proliferative (64–66) and cytotoxic (64, 66) responses to IL-2. Importantly, PI3K signaling can be triggered by diverse stimuli including cytokines other than IL-2 or IL-15 (67) chemokines (68) and NK activating receptors (69). The phenotype described in the PI3K deficient NK cells can thus be the consequence of the impairment of responses to other stimuli and not only IL-15. Moreover, direct activation of PI3K by IL-15 was not assessed in these studies neither *in vitro* nor *in vivo* leaving the question of a direct effect unanswered. The generation of phosphatidylinositol triphosphate by PI3K recruits a vast number of targets to the plasma membrane and leads to their activation. However, the downstream targets important for NK cell differentiation and activation have not been investigated. We recently discovered that the kinase mechanistic Target Of Rapamycin (mTOR), which can be activated downstream of PI3K, is a key signaling node activated by IL-15 and responsible for NK cell maturation and activation by pro-inflammatory signals (Marçais et al. manuscript in preparation). mTOR is an evolutionarily conserved serine/threonine kinase integrating various extracellular cues: metabolite and growth factors but also antigenic and inflammatory signals as recently described for T cells (70). mTOR takes part in two complexes: mTORC1 and mTORC2 differing by their constituting members and the targets they phosphorylate. We followed phosphorylation of key mTOR targets by flow cytometry and showed that mTOR activity is developmentally regulated with a progressive shut down upon differentiation and BM egress. In contrast, mTOR activity is strongly induced when NK cells are exposed to pro-inflammatory signals triggered by poly(I:C) injection. Of note, mTOR activation necessitated high IL-15 concentrations; instead, STAT5 phosphorylation was readily triggered by low doses of IL-15. This could provide a first molecular basis to explain the dual effect of IL-15 on NK cells. We also showed that IL-15 controls mTOR activity both *in vitro* and *in vivo*. This is confirmed by the observation that NK cells harvested from mice with NK cell specific mTOR deletion are arrested at the immature $CD11b^{low}$ $CD27^{high}$ stage and their activation in response to poly(I:C) or IL-15 is severely impaired. Interestingly, survival of mTOR deficient NK cells is not affected in accordance with previous studies suggesting that the pro-survival signals given by IL-15 are mediated by STAT5 (62). Preliminary results suggest that only a fraction of mTOR activity is controlled via PI3K. This would fit with the fact that the phenotype of mTOR deficient NK cells is much stronger than PI3K deficient cells. The identity of the relevant mTORC1 or mTORC2 downstream targets remains to be addressed.

Apart from STAT5, the transcription factors, downstream of IL-15 are not characterized. It is worth mentioning the fact that upon MCMV infection, NK cells activate the E2F pathway, a phenomenon that can be blocked using blocking IL-2/15Rβ antibodies (71). As IL-2 has been shown to mediate its proliferative effects through E2F activation (72), we can hypothesize that E2F is also involved in IL-15 induced proliferation. This has however not been formally tested. Moreover IL-15 stimulation leads to NF-κB p65-mediated increase in Myc expression in a context of IL-15 driven leukemia (28).

IL-2

As discussed above, a number of IL-15 effects are recapitulated by *in vitro* treatment with IL-2. In particular, it has long been known that activated cytotoxic NK cells from BM culture can be generated after exposure to IL-2 (73, 74). However, when IL-2 and IL-15 were compared, 10 to 50 times more IL-2 than IL-15 was needed to activate NK cells (37). This is due to the fact that NK cells do not express the high affinity IL-2 receptor due to their lack of IL-2Rα expression at steady-state. Instead, trans-presentation of IL-15 allows sensing of nanomolar quantities by cells expressing only IL-2/15Rβ and γc (54). Whether IL-2 *in vitro* effects are relevant *in vivo* is difficult to evaluate. Indeed, effect on NK cells of mutations affecting IL-2 signaling are difficult to interpret since they result in overt auto-immunity due to the role of IL-2 in the generation and maintenance of Treg cells. To avoid this caveat, mice deficient both for IL-2 and T cells have been generated. NK cell differentiation and numbers are normal in these $Rag^{-/-}$ $Il2^{-/-}$ double deficient mice. IL-2 is thus not needed for maintenance of NK cell homeostasis in the absence of T and B cells (13). This is concordant with the fact that, unlike IL-15, which is produced under homeostatic conditions, IL-2 production mainly results from stimulation of the immune system. This cytokine could nevertheless play a role during NK cell priming following inflammatory challenge. Two studies even suggested that IL-2 could play a non-redundant role in this process (75, 76). Indeed, Granucci et al. described a non-redundant role for IL-2 produced by DCs in the first hours following bacterial challenge (76). In this study, DC-derived IL-2 was important for the induction of IFN-γ secretion by NK cells while induction of cytotoxicity was independent of IL-2. A direct contact between the NK and the DC was needed, suggesting that other molecules were involved. This interaction was functionally relevant for bacterial clearance and anti-tumor response. One caveat of this study is however the use of IL-2 deficient DCs differentiated from BM harvested from IL-2 deficient hosts in which T cell dependent auto-immunity develops. Another study described that, following *Leishmania major* infection, the T cell-derived IL-2 was necessary for the induction

of IFN-γ secretion by NK cells (75). These findings are challenged by the fact that NK cell priming does not happen in the absence of IL-15 (29, 34, 35). A possibility to reconcile these studies would be to imagine cooperation between the two cytokines, both being needed. In this context, cytotoxicity induction would be under IL-15 control specifically since IL-2 only impacts IFN-γ production in these models (75, 76). The fact that 2 cytokines signaling through the same IL-2Rβ/γc-STAT5-mTOR axis lead to such dissociated effects could be due to difference in the spatio-temporal availability as well as the mode of delivery of these cytokines to NK cells. In any case, this issue remains open for further investigations.

Interest for the IL-2 dependent control of NK cells has recently been renewed by a series of studies proposing that NK cell activity was kept in check by Treg cells buffering excess IL-2 produced by activated T cells (77, 78). In these studies, the authors show that systemic Treg cell depletion leads to an increase in CD4 T cell derived IL-2. This increased IL-2 availability was correlated to increased NK cell IFN-γ production (78) and cytotoxicity toward missing-self targets (77) and could be abrogated by blocking IL-2 antibody treatment. Similar findings have been reported upon transfer of *in vitro* pre-activated NK cells in an irradiated host (79). Mechanistically, Gasteiger et al. linked this increased responsiveness to a better capacity to generate conjugates with target cells after even very short-term exposure to IL-2. Interestingly, this increased cytotoxic capacity was only evidenced against missing-self targets, IL-2 being unable to increase cytotoxicity toward cells expressing both inhibitory and activating ligands. The same group also described phenotypical changes of the NK cell population with the progressive emergence of a $CD127^+$ immature NK cell population following Treg depletion (80). A similar population also accumulated in tumor-bearing or chronically infected animals. This population was able to up-regulate IL-2Rα upon low dose IL-12 stimulation confirming previous findings (81). The authors interpreted this result as an increase in the capacity to sense and use IL-2. However, expression of IL-2Rα also renders cells more sensitive to IL-15 (54), the cytokine involved in the homeostasis of this population is thus debatable.

At this stage, we can conclude that DCs and monocytes probably have a prominent role in NK cell activation, through IL-15 trans-presentation. However, this does not exclude the fact that other closely related cytokines like IL-2 and other cell types like T cells or NK cells themselves can contribute and in some conditions replace the IL-15 priming.

IL-21

IL-21R was discovered independently by two groups, it is homolog to IL-2Rβ and its ligand, IL-21, homolog to IL-2, IL-4, and IL-15 with the strongest similarity with the latest (82, 83). Upon IL-21 binding, IL-21R pairs with γc and signals through JAK1 and STAT5 (82). IL-21 is expressed by activated CD4 T cells while IL-21R is found on lymphoid cells (83, 84). Its first effects described on human NK cells were a potentiation of differentiation from BM progenitors and activation of mature NK cells (83). These effects were in accordance with further studies showing that IL-21 inhibits IL-15 effects on NK cell proliferation but potentiates IL-15 driven NK cell terminal differentiation, i.e., cytotoxicity and IFN-γ secretion (84–87). Forced expression of IL-21 *in vivo* by hydrodynamic plasmid delivery decreases, in an NK cell dependent manner, the number of lung metastasis obtained after tumor cell lines injection (85). Given that IL-21 boosts T cell proliferation *in vitro*, it was suggested that its production by activated T cells could help shutdown the NK cell response once adaptive immunity was functional (84). However no *in vivo* data came to confirm this hypothesis. IL-21 produced by CD4 T cells is essential to prevent CD8 T cell exhaustion during chronic viral infections (88–90). In addition to direct positive effects on antiviral T cells, IL-21 restricts virus-driven Treg cell expansion and their suppressive effect on CD8 T cells (91). Whether IL-21 also controls the function of NK cells during chronic infections remains to be formally tested even though *ex vivo* treatment of NK cells from HIV-infected patients with IL-21 improves their effector function (92, 93).

IL-7

IL-7, another member of the γc family of cytokines signaling via STAT5, is well known for its role during early steps of B and T cell development in the BM and thymus respectively (94). The fact that early pre-pro NK cells and immature NK cells express high levels of IL-7Rα (6) is puzzling since NK cell development and acquisition of effector functions is perfectly normal in the absence of IL-7 (13, 95). It should be noted that IL-7Rα is also used in combination with the Cytokine Receptor-like factor 2 to form the thymic stromal lymphopoietin receptor (TSLPR). Whether this cytokine plays a role in early stages of NK cell physiology is unknown. It has however been shown to regulate CD8 T cell viability (96). A peculiar thymic IL-7-dependent NK cell subset has also been described (97). This subset is present in minute amount in mice thymi (between 10,000 and 100,000 cells), is virtually absent in $Il7^{-/-}$ animals, expresses IL-7Rα and depends on GATA-3 in contrast to BM derived NK cells. Functionally, these NK cells are poorly cytolytic but secrete higher amount of cytokines than conventional NK cells. Given the very low abundance of this population, their function has not been investigated.

IL-4

IL-4 is also a member of the γc family of cytokines, well known for its pro-Th2 effects during T cell differentiation. Its absence does not affect NK cell generation and homeostasis (13). However, NK cells express the IL-4 receptor as evidenced by their sensitivity to IL-4 treatment *in vitro* (86). Of note is the strong ability of IL-4 to repress some key NK effector functions, such as cytokine production or cytotoxicity. Indeed, it has been demonstrated that IL-4 suppresses the inflammatory cytokine (IFN-γ, TNFα, and GM-CSF) production-increase that is induced following IL-12 treatment in human NK cells (98). Similarly, in mouse NK cells, IL-4 treatment induces a decrease in the cytokine-induced-cytolytic-activity toward tumor cells or immature DC. The mode of action of IL-4 did not involve a down regulation of perforin or granzyme-B by NK cells but could be mediated through NKG2D down regulation (86, 98). In line with these observations, the capacity of NK cells to shape the adaptive immune response and favor a polarized Th1 response through interactions with DCs is abrogated when NK cells are pretreated with IL-4 (99). IL-4 treated NK cells are unable to induce DC maturation and favor tolerogenic or Th2 responses (99).

As mentioned above, another measurable effect of IL-4 was to down regulate NKG2D and other NK cell markers expression *in vitro* and *in vivo* and as a result to decrease NKG2D dependent cell killing (86). Similarly IL-4 treatment has been shown to down regulate NKG2D and CCL5 expression by memory CD8 T cells (100–102). This is in contrast to the promotion, by IL-4, of innate memory-like CD8 T cells generation that has been recently described (103). In the mouse strain Balb/c, high frequency of IL-4-secreting PLZF$^+$ NKT cells is associated with increased proportion of memory phenotype CD8 T cells compared to C57Bl/6 mice (104). The generation of this memory population has been shown to be dependent on IL-4, as revealed by the lack of memory phenotype CD8 T cells in *Cd1d*$^{-/-}$ and *Il4r*$^{-/-}$ mice. IL-4 produced during the course of a Th2 response is thought to act in a similar way, as it induces a strong proliferation of memory phenotype as well as naive CD8 T cells (105). The stimulation of memory CD8 T cells by IL-4 induces a strong up-regulation of the Eomes transcription factor (104). Given the implication of Eomes in NK cell differentiation, it is tempting to speculate that IL-4 could have far reaching effects on NK cell biology.

IL-12 FAMILY

The IL-12 family of cytokines is constituted by heterodimeric cytokines presenting a four α-helix bundle structure and belonging to the IL-6 super-family (106). The constituting heterodimers of this family are formed by combination of two possible β-chains and three possible α-chains. Indeed, the cytokine β-chains are components of two cytokines (p40 of IL-12 and IL-23 and Ebi3 of IL-27 and IL-35) while α-chains include p35, components of IL-12 and IL-35, p19 component of IL-23, and p28 component of IL-27. On the receptor side, IL-12 is recognized by an IL-12Rβ1/β2 receptor, IL-23 by an IL-12Rβ1/IL-23R heterodimer, IL-27 by an IL-27R/gp130 complex, and IL-35 by a gp130/IL-12Rβ2. They have activating as well as inhibitory roles on the immune system, IL-12 and IL-23 being seen as more pro-inflammatory while IL-27 and IL-35 have been more described as inhibitory. Moreover, given the receptors and ligands promiscuity, some members can compete with others generating a complexity far from being understood.

IL-12

DISCOVERY/RECEPTORS

IL-12 was purified at the end of the 80s from the supernatant of EBV immortalized B cell lines and named NK cell stimulating factor (NKSF) for its ability to induce IFN-γ, cytotoxic activity, and proliferation of NK cells *in vitro* (107). NKSF was later renamed IL-12 and is constituted of two polypeptides: IL-12p40 and p35 covalently linked by disulfide bonds, and binding to a heterodimeric receptor composed of IL-12Rβ1 and β2. Importantly, NK cell constitutively express both chains of the IL-12R (108).

REGULATION/PRODUCTION

IL-12 is produced by several types of Antigen Presenting Cells, including DCs (109, 110) and activated macrophages (48, 111). *In vivo*, IL-12 is produced early after viral infection (112). The inducing signals include pathogen derivative (113), sensed by TLRs and the RIG I pathway, but also molecules expressed by activated T cells like CD40L (109), or the NK cell derived IFN-γ (114–116). The exact mechanism leading to IL-12 production can be more complex as is the case following CpG stimulation. Indeed, upon CpG injection *in vivo*, IL-12 is induced by IL-15 after a cross-talk between conventional DCs and plasmacytoid DCs (117). Interestingly, IL-12 delivery seems to involve the formation of a synapse between the NK cell and the DC involving the polarization of the DC's secretory apparatus toward the NK cell (118).

SIGNALING

Upon binding, IL-12Rβ2 becomes tyrosine phosphorylated and provides docking sites for the kinases tyk2 and Jak2 leading to the phosphorylation and activation of STAT4 (119–121). The importance of tyk2 and STAT4 in NK cells is manifested by the sharp decrease of IFN-γ production in mice deficient for these molecules (40, 121). One report also suggests that some of IL-12 effects are mediated by PKCθ (122), however no further study has confirmed this point.

ROLE

The major role of IL-12 *in vivo* is to induce IFN-γ production, while a marginal effect was described at first on early proliferation and development of cytotoxicity (40, 112, 123). Indeed, IFN-γ production is reduced 20-fold in IL-12p35 deficient mice while cytotoxicity is intact (40), antibody mediated blocking of the cytokine leads to similar results (112). Moreover, microscopy studies have shown a perfect time- and location-dependent correlation between IL-12 production by DCs and IFN-γ production by NK cells in *Listeria* induced granuloma (124). As IFN-γ can itself promote IL-12p40 expression, it generates a positive feedback loop promoting inflammation and the differentiation of monocytes into DCs (114, 124). More recently, it has been shown that IL-12 in combination with another cytokine, IL-18, helps optimal NK cell expansion (125, 126) an effect which is not dependent on IFN-γ secretion. Moreover, in some settings, IL-12/IL-18 can drive an IL-15 independent response of NK cells (127, 128). However these results are controversial, indeed, another study found no or only a very minor role of IL-18 and IL-12 for the promotion of the expansion of Ly49H$^+$ NK cells during MCMV infection *in vivo* (129). Moreover, the settings used in one study are very peculiar since they involve adoptive transfer of WT NK cells in *Il15*$^{-/-}$ × *Il15rα*$^{-/-}$ mice to unmask the IL-12 dependent proliferative effects (128).

At the molecular level, induction of IFN-γ by IL-12 secretion relies on increased transcription as determined by run-on experiments (130). This transcriptional response is abrogated in tyk2 deficient mice (121). IFN-γ mRNA, like many other cytokine messengers, bears AU rich elements in its 3′UTR, which renders it unstable (131) moreover, it is also a target for miRNA mediated regulation (132). Hence, a large part of its expression relies on post-transcriptional regulation. In this context, it has been shown that IL-18 stabilizes the IFN-γ mRNA through the activation of a MAPK p38 dependent pathway (133) (**Figure 3**). This effect explains at least in part the formidable synergy existing between IL-12 and IL-18 on induction of IFN-γ secretion (134). Interestingly, IL-12 in combination with IL-18 can also trigger IFN-γ secretion by memory T cells *in vivo* in an antigen independent fashion underlying acquisition of "innate-like" capacities by these cells (38, 135). Conversely, several groups have been able to induce

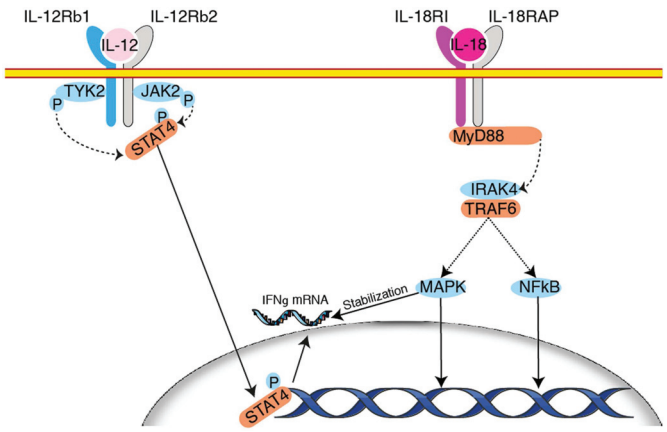

FIGURE 3 | Synergy between IL-12 and IL-18 for the induction of IFN-γ production. Engagement of the heterodimeric IL-12 receptor leads to STAT4 phosphorylation after recruitment of the kinases Tyk2 and JAK2. STAT4 transctivates IFN-γ transcription. Upon binding of IL-18 to its receptors there is activation of the MAPK pathway downstream the adapter MyD88. This leads to the stabilization of IFN-γ mRNA and enhances IFN-γ secretion by NK cells. Other mechanisms may also contribute to the synergy between IL-12 and IL-18.

long-term survival of an NK cell population by transferring NK cells briefly activated *in vitro* in the presence of IL-12 and IL-18 in sublethally irradiated (79) or $Rag^{-/-}$ hosts (136). This echoes the concept of NK cell memory proposed in 2006 by the group of von Andrian (137). Indeed, under particular circumstances, a fraction of the activated NK pool survives the resolution of the response and is able to mount recall responses (126). This property is severely impaired in the absence of IL-12 (126). How exposure to these cytokines imprints long-term survival onto NK cells in these particular settings is not understood and could constitute fertile ground for further discoveries.

As previously mentioned, IL-12, in synergy with IL-18, has the capacity to induce IL-2Rα on NK cells (81, 86), the $CD127^+$ population of NK cells being extremely sensitive to this stimulation (80). This property may help to explain the sustained IL-2-dependent proliferation of IL-15/IL-12/IL-18 pre-activated NK cells after *in vivo* transfer (79). IL-18 is not the only cytokine synergizing with IL-12. Indeed, early studies described a synergy between IL-12 and IL-2 (107). Similarly to IL-18, the synergy between IL-12 and IL-2 also involves regulation of IFN-γ mRNA half-life (130).

IL-18

IL-18 is a cytokine that was originally identified as an IFN-γ inducible factor (IGIF) (138) and it appears to share its biologic functions with IL-12, including enhancement of the NK cell activity (139). Its absence leads to decreased NK cell response in a variety of models (140, 141). It is part of the IL-1 family which comprises 11 members (142). It is produced as an inactive pro-IL-18 precursor. In contrast to other IL-1 family member such as IL-1β, proIL-18 is constitutively expressed (143). ProIL-18 requires cleavage by active caspase-1 in the inflammasome complex to generate biologically active IL-18 (144, 145). Other proteases [caspase-8 (146, 147), proteinase-3 (148), granzyme-B (149)] have been reported to cleave proIL-18 and generate bioactive cytokine. The physiologic producers of IL-18 include myeloid cells such as activated macrophages (138, 150), dendritic cells (151), neutrophils (152), and $Ly6C^+CCR2^+$ inflammatory monocytes (38). In addition, IL-18 is expressed in numerous non-hematopoietic lineages (153). IL-18 mature form is a leaderless protein secreted via a poorly understood mechanism (154). The ability of IL-18 released by macrophages or DCs to activate the production of IFN-γ by NK cells is dependent on cell-to-cell contact (155–159). In agreement with this cell-to-cell contact requirement, IL-18 secretion by DC is polarized and occurs at the immunological synapse formed between the DC and the NK cell (160). Furthermore, another regulatory step may include the presence of a membrane-bound form of IL-18 as an intermediate between the cytosolic pro IL-18 and the mature soluble IL-18 (161). Finally, IL-18 signaling can be antagonized by IL-18BP, which is limiting the systemic effects of this cytokine (162).

Upon IL-18 binding, its primary receptor, IL-18R1, dimerizes with a second receptor subunit: IL-18R accessory protein (IL-18RAP). This recruits myeloid differentiation primary response protein 88 (MyD88) and initiates signaling through IL-1R-associated kinase 4 (IRAK4) and TNFR-associated factor 6 (TRAF6), leading to activation of the NF-κB and MAPK pathways. The importance of IL-18 for NK cells is underlined by the fact that at steady state, NK cells are the only hematopoietic cells analyzed by the Immunological Genome Project to contain consequent levels of transcripts for IL-18R1 and IL-18RAP (www.immgen.org). This expression grants them with exquisite sensitivity to IL-18 stimulation (150). Moreover, NK from IL-18R1-deficient mice

have decreased IFN-γ secretion and cytotoxic capacities (163). A similar phenotype is observed in IRAK4 deficient NK cells (164). Other pathways may also be activated as suggested by impaired IFN-γ secretion in response to IL-12/18 stimulation in p110γ or δ deficient NK cells (65, 66). IL-18 is critical for IFN-γ production by NK cells during numerous bacterial (165), fungal (166), parasites (127), and viral (167) infections. In addition to regulating IFN-γ production, IL-18 takes part to the priming of NK cells (29, 168), and leads to the acquisition of novel migratory function through up-regulation of CCR7 (155, 169). It has also been shown that IL-18 induces the release of CC chemokine Ligand 3 (CCL3) by NK cells, which in turns recruits inflammatory monocytes in the intestine and contribute to local inflammation (170).

The role of IL-18 in regulating the anti-tumoral activity of NK cells is unclear and might be highly dependent of the other signals received by NK cells concomitantly to IL-18. Indeed IL-18 has been shown to promote tumor immuno-suppression and tumor growth by converting Kit^- NK cells into Kit^+ NK cells. NK cells from this Kit^+ subset have the potential to lyse DCs leading to a reduction in the tumor immuno-surveillance (171). In contrast, others studies have demonstrated an anti-tumor effect of IL-18 (172, 173) in part through the generation of "helper" NK cells producing CCL3 and CCL4, which results to the local recruitment of DCs and effector CD8 T cells (174).

As detailed above, the main effect of IL-18 on NK cells is to synergize with IL-12 to induce IFN-γ production. However, in some systems, IL-12 can be dispensable while IL-18 is not (29, 175). Importantly, if systemic IFN-γ production depends on IL-12/IL-18 synergy, local IFN-γ production in the liver can be preserved in the absence of IL-18 and be sufficient to allow host survival upon MCMV infection (167).

TRANSFORMING GROWTH FACTOR-β

PRODUCTION

Transforming growth factor-β is a cytokine with a pivotal role in the regulation of the immune system. TGFβ1 is the predominant TGFβ isoform expressed in the immune system. TGFβ associates non-covalently with the latency-associated protein (LAP), forming a complex called the small latent complex (SLC). The SLC can be secreted as such or in association with latent TGFβ-binding protein (LTBP) as a large latent complex (LLC). TGFβ must be released from the complexes to bind to TGFβ receptors (176). This can be achieved through different mechanisms that remain mostly unclear. TGFβ binding proteins control ligand access but can also act as ligand reservoirs (177). Virtually all cells of the immune system can produce TGFβ. TGFβ is regulated at transcriptional, post-transcriptional, and post-translational levels. It is therefore difficult to precisely determine the sources of active TGFβ during immune responses.

SIGNALING

Transforming growth factor-β mediates its biological functions through binding to type I and II transmembrane serine/threonine kinase receptors. TGFβ1 signals mostly via TGFBR1 (type I receptor) and TGFBR2 (type II receptor). TGFBR1 is not necessary for binding to TGFβ but initiates signaling. Two signaling pathways have been described that are dependent or not on smad transcription factors. Receptor-associated smads (mostly R-smad 2 and 3 in the immune system) are sequestered in the cytoplasm in the absence of signaling. Upon phosphorylation by TGFBR1, R-smad 2 and 3 interact with the common mediator smad-4 and are translocated into the nucleus. Smad complexes recruit other transcription factors to activate or repress the expression of a wide range of genes. Various Smad-independent TGFβ signaling pathways operate in a context-dependent manner and contribute to cell-specific biological responses. TGFβ may thus activate small GTPases, MAP kinases, and the PI3K pathway (177). In T cells, a JNK-c-Jun pathway has been shown to suppress the expression of Eomes in Th17 cells in response to TGFβ (178). In NK cells, smad-independent TGFβ signaling pathways have not been addressed.

IMPACT OF TGFβ ON NK CELLS

The addition of recombinant TGFβ in cultures of mouse spleen cells or human PBMC with IL-2 *in vitro* has long been shown to reduce NK cell proliferation and cytotoxicity (179–182). Administration of TGFβ also depresses NK cell activity in mice (183), and reduces their proliferation during antiviral responses (184) while blocking TGFβ increases NK cell cytotoxic activity (185). It was later found that TGFβ also counteracts IL-12 mediated cytokine production by mouse NK cells (186, 187) and human NK cells (188, 189). TGFβ not only counteracts the effects of IL-2 and IL-12 but also reduces IFN-γ production in response to the engagement of the Fc receptor CD16 on human NK cells (190). Finally, TGFβ also shapes the NK cell surface by reducing the expression of NK cell receptors NKG2D and NKp30 (191, 192) and changes their trafficking properties by modulating the expression of chemokine receptors (193).

When does TGFβ act on NK cells *in vivo*? Early studies show that TGFβ is produced during viral infections, especially by T cells at late stages of infection, which could help limiting NK cell cytotoxicity (194). More recent studies show that the transgenic expression of a dominant negative form of the TGFBR2 receptor in CD11c positive cells (including dendritic cells and NK cells) dramatically increases the number of mature NK cells (195), suggesting that TGFβ negatively regulates NK cell development and maturation at steady-state, especially during infancy (196). The smad-dependent pathway has been shown to be important to limit NK cell IFN-γ production by repressing the expression of T-bet (189). Whether this pathway also limits NK cell proliferation and cytotoxicity induced by pro-inflammatory cytokines remains to be determined. Early studies have suggested that TGFβ acts very rapidly, perhaps in a smad3 independent manner to decrease tyrosine phosphorylation induced by IL-2 (197).

IL-10

IL-10 was described as a Th2 cytokine that inhibited Th1 cytokine synthesis (198). It is now known to be produced by macrophages, DCs, B cells, various subsets of T cells, and NK cells themselves (199–202). NK cells constitutively express both chains of IL-10 receptor (Immgen data). Several diverging effects of IL-10 on NK cells have been described (203–207). Most of these effects seem to be indirect, indeed, IL-10 *in vitro* treatment of purified NK cells does not have noticeable effects (86). However, to the best of our

knowledge, this has not been thoroughly tested using chimeras or transfer of IL-10R deficient or sufficient NK cells in IL-10 sufficient hosts. The experiments of *in vivo* blockade using antibodies being non-informative about the responding cell type (208, 209).

IFN I

IFN-α/β or type I IFN (IFN I) were originally identified as proteins responsible for induction of cellular resistance to viral infections. They are produced by various immune and non-immune cell types. According to a recent study, the capacity of mononuclear phagocytes (i.e., DCs and macrophages) to express IFN I and subsequently IL-15 after microbial challenge could be imprinted by previous contact with the microbial flora (210). However this results still awaits confirmation. IFN I effects on NK cells have been known for a long time. Indeed, IFN I induce NK cell proliferation and cytotoxicity (211). However, IFN I receptor (IFNAR) deficiency can be compensated by recombinant IL-15 injection (40). The bulk of IFN I effects are thus probably mediated through release of IL-15 as recently confirmed by a systems biology analysis of the response to MCMV viral infection (71). In this study, the authors show that IFN-stimulated genes were not strongly up regulated by NK cells, suggesting that, at least at the transcriptional level, these cells were not the primary targets of IFN I. This lower responsiveness was associated with a lower expression of STAT1 by NK cells. IFN I dependent effects on NK cells are however not absent and some studies have evidenced a direct role of IFN I on the induction of NK cell cytotoxicity (212, 213).

CONCLUDING REMARKS

Natural killer cell development and function depend on a multiplicity of cytokines which have complementary as well as overlapping functions. A complete understanding of their action will require the precise identification of the cell types producing them, the time window during which they are produced and the signaling events that their receptors engage in NK cells. These various parameters and the outcome on NK cells could be very different depending on the infectious agent. The role of some cytokines such as IL-2 may thus be important only with particular pathogens and efforts should therefore be made to diversify the models of infection. Moreover, many cytokine effects are only seen when combining them, as best exemplified by IL-12 and IL-18. It is therefore essential to define the relevant cytokine combinations in different niches and to delineate the signaling pathways they induce as well as their combined effects on NK cells. How NK cells integrate signals from activating and inhibitory cytokines and which molecules act as "integrators" are important issues to address. Recent technological advances such as mass cytometry will be instrumental for this purpose in that they allow the simultaneous measurement of up to 100 parameters using very low cell numbers. The latter technique can be applied to the study of cell signaling using phospho-specific antibodies raised against various molecules of the transduction machinery. Recently generated NK-specific Cre-expressing mouse lines will also be important to discriminate between direct vs. indirect effects of various cytokines on *in vivo* NK cell physiology.

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3. Article 4 : T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow

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T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow

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Trail$^+$DX5$^-$Eomes$^-$ natural killer (NK) cells arise in the mouse fetal liver and persist in the adult liver. Their relationships with Trail$^-$DX5$^+$ NK cells remain controversial. We generated a novel Eomes-GFP reporter murine model to address this question. We found that Eomes$^-$ NK cells are not precursors of classical Eomes$^+$ NK cells but rather constitute a distinct lineage of innate lymphoid cells. Eomes$^-$ NK cells are strictly dependent on both T-bet and IL-15, similarly to NKT cells. We observed that, in the liver, expression of T-bet in progenitors represses Eomes expression and the development of Eomes$^+$ NK cells. Reciprocally, the bone marrow (BM) microenvironment restricts T-bet expression in developing NK cells. Ectopic expression of T-bet forces the development of Eomes$^-$ NK cells, demonstrating that repression of T-bet is essential for the development of Eomes$^+$ NK cells. Gene profile analyses show that Eomes$^-$ NK cells share part of their transcriptional program with NKT cells, including genes involved in liver homing and NK cell receptors. Moreover, Eomes$^-$ NK cells produce a broad range of cytokines, including IL-2 and TNF in vitro and in vivo, during immune responses against vaccinia virus. Thus, mutually exclusive expression of T-bet and Eomes drives the development of different NK cell lineages with complementary functions.

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Abbreviations used: ILC, innate lymphoid cell; TF, transcription factor.

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 innate lymphoid cells (ILCs; Spits et al., 2013). They are defined by their perforin-dependent cytotoxic properties that can be enhanced upon activation by IL-15 (Verbist and Klonowski, 2012). Moreover, they 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 reaction (Vivier et al., 2008). This property is shared with other innate lymphocytes such as NKT cells, γδ T cells, and adaptive lymphocytes such as memory CD8 T cells that behave like innate lymphocytes during the first phases of infections (Schoenborn and Wilson, 2007).

C. Daussy, F. Faure, and K. Mayol contributed equally to this paper.

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NK cells develop in the BM from pre-pro NK cells and NK cell precursors (Carotta et al., 2011; Fathman et al., 2011). Acquisition of the NK1.1 epitope marks their commitment to the NK cell lineage. Next, they undergo a sequential maturation program that includes four discrete steps marked by surface levels of CD27 and CD11b. The most immature NK cells do not express CD27 and CD11b and are found mainly in the liver (Chiossone et al., 2009). $CD11b^{-}$ $CD27^{+}$ NK cells express high levels of NKG2A and low levels of Ly49 receptors. They are found mainly in BM and LN. Upon acquisition of CD11b, NK cells massively proliferate in the BM (Kim et al., 2002b). $CD11b^{+}$ $CD27^{+}$ and $CD11b^{+}$ $CD27^{-}$ correspond to mature NK cells mainly found at the periphery, display the full repertoire of Ly49 receptors, and have the highest cytotoxic potential (Hayakawa and Smyth, 2006). KLRG1 expression in $CD11b^{+}$ $CD27^{-}$ NK cells marks cellular senescence (Huntington et al., 2007). At the $CD11b^{+}$ $CD27^{+}$ stage, NK cells acquire high expression of S1PR5 that induces their exit from the BM to the periphery (Walzer et al., 2007b). In parallel, they acquire expression of CX3CR1 (Grégoire et al., 2007) and progressively lose expression of CXCR3 and CXCR4 (Mayol et al., 2011), which have an impact on their trafficking. NK cells can also develop in the thymus (Vosshenrich et al., 2006) and NK cell precursors have been identified in human LNs (Freud et al., 2005), suggesting that NK cells may also develop at the periphery. Whether they develop through the same pathway as BM NK cells remains to be determined.

NK cell development is under the control of several transcription factors (TFs). The sequence of their respective actions is difficult to define as they often cross-regulate each other. E4BP4 (Gascoyne et al., 2009; Kamizono et al., 2009), Runx3 (Cruz-Guilloty et al., 2009; Lai and Mager, 2012), and ETS1 (Ramirez et al., 2012) act very early during NK cell development by inducing the expression of important downstream TFs, such as Id2 (Yokota et al., 1999) and Tox (Aliahmad et al., 2010), that repress many lymphoid genes and are also required for NK cell development. The T-box family TF T-bet and Eomesodermin (Eomes) are both expressed in mature NK cells (Gordon et al., 2012). They are believed to bind to the same DNA sequence but probably have both redundant and specific activities. Intlekofer et al. (2005) showed that mice with compound mutations of the genes encoding the TFs T-bet and Eomes were nearly devoid of several lineages dependent on IL-15, including memory CD8 T cells and mature NK cells, and that their cells had defective cytotoxic effector programming. They further showed that T-bet and Eomes cooperate to induce high expression of CD122, the β chain of IL-15. More recently they showed that Eomes-deficient mice lack all mature NK cells defined by high expression of integrin α2 (recognized by the DX5 antibody), whereas T-bet–deficient mice lack a population of liver NK cells with a $Trail^{+}$ $DX5^{-}$ phenotype (Gordon et al., 2012). $Trail^{+}$ $DX5^{-}$ NK cells had been previously described and shown to originate in the fetal liver and to progressively decrease after birth. They can secrete both IFN-γ and IL-13 but

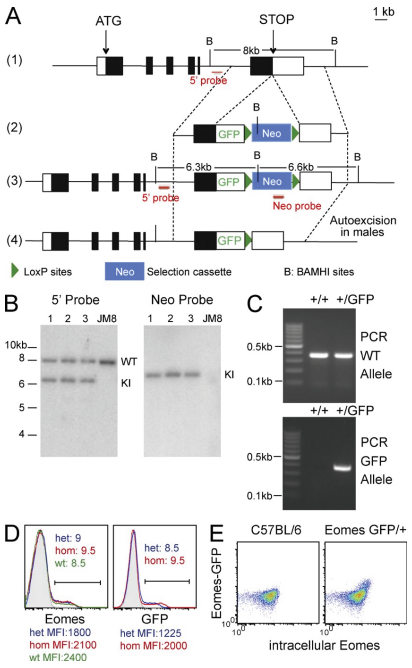

Figure 1. Generation and analysis of Eomes–GFP knockin reporter mice. (A) Outline of the Eomes-locus (1) and of the insertion. A modifying vector (2) was used to insert an ires-GFP cassette, followed by a Neo selection loxP-flanked cassette auto-excisable in males downstream the Eomes STOP codon (3). After Cre-induced recombination, the Neo cassette is removed, leaving only the ires-GFP (4). Different DNA probes (shown in red) were used to check for correct recombination in ES cells by Southern blotting after BAMHI (B) digestion. (B) Southern blots of control and recombined ES cells DNA digested with BAM HI and hybridized with the indicated probes. The bands corresponding to WT and KI Eomes alleles are indicated. (C) Screening of mice bearing the Eomes-GFP allele. PCR of tail DNA with WT or knockin-specific PCR primers is shown. (D) Flow cytometric analysis expression of intracellular Eomes (left) and GFP (right) in spleen lymphocytes of C57BL/6, $Eomes^{GFP/+}$, and $Eomes^{GFP/GFP}$ mice as indicated. Numbers within histograms correspond to percentages of Eomes or GFP-positive cells in the indicated mouse genotypes. Data are representative of 20 mice in 10 experiments. (E) Co-expression of Eomes and GFP in $Eomes^{GFP/+}$ mice. Data are representative of two independent experiments.

are less cytotoxic than $DX5^{+}$ NK cells (Takeda et al., 2005). Their role in immunity is still unclear. Gordon et al. (2012) proposed that $Trail^{+}$ $DX5^{-}$ NK cells correspond to immature NK cells that further differentiate into $Trail^{-}$ $DX5^{+}$ upon acquisition of Eomes expression. In this linear model of differentiation, $Trail^{+}$ $DX5^{-}$ would depend on T-bet for developmental stability. However, several observations argue against this model. First, in the BM—the main site of NK cell development and therefore expected to contain a large pool of

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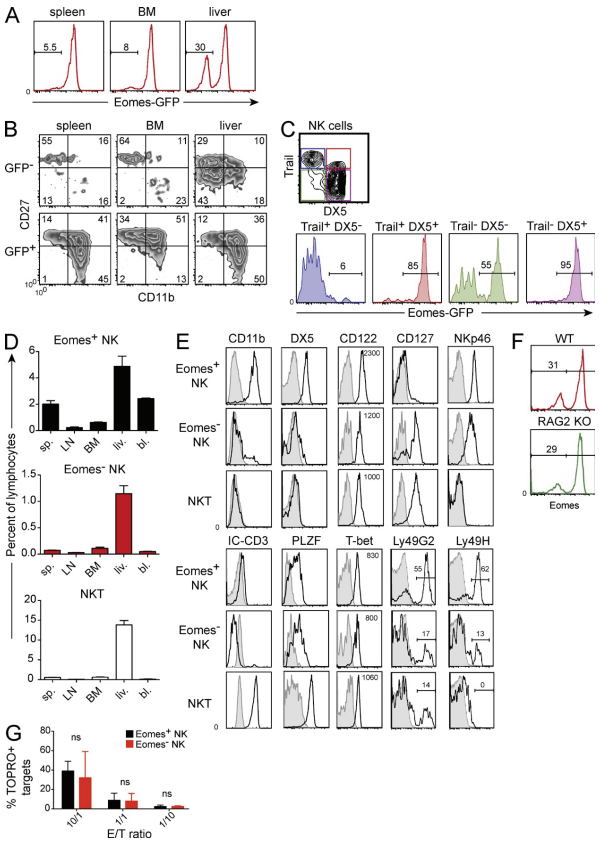

Figure 2. Eomes⁻ NK cells are enriched in the liver and are similar to NKT cells. (A) Flow cytometric analysis of Eomes-GFP expression in NK cells (NK1.1⁺ CD3⁻) isolated from different organs of Eomes$^{GFP/+}$ mice as indicated. Data are representative of 20 mice in 10 experiments. (B) Flow cytometric analysis of CD11b and CD27 expression in gated Eomes-GFP⁻ and Eomes-GFP⁺ NK cells as indicated. Data are representative of 10 mice in 5 experiments. (C) Flow cytometric analysis of Trail, DX5, and GFP expression in NK cells (NK1.1⁺ CD3⁻) of Eomes-GFP mice. Bottom histograms show the frequency of Eomes-GFP⁺ cells (numbers) within the colored gates indicated on the top. Data are representative of 6 mice in 3 experiments. (D) Flow cytometry analysis of Eomes⁻, Eomes⁺, and NKT cells (identified using α-GalCer loaded CD1d tetramers) in different organs of Eomes-GFP⁺ mice. Results show the mean frequency of the indicated cell types ± SD. Two experiments and **n** = 3 mice are shown. (E) Expression of various cell surface or intracellular proteins by gated hepatic Eomes⁻, Eomes⁺, and NKT cells as indicated. Numbers indicate mean fluorescence intensities. Data are representative of 6 mice in 3 experiments. (F) Flow cytometric analysis of intracellular Eomes expression in gated NK cells of WT versus RAG2 KO mice. Data are representative of two experiments with two mice per group in each experiment. (G) Hepatic lymphocytes were isolated from Eomes-GFP mice and cultured overnight with IL-15. The next day, Eomes⁻ and Eomes⁺ NK cells were sorted by flow cytometry and cultured for 4 h with CFSE-labeled YAC1 cells. The percentage of TOPRO3⁺ YAC1 cells is shown. Data are mean ± SD results of 4 mice in 2 experiments.

immature NK cells—there are very few Trail⁺ DX5⁻ Eomes⁻ NK cells. Second, Peng et al. (2013) recently reported that liver DX5⁻ NK cells did not convert into DX5⁺ NK cells upon adoptive transfer. Third, GATA3 has been shown to be important for the occurrence of liver but not BM NK cells, suggesting that liver and BM NK cells derive from different developmental pathways (Samson et al., 2003). The origin, developmental pathway, and functions of Trail⁺ DX5⁻ Eomes⁻ NK cells thus remain unclear.

To clarify these issues, we generated a novel GFP reporter mouse model that allows the identification and tracing of Eomes-expressing cells. We used Eomes-GFP mice to analyze the relationship between Eomes-positive and -negative NK cells. Our results clearly demonstrate that Eomes⁻ NK cells develop in the liver and are not precursors of Eomes⁺ NK cells. Rather, Eomes⁻ NK cells constitute a novel subset of group 1 ILCs that resemble NKT cells in terms of trafficking machinery and cytokine expression, even though they are most closely related to Eomes⁺ NK cells in terms of global transcription. The development of Eomes⁻ NK cells is dependent on T-bet and IL-15. Their development is impaired in the BM because of an active repression of T-bet in the BM microenvironment that allows the development of classical Eomes⁺ NK cells instead. Reciprocally, the liver environment permits early expression of T-bet that represses Eomes and instructs the development of Trail⁺ DX5⁻ Eomes⁻ NK cells. Eomes⁻ NK cells constitutively express mRNA encoding IL-2 and TNF and secrete these cytokines in the liver during antiviral immune responses, whereas Eomes⁺ NK cells only secrete IFN-γ. These data support complementary functions of NK cell subsets during immune responses.

RESULTS

A large fraction of liver NK cells does not express Eomes and displays an immature phenotype

To investigate the dynamics of Eomes expression in the immune system, we generated Eomes-GFP reporter mice by

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inserting an Ires-GFP cassette in the 3′ untranslated region of Eomes (Fig. 1, A–C). Unlike previous models (Arnold et al., 2009), the insertion of the reporter cassette did not alter endogenous Eomes protein expression and regulation, even when both Eomes alleles carried the GFP reporter (Fig. 1 D). Moreover, GFP expression faithfully reproduced the endogenous expression of Eomes (Fig. 1 E). The mean GFP fluorescence intensity was higher in spleen cells carrying two copies of Eomes-GFP alleles than in cells with only one copy, indicating a biallelic expression of Eomes (Fig. 1 D). Eomes-GFP was expressed in different spleen T cell subsets (unpublished data) and in NK cells (Fig. 2 A). The fraction of Eomes-GFP–negative NK cells was variable depending on the anatomical site. The liver contained the highest fraction (□ 20–40%) of Eomes-GFP–negative NK cells, whereas the other organs contained 1–10% Eomes-GFP–negative cells (Fig. 2 A), which was in accordance with previously published results (Gordon et al., 2012). Eomes-GFP–negative NK cells displayed an immature phenotype on the basis of CD11b/CD27 staining (Fig. 2 B). Reciprocally, most CD11b$^-$ CD27$^-$ NK cells were GFP$^-$ in all organs. Previous articles reported the existence of Eomes-negative NK cells with a Trail$^+$ DX5$^-$ phenotype in the liver (Takeda et al., 2005; Gordon et al., 2012). We confirmed that most Eomes-GFP–negative NK cells were Trail$^+$ DX5$^-$ in the liver, but importantly a small fraction of Eomes-GFP$^+$ is also Trail$^+$ DX5$^-$, and conversely a fraction of Eomes-GFP–negative is Trail$^-$ DX5$^+$ (Fig. 2 C). The expression of Eomes is therefore not strictly correlated with that of Trail and DX5.

Eomes$^-$ NK1.1$^+$ CD3$^-$ cells are bona fide NK cells but resemble NKT cells

The localization of Eomes$^-$ NK cells was highly skewed toward the liver (Figs. 2, A and D). This distribution was similar to that of NKT cells and different from that of classical Eomes$^+$ NK cells (Fig. 2 D). To explore further the similarity between NKT cells and Eomes$^-$ NK cells, we compared the expression of various cell surface or intracellular molecules between Eomes$^-$ versus Eomes$^+$ versus NKT cells. The lack of Eomes expression associated with low expression of both CD11b and CD49b (recognized by the DX5 antibody) was a feature of both Eomes$^-$ NK cells and NKT cells (Fig. 2 E). Moreover, NKT cells and Eomes$^-$ NK cells showed a striking similarity in terms of expression of cytokine receptors and receptors of the Ly49 family, with a high expression of CD127 (IL-7 receptor) and a lower expression of CD122 (IL-15 receptor β) and Ly49G2 and Ly49H compared with Eomes$^+$ NK cells (Fig. 2 E). These data led us to hypothesize that Eomes$^-$ NK cells could be NKT cells masquerading as NK cells because of low CD3/TCR expression, a phenomenon which we previously reported for γδ T cells (Stewart et al., 2007). However, a series of evidence argued against this possibility. First, unlike NKT cells, Eomes$^-$ and Eomes$^+$ NK cells expressed the NK cell marker NKp46 (Walzer et al., 2007a). Reciprocally, only NKT cells expressed intracellular CD3 (Fig. 2 E). Second, NKT cells expressed much higher levels of

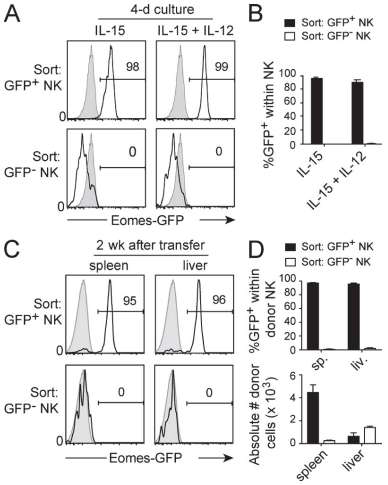

Figure 3. Eomes$^-$ NK cells are not precursors of Eomes$^+$ NK cells. (A and B) Eomes$^-$ and Eomes$^+$ NK cells were sorted from Eomes$^{GFP/+}$ mice as detailed in the Materials and methods. They were cultured in vitro in the indicated conditions and their GFP expression was measured 4 d later. (A) Representative FACS analysis of GFP expression in live 7AAD$^-$ NK cells after culture. (B) Mean ± SD percentage of GFP expression after culture (4 mice in 2 experiments). (C and D) Sorted Eomes-GFP$^-$ and Eomes-GFP$^+$ NK cells were adoptively transferred to congenic CD45.1 C57BL/6 mice. 2 wk later, transferred NK cells were identified as CD45.2$^+$ CD45.1$^-$, they were counted, and their GFP expression was measured in the spleen and liver. (C) Representative FACS analysis of GFP expression after transfer. (D, Top) Mean ± SD percentage of GFP expression in donor NK cells after transfer for 9 mice in 3 experiments. (D, Bottom) Mean ± SD number of recovered cells after transfer for 9 mice in 3 experiments.

PLZF, the master NKT cell TF (Savage et al., 2008; Kovalovsky et al., 2008), than Eomes$^-$ and Eomes$^+$ NK cells (Fig. 2 E). Third, the fraction of Eomes$^-$ cells within the NK cell gate was similar in WT and RAG2$^{-/-}$ mice (Fig. 2 F). Fourthly, both Eomes$^-$ and Eomes$^+$ NK cells were capable of killing YAC1 cells efficiently after overnight stimulation with IL-15 (Fig. 2 G). Thus, Eomes$^-$ NK cells are bona fide NK cells that share several features with NKT cells.

Liver Eomes$^-$ NK cells are not precursors of Eomes$^+$ NK cells under physiological conditions

Previous studies have suggested that Eomes$^-$ NK cells were immature precursors of Eomes$^+$ NK cells on the basis of adoptive transfers of Trail$^+$ DX5$^-$ (Gordon et al., 2012) NK cells or Trail$^+$ (Takeda et al., 2005) NK cells into lymphopenic mice. To address this point more directly, we took advantage of Eomes-GFP reporter mice and FACS-sorted Eomes-GFP$^-$ or Eomes-GFP$^+$ NK cells. We first cultured these cells in vitro for 4 d in the presence of different cytokines. Eomes-GFP$^-$ NK cells survived well but did not up-regulate Eomes expression upon

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culture with IL-15. IL-12 in the presence or absence of IL-18 did not induce Eomes expression by Eomes$^-$ NK cells (Fig. 3, A and B; and unpublished data). Second, we adoptively transferred Eomes-GFP$^+$ and Eomes-GFP$^-$ into un-irradiated syngeneic CD45.1$^+$ host mice. 2–3 wk after transfer, Eomes-GFP$^-$ NK cells were found mainly in the liver, demonstrating the skewed tropism of these cells (Fig. 3 D, bottom). Moreover, no significant up-regulation of GFP was detected, even within cells homing to the spleen (Fig. 3, C and D). This was also true when donor Eomes-GFP$^-$ NK cells were sorted from the spleen or when recipients were irradiated with a sublethal dose (unpublished data). Reciprocally, Eomes-GFP$^+$ NK cells preferentially homed to the spleen and remained GFP$^+$ (Fig. 3, C and D). Altogether, these results suggest that Eomes$^-$ NK cells are not precursors of Eomes$^+$ NK cells in homeostatic conditions and rather correspond to a distinct lineage of ILCs.

Liver Eomes$^-$ NK cells arise in the liver and are dependent on T-bet and IL-15 but not IL-7 and TGF-β for their development

NK cells are thought to develop mainly in the BM in response to IL-15–dependent signals (Colucci et al., 2003). As Eomes$^-$ NK cells are highly enriched in the liver, we sought to determine their site of development. Their high CD127 expression was reminiscent of thymic NK cells (Vosshenrich et al., 2006). However, the normal representation of Eomes$^-$ NK cells in thymectomized mice (Fig. 4 A) excluded a thymic development of these cells. Unlike other NKp46$^+$ innate lymphocytes, CD127 expression was also dispensable for their development (Fig. 4 B). Previous studies have shown that Trail$^+$ DX5$^-$ NK cells are highly enriched in the fetal liver. Accordingly, we found that the livers of newborn mice were highly enriched in Eomes-GFP$^-$ NK cells (Fig. 4 C, mean percentage of Eomes$^-$ NK cells at birth: 90%). In contrast, in the BM and spleen of newborn mice, most NK cells were already Eomes$^+$, which is another supportive piece of evidence of the dichotomy between Eomes$^-$ and Eomes$^+$ NK cell lineages (Fig. 4 C). In the BM, Eomes was not expressed in recently described NK cell progenitors (Fig. 4 D) but was induced upon acquisition of CD27 and NK1.1 (Fig. 2). The frequency of Eomes-GFP$^-$ cells among liver NK cells progressively decreased after birth (Fig. 4 E). Altogether, these data strongly support the existence of a liver NK cell developmental pathway restricting Eomes expression.

The lack of Eomes expression in liver Eomes$^-$ NK cells led us to test the role of T-bet in their development. We found that T-bet$^{-/-}$ mice lacked both NKT cells and Eomes$^-$ NK cells, in accordance with previous observations (Townsend et al., 2004; Gordon et al., 2012), and further showing the similarity between Eomes$^-$ NK cells and NKT cells (Fig. 4 F). NKT cells are dependent on both IL-15 (Ranson et al., 2003) and TGF-β (Doisne et al., 2009) for their development. To test the role of these cytokines in the development of Eomes$^-$ NK cells, we used IL-15$^{-/-}$ mice and CD11c-dnTGFβRII mice. The latter mice express a dominant-negative form of the TGF-β receptor preventing TGF-β signaling in CD11c-positive

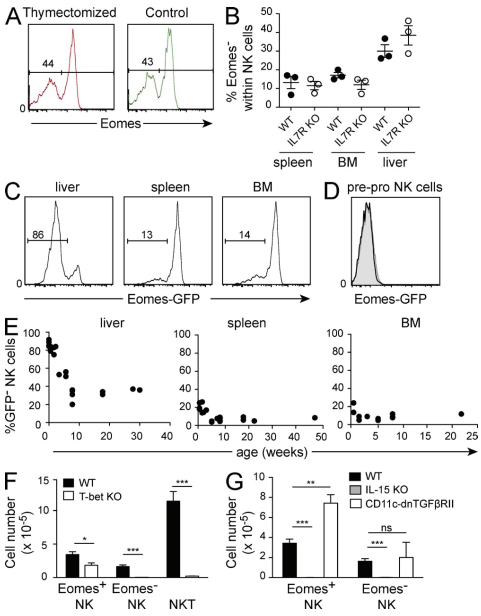

Figure 4. Eomes$^-$ NK cells develop in the liver and are dependent on T-bet and IL-15 but not TGF-β and IL-7. (A) Flow cytometric analysis of intracellular Eomes expression in liver NK cells isolated from control or thymectomized mice. Numbers show the frequency of Eomes$^-$ cells within NK cells. Data are representative of 6 mice in 3 experiments. (B) Mixed WT CD45.1:IL7R$^{-/-}$ CD45.2 BM chimeric mice were generated and analyzed by flow cytometry 6 wk after reconstitution for Eomes expression in liver NK cells originating from each donor marrow. (C) FACS analysis of GFP expression in NK cells from the indicated organ of newborn Eomes-GFP mice. Data are representative of $n = 3$ mice in two experiments. (D) GFP expression was measured by flow cytometry in pre-pro NK cells gated as CD3$^-$CD19$^-$Ly6D$^-$CD11b$^-$NK1.1$^-$CD135$^-$CD122$^-$CD244$^+$CD127$^+$. Data are representative of 6 mice in 3 experiments. (E) Percentage of GFP$^-$ cells in NK cells from BM, spleen, and liver of Eomes-GFP mice of the indicated age as determined by flow cytometry. Each dot represents one mouse. (F and G) Cell number of Eomes$^-$, Eomes$^+$, NK cells, and NKT cells in the liver of the indicated mouse strains. Bar graphs represent the mean ± SD of 3–5 mice in each group in two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, paired Student's t test.

cells (Laouar et al., 2005), including liver Eomes$^-$ and Eomes$^+$ NK cells (unpublished data). The number of liver Eomes$^-$ NK cells was strongly reduced in IL-15$^{-/-}$ but not CD11c-dnTGFβRII mice (Fig. 4 G), showing that Eomes$^-$ NK cells are dependent on IL-15 but not TGF-β for their development. Eomes$^+$ NK cells were also absent from IL-15 KO mice but increased in CD11c-dnTGFβRII mice. Thus, Eomes$^-$ NK cells and NKT cells share the dependence on T-bet and IL-15 for their development but only NKT cells are dependent on TGF-β.

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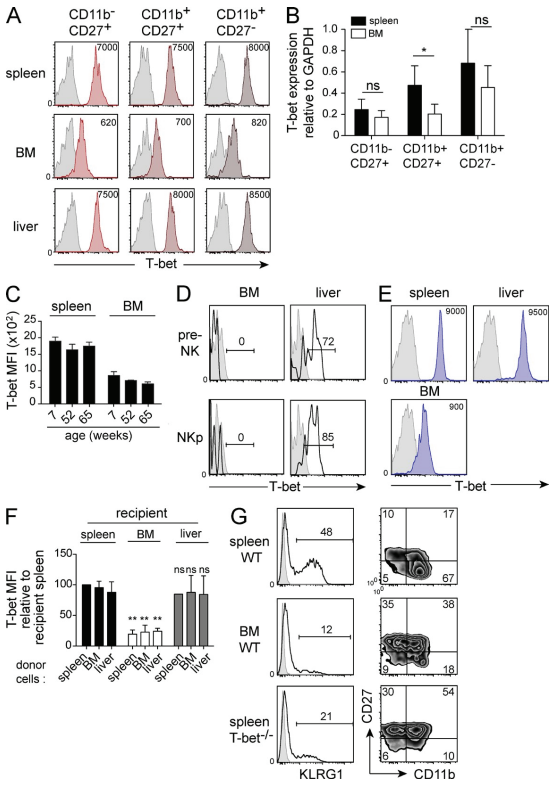

Figure 5. T-bet expression is actively repressed in the BM. (A) Flow cytometric analysis of intracellular T-bet expression in WT NK cells of the indicated subset and isolated from the indicated organs. Numbers indicate mean fluorescence intensity of T-bet staining. Data are representative of 20 mice in 10 experiments. (B) NK cells of the indicated phenotype were sorted from the spleen or the BM and the amount of T-bet mRNA was quantified by quantitative RT-PCR, relative to GAPDH mRNA. Data are mean ± SD of three independent experiments. *, $P < 0.05$, paired Student's *t* test. (C) T-bet expression in spleen or BM NK cells of WT mice of the indicated age. $n = 3$ mice in each group in two independent experiments. (D) Flow cytometric analysis of intracellular T-bet expression in pre-pro NK cells and NK precursors identified as shown in Fig. S1. Data are representative of 6 mice in 3 experiments. (E) FACS measurement of T-bet expression in NK1.1+ T cells isolated from the BM, spleen, or liver as indicated. Numbers indicate T-bet MFI. Data are representative of 10 mice in 5 experiments. (F) Spleen, liver, or BM cells from CD45.1 C57BL/6 mice were adoptively transferred into C57BL/6 mice. 3 d later, transferred cells were identified as CD45.1+ CD45.2− in the spleen, liver, and BM of recipient mice and their intracellular T-bet expression was measured. Results show T-bet MFI relative to recipient spleen NK cells. Bar graphs show mean ± SD of 4 mice in 4 experiments. Statistics were calculated to compare migration to the BM or liver versus migration to the spleen. **, $P < 0.01$, paired Student's *t* test. (G) Flow cytometric analysis of the indicated surface markers on gated NK cells from the spleen or the BM of WT and T-bet KO mice as indicated. Data are representative of 6 mice in 3 experiments.

T-bet expression is repressed in the BM allowing the development of Eomes+ NK cells

Having established the existence of two distinct pathways of NK cell development, we sought to identify the factors that instruct the commitment of precursor cells to each pathway. As T-bet is essential for the Eomes− pathway, we hypothesized that T-bet could be differentially regulated in the BM and liver during early NK cell development. Remarkably, we found that T-bet expression was very low in BM NK cells compared with NK cells from all peripheral sites tested (Fig. 5 A), irrespective of the maturation status (Fig. 5 A). T-bet mRNA levels were similar between BM and spleen NK cells, suggesting that a posttranscriptional mechanism accounts for the difference in T-bet protein levels. The difference in T-bet levels between BM and spleen NK cells was true for different mouse strains (unpublished data) and for mice of different ages (Fig. 5 C). NK cell progenitors identified using the gating strategy shown in Fig. S1 (pre-pro NK cells and NKp) also expressed low levels of T-bet in the BM compared with those found in the liver (Fig. 5 D). Moreover, NK1.1+ T cells present in the BM also expressed lower levels of T-bet compared with those found in the periphery (Fig. 5 E). The low level of T-bet in BM NK cells was not due to an absence of T-bet induction in developing NK cells but rather to an active repression by the BM microenvironment. Indeed, when we adoptively transferred spleen or liver CD45.1+ NK cells into normal CD45.2 recipient mice, the level of T-bet in transferred NK cells that recirculated to the BM rapidly decreased to reach the same levels as that of recipient BM NK cells (Fig. 5 F). Reciprocally, when transferred, BM NK cells up-regulated T-bet expression upon migration to the spleen or to the liver while remaining T-bet^{low} when homing to the BM (Fig. 5 F). Low T-bet expression in BM NK cells correlated with a modified pattern of expression of T-bet target genes. Indeed, WT BM NK cells were very similar to T-bet−/− spleen NK cells in terms of KLRG1, CD11b, and CD27 expression levels (Fig. 5 G).

To test the physiological relevance of BM-induced T-bet repression in NK cell development, we used transgenic mice expressing T-bet under the control of the CD2 promoter (Ishizaki et al., 2007). In these mice, T-bet is overexpressed

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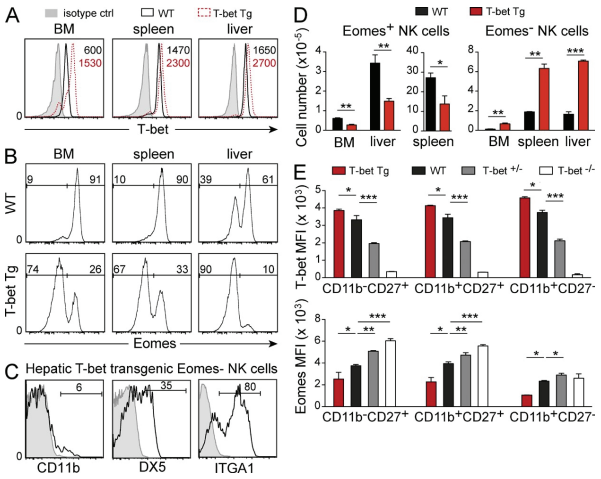

Figure 6. Overexpression of T-bet induces the neo development of Eomes⁻ NK cells at the expense of the Eomes⁺ NK cell population. (A) Flow cytometric analysis of intracellular T-bet expression in WT or T-bet transgenic NK cells compared with isotype control in different organs. Numbers indicate mean fluorescence intensity of T-bet staining. Data are representative of 5 mice per group. (B) Flow cytometric analysis of intracellular Eomes expression in NK cells from the indicated organs of WT and T-bet transgenic mice. Numbers indicate percentages of Eomes⁻ and Eomes⁺ cells among NK cells. Data are representative of 5 mice per group. (C) Expression of CD11b, DX5, and ITGA1 by liver Eomes⁻ NK cells of T-bet transgenic mice. Data are representative of 4 mice in 2 experiments. (D) The number of Eomes⁻ and Eomes⁺ NK cells in different organs of WT and T-bet transgenic mice was determined by flow cytometry. Bar graphs represent mean ± SD. $n = 3$ in each group. (E) Flow cytometric analysis of T-bet (top) and Eomes (bottom) expression in spleen NK cells of the indicated mouse strains. Results show the mean ± SD florescence intensity for at least 3 mice in each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, paired Student's *t* test.

both in NK and T cells in the BM and spleen (Fig. 6 A; Ishizaki et al., 2007). Results presented in Fig. 6 (B–D) showed that ectopic T-bet expression induced the neo development of a large population of Eomes⁻ NK cells in all organs, including the BM. T-bet transgenic Eomes⁻ NK cells expressed low levels of CD11b and DX5 and high levels of ITGA1 (Fig. 6 C), similar to WT Eomes⁻ NK cells (Fig. 2 and Fig. 7). Moreover, the size of the Eomes⁺ NK cell population decreased proportionally (Fig. 6 D). These results demonstrate that early induction of T-bet instructs the development of Eomes⁻ NK cells at the expense of Eomes⁺ NK cells.

One possible mechanism of T-bet action could be the repression of Eomes expression. To test this hypothesis, we compared the level of Eomes in Eomes⁺ NK cells isolated from mice expressing different doses of T-bet: T-bet transgenic, WT, T-bet$^{+/-}$, and T-bet$^{-/-}$ mice. Remarkably, the level of Eomes in Eomes⁺ NK cells was inversely proportional to the level of T-bet, irrespective of the CD11b/CD27 NK cell subset analyzed (Fig. 6 E, compare top and bottom). In particular, T-bet$^{-/-}$ NK cells expressed very high level of Eomes whereas T-bet transgenic NK cells expressed very low levels of Eomes. Altogether, these results show that early induction of T-bet in liver NK cell progenitors instructs the development of Eomes⁻ NK cells via a mechanism that involves the repression of Eomes expression.

Global gene profile analyses reveal that NKT cells are more closely related to Eomes⁻ than to Eomes⁺ NK cells

Having shown the existence of two alternate NK cell developmental pathways, we sought to better understand their respective genetic program. We compared gene expression profile between liver Eomes⁻, Eomes⁺ NK cells, and NKT cells. NKT cells were included in this analysis as we previously found similarities between Eomes⁻ and NKT cells. Globally, Eomes⁻ and Eomes⁺ NK cells were more related to each other than to NKT cells (Fig. 7 A). This segregation is due to the differential expression of T cell–specific (TCR–CD3 complex) transcripts and NK cell–specific (such as NCR1 encoding for NKp46 and KLRA8 encoding for Ly49H) transcripts by NKT and NK cell subsets, respectively (Fig. 7 B, red boxes). However, NKT cells were more closely related to Eomes⁻ than to Eomes⁺ NK cells in terms of global transcription (Euclidean distance 52,3 between NKT and Eomes⁺ vs. 43,2 between NKT and Eomes⁻). When looking at differential gene expression between NKT versus Eomes⁺ NK cells (y axis) and Eomes⁻ versus Eomes⁺ NK cells (x axis), most genes were regulated similarly in Eomes⁻ NK cells and NKT cells (hence found on the diagonal of the figure), confirming the similarity between Eomes⁻ and NKT cells (Fig. 7 B). This included genes down-regulated compared with Eomes⁺ NK cells (Eomes and others) and genes up-regulated compared with Eomes⁺ NK cells (Fig. 7 B, green boxes; and Table S1). When looking at these genes, several interesting observations can be made. First, many of them were involved in cell trafficking. Eomes⁻ NK cells and NKT cells expressed high levels of CXCR6, ITGA1, ITGAV, and ITGB3 but did not express CD62L, CX3CR1, ITGB7, or S1PR5 (Fig. 7, C and D; and Table S1). Expression of CXCR6 and lack of CX3CR1, CD62L, ITGB7, S1PR1, and S1PR5 could explain their lack of recirculation and liver homing. Interestingly, Eomes⁻ NK cells express low levels of KLF2, which is known to induce CD62L, ITGB7, and S1PR1 (Carlson et al., 2006), which are required for lymphocyte recirculation (Arbonés et al., 1994; Wagner et al., 1998; Cyster and Schwab, 2012), suggesting that KLF2 is a target of Eomes. Second, Eomes⁻ NK cells expressed low levels of perforin and granzyme A/B (Table S1 and Fig. 8), but instead high levels of granzyme C and Trail, suggesting that both NK cell subsets kill target cells using

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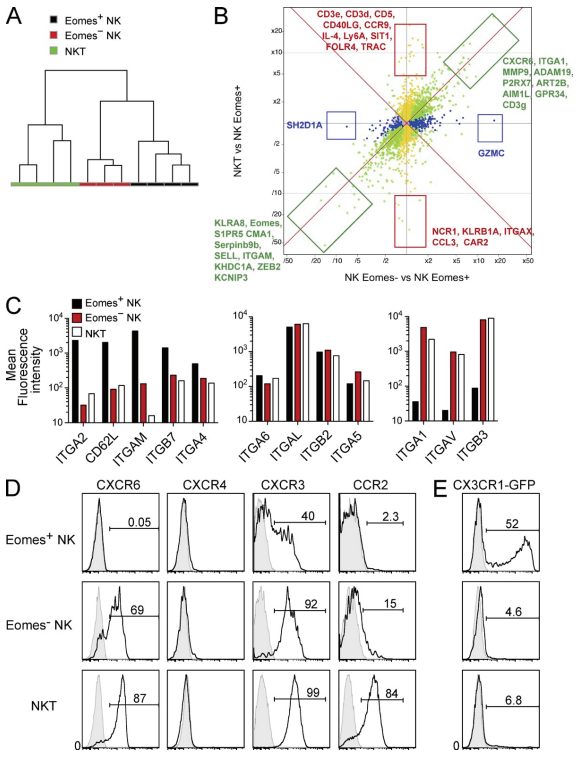

Figure 7. Gene profiling analysis of Eomes⁻ and Eomes⁺ NK cells. (A) Hierarchical clustering with Ward's agglomeration method of microarray data for the indicated populations (three to four samples in each group). (B) Comparison of differential gene expression between NKT versus Eomes⁺ NK cells (y axis) and Eomes⁻ versus Eomes⁺ NK cells (x axis). Orange dots correspond to genes differentially expressed, at a multiple testing adjusted value of 0.05, between NKT and Eomes⁺ NK cells, blue dots to genes differentially expressed between Eomes⁻ and Eomes⁺ NK cells, and green dots to genes differentially expressed in both conditions. The names of some of the most informative genes (boxes) are indicated. (C and D) Flow cytometric analysis of indicated receptors involved in cell trafficking in Eomes⁻, Eomes⁺ NK cells, and NKT cells. In C, mean fluorescence intensities of stainings for each molecule are shown, whereas in D, FACS histograms are displayed. (E) Flow cytometry analysis of GFP expression in NK and NKT cell subsets from CX3CR1-GFP reporter mice. Data are representative of 6 mice in 3 experiments.

different mechanisms. Eomes⁻ and Eomes⁺ NK cells could also recognize different types of target cells as they are clearly equipped with different sets of NK cell and other surface receptors (Table S1 and Fig. 8). Third, Eomes⁻ NK cells expressed constitutive levels of mRNA for different cytokines, including TNF and IL-2, and cytokine receptors (IL-7R, IL-17RD, IL-21R, and TGFBR), suggesting that they could be important in immune regulation. Fourthly, several genes involved in the Notch signaling pathway were enriched in Eomes⁻ NK cells (Table S1), suggesting that Notch could be important for their development as is the case for NKp46⁺ RORγt⁺ ILCs (Rankin et al., 2013). However, unlike the latter cells, Eomes⁻ NK cells do not express RORγt or constitutive levels of IL-22 (Table S1 and Fig. 8).

Eomes⁻ NK cells secrete a broad array of cytokines and contribute to immune responses against intracellular bacteria

A previous study showed that Trail⁺ DX5⁻ NK cells secreted large amounts of IFN-γ and low amounts of IL-13 in response to different stimuli (Takeda et al., 2005). Based on the microarray results, we wanted to revisit this point and assessed the capacity of liver Eomes⁻ and Eomes⁺ NK cells to produce different cytokines at the single cell level in response to in vitro or in vivo stimuli. Upon stimulation with PMA/ionomycin, Eomes⁻ NK cells secreted a broad pattern of cytokines and chemokines including IL-2, IL-4, GM-CSF, TNF, CCL3, and IFN-γ (Fig. 9 A). IL-4 intracellular staining was weak but specific as shown using IL-4$^{-/-}$ NK cells (unpublished data). Their secretion of IL-2 that correlated with their constitutive expression of IL2 mRNA was particularly striking, even higher than that of NKT cells. Eomes⁻ NK cells did not express IL-17 or IL-10 (unpublished data). The cytokine secretion pattern of Eomes⁻ NK cells was very similar to that of NKT cells but in sharp contrast to that of Eomes⁺ NK cells which mainly secrete IFN-γ and CCL3. We then stimulated hepatic lymphocytes with cross-linking antibodies directed against NKp46, NK1.1, and NKG2D or with IL-12 combined with IL-18. Again, we found that Eomes⁻ NK cells secreted a broader pattern of cytokines than Eomes⁺ NK cells. In particular, they secreted TNF in response to activation via NKp46, NK1.1, or NKG2D (Fig. 9 B). Next, we treated mice with poly(I:C), a well-known activator of NK cells, or with α-GalCer, a glycolipid which strongly activates invariant

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NKT cells, and cytokine secretion was measured ex vivo. In these conditions, only IFN-γ was detected. Using both stimuli, Eomes$^-$ NK cells expressed more IFN-γ than Eomes$^+$ NK cells (Fig. 9 C). In particular, α-GalCer injection induced a coordinated secretion of IFN-γ by NKT cells and Eomes$^-$ NK cells, suggesting that Eomes$^-$ NK cells and NKT cells are located in the same hepatic niches. Finally, we assessed the cytokine response of hepatic lymphocytes after mouse infection with *Francisella tularensis*, a highly pathogenic intracellular bacterium, or with Vaccinia virus (VV). 48 h after infection with *F. tularensis*, hepatic NKT cells, Eomes$^-$ NK cells, and Eomes$^+$ NK cells were all activated and produced IFN-γ (Fig. 9 D). Upon VV infection, hepatic lymphocytes did not secrete much IFN-γ. Instead, Eomes$^-$ NK cells and NKT cells, but not Eomes$^+$ NK cells, secreted IL-2 and TNF early after infection, whereas Eomes$^+$ NK cells secreted CCL3. Thus, Eomes$^-$ NK cells contribute to the immune response against intracellular pathogens, such as *F. tularensis* or VV, by secreting a broad range of cytokines complementary to those produced by Eomes$^+$ NK cells in the case of VV infection.

DISCUSSION

Research in the past few years has allowed the identification of several innate lymphoid subsets. They have been classified in three groups depending on the type of cytokines they produce (Spits et al., 2013). Group 1 ILCs express TH1 type cytokines such as IFN-γ and TNF. They include NK cells and ILC1, which are present in inflamed mucosal tissue (Vonarbourg et al., 2010) and differentiate from NCR$^+$ ILC3s (Rankin et al., 2013) under the influence of IL-12 and IL-15. The data we present here classify liver Eomes$^-$ NK cells as a novel subset of T-bet/IL-15–dependent group 1 ILC. These cells presumably derive from Id2$^+$ common ILC precursors. They differentiate mostly in the liver and perhaps also in other organs. Our results show that all peripheral NK cells and precursors express high levels of T-bet, suggesting that Eomes$^+$ NK cells only develop in the medullary environment whereas all other organs may rather drive the development of Eomes$^-$ NK cells.

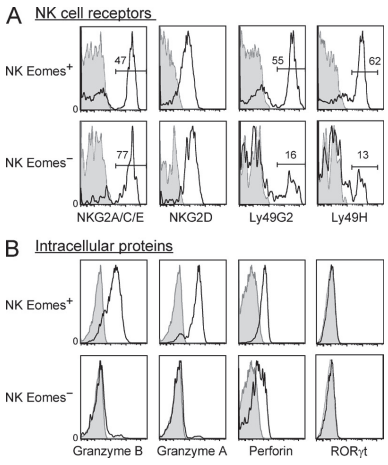

Figure 8. Expression of NK cell receptors and intracellular proteins by Eomes$^-$ versus Eomes$^+$ NK cells. Flow cytometric analysis of surface NKG2A/C/E, NKG2D, Ly49C/I, Ly49G2, and Ly49H (A) and intracellular perforin, granzyme A and B, and RORγt expression (B) in spleen NK cells of the indicated subsets. Data are representative of 6 mice in 3 experiments.

Using a novel model of Eomes-GFP reporter mice, we found that Eomes$^-$ NK cells did not give rise to Eomes$^+$ NK cells upon transfer to normal or irradiated recipient mice. This result strongly supports the existence of two alternative NK cell developmental pathways that segregate in time and space. Eomes$^-$ NK cells appear earlier than Eomes$^+$ NK cells, during fetal liver hematopoiesis, whereas Eomes$^+$ NK cells constitute the main medullary subset, arising directly after birth and rapidly populating the periphery. Our results are in apparent contradiction with a previous study which showed that upon adoptive transfer into RAG$^{-/-}$ γc$^{-/-}$ immunoincompetent mice, Trail$^+$ NK cells gave rise to Trail$^-$ DX5$^+$ Eomes$^+$ NK cells (Gordon et al., 2012). One possible explanation for this discrepancy could be a minute contamination of the Trail$^+$ population sorted by Gordon et al. (2012) with a few Eomes-positive cells. As Eomes$^+$ NK cells express higher levels of CD122 and proliferate more than Eomes$^-$ NK cells (unpublished data), they would rapidly outnumber Eomes$^-$ NK cells in recipient mice. This emphasizes the fact that Eomes expression does not strictly correlate with the expression of any surface marker and underlies the superiority of the Eomes–GFP reporter system to tackle this question. How to discriminate between live Eomes$^-$ and Eomes$^+$ NK cells in the absence of GFP reporter or intracellular staining for Eomes? Our microarray analysis pointed to ITGA1 as a potential marker of Eomes$^-$ NK cells, confirming a previous study (Peng et al., 2013). This is indeed the case in the liver, but not all Eomes$^-$ NK cells are ITGA1$^+$ in the spleen and vice versa (unpublished data). Thus, only an intracellular staining for Eomes unambiguously discriminates between Eomes$^-$ and Eomes$^+$ NK cells in all organs. The absence of a reliable marker for both NK cell subsets may be due to a redundant action of Eomes and T-bet on the expression of many of the surface markers commonly used to study NK cells. However, Eomes and T-bet may also cooperate to increase the likelihood of expression of other markers such as CD11b and DX5. Hence, we propose a model (Fig. 9 E) in which, depending on early expression of T-bet, Id2$^+$ precursors may give rise either to Eomes$^-$ NK cells with a CD11b$^-$ CD27$^{-/low}$ Trail$^{+/-}$ DX5$^{-/low}$ phenotype or to Eomes$^+$ NK cells. Eomes$^+$ NK cells probably start their maturation process directly as CD11b$^-$ CD27$^+$ even though a small fraction of them may go through an earlier CD11b$^-$ CD27$^-$ stage (Chiossone et al., 2009). They subsequently acquire CD11b expression before terminal maturation. T-bet expression, which is not influenced by the level of Eomes, is acquired upon migration of Eomes$^+$ NK cells to

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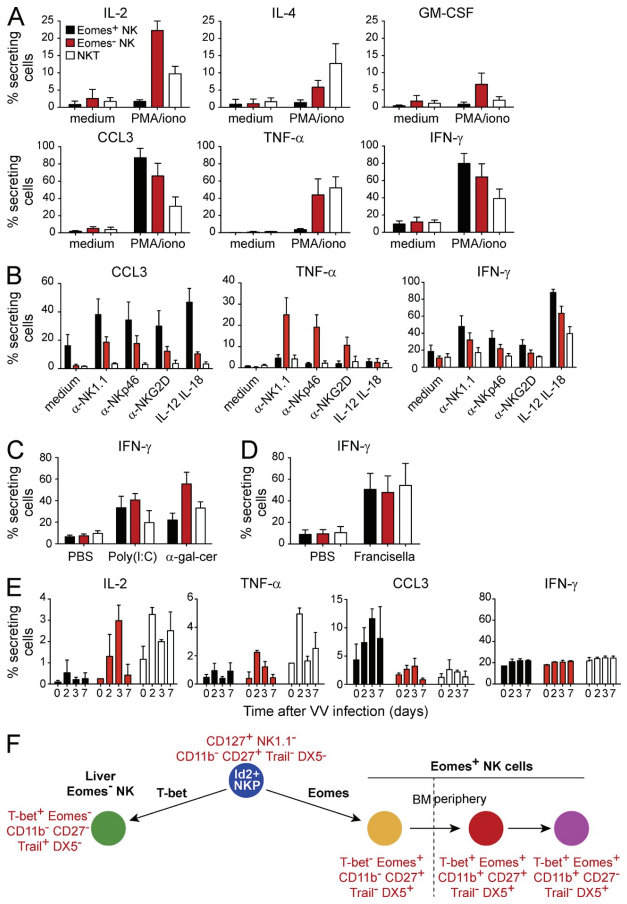

Figure 9. Eomes⁻ NK cells secrete a broad range of cytokines upon in vivo or ex vivo stimulation. (A) Hepatic lymphocytes from WT mice were cultured in medium supplemented or not with PMA and ionomycin for 6 h in the presence of Golgi traffic inhibitors. Expression of the indicated cytokines by gated Eomes⁻ NK cells, Eomes⁺ NK cells, and NKT cells was then measured by flow cytometry after intracellular stainings. Results show the mean ± SD percentage of cytokine-expressing cells calculated from $n = 6$ mice in three experiments. (B) Cytokine secretion was measured as in A, but cells were cultured with IL-12 + IL-18, cross-linking anti-NKp46, anti-NK1.1, or anti-NKG2D antibody. $n = 4$ mice in two experiments. Bar graphs show the mean ± SD. (C–E) WT mice were injected with PBS, poly(I:C), α-GalCer, *F. tularensis*, or Vaccinia virus as indicated. 3 h later (C), 48 h later (D), or at different time points as indicated (E), hepatic lymphocytes were cultured in medium for 6 h in the presence of Golgi traffic inhibitors and cytokine secretion was measured after intracellular staining. Data show the mean ± SD of two independent experiments with at least 4 mice per group. (F) Model of development and maturation of NK cells. In this model, Id2⁺ pre-pro NK cells differentiate into T-bet⁺ Eomes⁻ NK cells in the liver and T-bet⁻ Eomes⁺ in the BM. T-bet expression is repressed in the BM by factors that remain to be identified and this phenomenon allows Eomes expression. Once in the periphery, Eomes⁺ NK cells acquire expression of T-bet, which induces their terminal maturation. Liver-derived NK cells express high levels of Trail and CD127, and low levels of DX5, CD11b, and CD27. BM-derived NK cells express high levels of CD27 and CD127 at early steps of development and progressively acquire CD11b before losing CD27 expression.

the periphery and is involved in the completion of their differentiation process (Townsend et al., 2004; Soderquest et al., 2011). Importantly, our model of NK cell development does not exclude the possibility that Eomes⁻ NK cells may give rise to Eomes⁺ NK cells under certain conditions. Indeed, the plasticity of ILC subsets has already been appreciated (Rankin et al., 2013), and there may be special microenvironments that could induce the conversion of Eomes⁻ NK cells to Eomes⁺ NK cells. For example, IL-4 has been shown to strongly induce Eomes expression in CD8 T cells and could therefore induce Eomes expression in Eomes⁻ NK cells (Weinreich et al., 2010). A highly lymphopenic environment (e.g., RAG$^{-/-}$ γc$^{-/-}$ mice) could also artificially induce this conversion.

We found that T-bet was essential for the development of Eomes⁻ NK cells, confirming previous findings by Gordon et al. (2012). T-bet is therefore a master of development for various ILCs including gut ILC1, ILC3 (Powell et al., 2012; Sciumé et al., 2012; Klose et al., 2013; Rankin et al., 2013), and now liver Eomes⁻ NK cells. T-bet is also required for the development of NKT cells (Townsend et al., 2004). T-bet probably deeply imprints the genetic program of these various lymphoid subsets as illustrated by the important similarity between NKT cells and Eomes⁻ NK cells in terms of global gene expression. ChIP-Seq experiments will be required to understand which genes are directly regulated by T-bet or indirectly by other TFs modulated by T-bet. A previous study in CD4 T cells identified that T-bet induced a modification of the chromatin at the Eomes locus (Zhu et al., 2012). This correlates with our finding that T-bet expression inversely correlated with that of Eomes. Thus, early T-bet expression in liver

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NK cell progenitors could suppress Eomes expression and drive the differentiation of Eomes$^-$ NK cells. Reciprocally, low T-bet expression in BM NK cells is probably not sufficient to repress Eomes and therefore allows the development of Eomes$^+$ NK cells. Importantly, once Eomes is expressed, T-bet can probably no longer repress its expression. This may explain why mature peripheral NK cells that express high levels of T-bet express levels of Eomes similar to those expressed by BM NK cells.

The factors that repress T-bet expression in developing or recirculating NK cells in the BM remain to be determined. Our unpublished data exclude a role for major immune regulators such as IL-10, TGF-β, MHC-I, IL-4, MyD88, or type I IFN in this phenomenon. Similarly, the factors that induce T-bet at the periphery are unknown. Our microarray analysis showed that several members of the Notch signaling pathways were more expressed in Eomes$^-$ NK cells compared with Eomes$^+$ NK cells. It is therefore tempting to speculate that Notch could induce T-bet expression in peripheral NK cells, as previously suggested for NCR$^+$ ILC3s (Rankin et al., 2013). Other good candidates include IL-12 and IFN-γ that are known to induce T-bet expression in T cells (Lazarevic and Glimcher, 2011).

Eomes$^-$ and Eomes$^+$ NK cells display important differences in terms of gene expression. As Eomes$^+$ NK cells express both Eomes and T-bet whereas Eomes$^-$ NK cells only express T-bet, this may reflect a nonredundant activity of Eomes compared with T-bet. In fact, mouse T-bet and Eomes have very similar DNA binding domains (90% identical). However, the other protein domains are totally different, which may explain the different functions of these TFs. In particular, they may recruit different co-activators or co-repressors of transcription. Eomes may be uniquely capable to induce the expression of Ly49 receptors and may also cooperate with T-bet to induce expression of perforin and IL2Rβ in NK cells. In contrast, Eomes could also repress a series of T cell genes, such as those encoding for IL7R or IL-2, and others such as Trail and ITGA1. Eomes$^-$ and Eomes$^+$ NK cells may therefore have different and perhaps complementary functions during immune responses. Eomes$^-$ NK cells express high levels of CXCR3 and CXCR6 similarly to NKT cells (Kim et al., 2002a). These receptors are involved in the trafficking/homing of NK and NKT cells in the liver (Wald et al., 2006; Germanov et al., 2008). A previous study established that liver NK and NKT cells did not recirculate outside the liver (Thomas et al., 2011), which was later confirmed (Peng et al., 2013). Eomes$^-$ NK cells may therefore be important for liver immune responses, especially in newborn mice. Our results show that they can secrete a wide variety of cytokines, including IL-2, TNF, GM-CSF, IFN-γ, and CCL3, whereas conventional NK cells only secrete IFN-γ and CCL3. This is not only true in vitro in response to different stimuli but also ex vivo after in vivo infection with VV. The receptors that trigger activation of Eomes$^-$ NK cells remain to be identified, but our microarray analysis showed that Eomes$^-$ NK cells express high levels of DNAM1 (CD226) and CD160, which are known co-stimulators of NK cells during antiviral responses. Cytokine secretion by Eomes$^-$ NK cells may be important to amplify local immune responses and for the cross talk with other liver lymphocytes. In fact, we found that NKT cell activation with α-GalCer–induced Eomes$^+$ NK cells, but especially Eomes$^-$ NK cells, to produce IFN-γ, suggesting that Eomes$^-$ NK cells and NKT cells are located in the same hepatic niches and thus exposed to the same inflammatory environment. Moreover, several articles reported that CXCR6$^+$ (Paust et al., 2010) or DX5$^-$ (Peng et al., 2013) NK cells possess memory potential using models of contact hypersensitivity to haptens. Thus, Eomes$^-$ NK cells may be important for recall responses in the liver.

Eomes$^-$ NK cells could also be involved in the maintenance of the tolerogenic milieu in the liver under homeostatic conditions. Indeed, they express high levels of PD1-L, ICOSL, and IL-2, which may suppress T cell responses both directly (Terme et al., 2012) and indirectly via interaction with regulatory T cells (T reg cells). Interestingly, a recent study reported that T reg cells restrained the expansion of a population of spleen CD127$^+$CD25$^+$DX5$^-$Eomes$^-$ NK cells (Gasteiger et al., 2013). Thus, there could be a preferential cross talk between T reg cells and Eomes$^-$ NK cells. Consistent with this, RAG$^{-/-}$ T-bet$^{-/-}$ mice develop autoimmune-like syndromes, suggesting that T-bet–dependent innate lymphocytes may be important for limiting inflammation (Lazarevic and Glimcher, 2011).

Together, our findings establish the existence of two alternative pathways of NK cell development occurring in the BM and in the liver, respectively. Early T-bet levels are instrumental to determine the commitment of precursor cells to either lineage. The BM microenvironment restricts T-bet expression, thus allowing the development of classical Eomes$^+$ NK cells. Reciprocally, the liver environment permits high expression of T-bet early on during NK cell development, which results in Eomes repression and the development of Eomes$^-$Trail$^+$ DX5$^-$ Itga1$^+$ NK cells that are specialized in cytokine secretion and may have immunoregulatory functions.

MATERIALS AND METHODS

Mice, infections, and reagents

This study was performed in strict 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 Animal Experimentation Ethics Committee CECCAPP. WT C57BL/6 and BALB/c mice were purchased from Charles River. CD127$^{-/-}$ (Peschon et al., 1994), RAG2$^{-/-}$ (Hao and Rajewsky, 2001), T-bet$^{-/-}$ (Finotto et al., 2002), IL-4$^{-/-}$, CD11c-dnTGFβRII transgenic (Laouar et al., 2005), and CD2-T-bet transgenic (Ishizaki et al., 2007) mice have been previously described. IL-15$^{-/-}$ (Kennedy et al., 2000) and CX3CR1$^{gfp/gfp}$ (Jung et al., 2000) mice have been provided by S. Jung (Weizmann Institute, Rehovot, Israel) and J.P. Di Santo (Institut Pasteur, Paris, France). In some experiments, we also used C57BL/6 CD45.1 mice or C57BL/6 CD45.1 x CD45.2 mice that were bred in our animal house. Female mice 8–24 wk old were used unless specified. For infection, mice were injected intradermally with 5×10^3 cfu *F. tularensis* subspecies *novicida* strain U112 in 50 µl PBS or intraperitoneally with 2×10^5 PFU Vaccinia virus (Modified Vaccinia Virus Ankara). In some experiments, mice were injected with 150 µg poly(I:C) or 4 µg α-GalCer intraperitoneally. In some experiments, we used thymectomized mice. Thymectomies were performed as previously described (Pihlgren et al., 1996).

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Generation of Eomes-GFP reporter/knockin mice

A 179 kb bacterial artificial chromosome containing Eomes 3′ region was selected (clone number RP23-448K1; CHORI). Using Red/ET cloning (Gene Bridges), an ires-GFP-loxP-tACE-Cre-PGK-gb2-neo-loxP cassette was inserted downstream the Eomes STOP codon. This cassette allows GFP expression under the control of Eomes regulatory regions. The rest of the cassette allows selection with neomycin in both bacteria and eukaryotic cells and is auto-excisable in male mice thanks to Cre expression under the control of the testis-specific Tace promoter and the loxP sites (Guia et al., 2011). The modified Eomes region and 3 kb on each side of the STOP codon were subcloned into PACYC177 using Red/ET cloning and included NotI sites allowing the release of the Eomes fragment. JM8.A3 C57BL/6N ES cells (Pettitt et al., 2009) were transfected with the NotI linearized targeting construct and cultured using standard culture conditions for ES cells. After G418 selection (150 µg/ml geneticin G418), ES clones were screened for homologous recombination by PCR analysis and Southern blotting. The occurrence of an appropriate homologous recombination event at the 5′ side was screened by PCR with the following primers: 5′-TTGACCTTGACCCTATGAGGGCTAT-3′ (forward primer); 5′-TAGGAATGCTCGTCAAGAAGACAG-3′ (reverse primer, 4.4 kb with knock-in allele). Next, a Southern blot was performed for some ES clones to further verify the recombination event using BamHI restriction enzyme digestion and probes amplified with the following primers: 5′ probe forward, 5′-CCAGGAGGAGGTTGCTACAG-3′; 5′ probe reverse, 5′-CAAAAGGATGAGCTGTCCAAG-3′; Neo Probe forward, 5′-GATCGGCCATTGAACAAGAT-3′; Neo probe reverse, 5′-ATACTTTCTCGGCAGGAGCA-3′.

Cells from each targeted ES clones were separately injected into C57BL/6 blastocysts to generate chimeric male mice that were mated with C57BL/6 females to obtain germline transmission of the mutated allele. Germline offspring of the GFP/KI genotype were routinely screened by PCR using the following primers in the gene encoding EGFP: 5′-AAGCTGACCCTGAAGTTCATCTGC-3′ (forward primer) and 5′-AAGTTCACCTTGATGCCGTTCTTCT-3′ (reverse primer). This pair of primers amplifies a 374 bp fragment. To generate homozygous mice, heterozygotes were mated and offspring screened using GFP PCR above and another set of primers amplifying the WT region across Exon6 and 3′UTR: 5′-ACTACCATGGACATCCAGAATGAGC-3′ (forward in Exon6) and 5′-CAAAGAACACAACAAAACACCACCA-3′ (reverse in 3′UTR). This pair of primers amplifies a 359 bp fragment in the case of a WT allele.

Generation of BM chimera

C57BL/6 CD45.1 x CD45.2 mice were irradiated twice at 450 rad within a 4-h interval. 4 h after the last irradiation, they received an intravenous injection of a 1:1 mixture of BM cells from WT CD45.1 and *CD127*$^{-/-}$ CD45.2 mice. BM chimeras were analyzed 6–12 wk after reconstitution.

Adoptive transfers

For assessing T-bet levels. BM or spleen cells from CD45.1 mice were prepared and injected intravenously (2×10^7 cells of each genotype in PBS) into anesthetized CD45.2 C57BL/6 mice. 1 wk later, mice were sacrificed and transferred cells were analyzed in different organs by flow cytometry.

Fate mapping of Eomes-GFP□ NK cells. We sorted Eomes-GFP$^-$NKp46$^+$ and Eomes$^+$NKp46$^+$ from the liver and in some experiments from the spleen and BM. 20–50 × 10^4 cells were injected intravenously to recipient CD45.1 C57BL/6 congenic mice that were previously irradiated or not with a 500 rad dose. 2 wk later, mice were sacrificed and transferred NK cells were identified by their expression of CD45.2 in the spleen and liver. Their level of GFP was measured by flow cytometry.

Antibodies and flow cytometry

BM, blood, spleen, LN, and liver cells were isolated and stained as previously described. Cell counts were determined using an accuri C6 flow cytometer (BD). The following mAbs (eBioscience, BD, or BioLegend) were used: anti-CD19 (ebio1D3), anti-CD3 (145-2C11), anti-NK1.1 (PK136), anti NKp46 (29A1.4), anti-CD11b (M1/70), anti-CD27 (LG.7F9), anti-CD122 (5H4), anti-CD127 (A7R34), anti-CXCR3 (CXCR3-173), anti-CXCR4 (2B11) T-bet (ebio4B10), Eomes (Dan11mag), Ki67 (SolA15), KLRG1 (2F1), anti-CD49b (DX5), anti-ITGA1 (Ha31/8), anti-ITGAV (RMV7), anti-ITGA4 (9C10), anti-ITGB2 (C71/16), anti-ITGB3 (2C9), anti-ITGB7 (M293), anti-Trail (N2B2), anti-CD4 (GK1.5), anti-PLZF (Mags.21F7), anti-CD244 (ebio244f4), anti-Ly6D (49H4), anti-CD135 (A2F10), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD62L (Mel14), anti-RORγt (B2D), anti–granzyme A (3G8.5), anti–granzyme B (NGZB), anti-IL2 (JES6-5H4), anti-IL-4 (11b11), anti-GM-CSF (MP1-22E9), anti-CCL3 (DNT3CC), anti–TNF-α (MP6-XT22), anti–IFN-γ (XMG1), and relevant isotype controls. Intracellular stainings for T-bet, Eomes, RORγt, granzyme A, and granzyme B were performed using Foxp3 Fixation/Permeabilization Concentrate and Diluent. BrdU incorporation was measured using a kit (BD). For Eomes staining on Eomes-GFP spleen cells, the pH of all buffers was adjusted to 8 to preserve GFP fluorescence. To detect CXCR6 on NK cell surface, we used indirect staining with CXCL16-Fc-His tagged (R&D Systems), followed by anti-His (Miltenyi Biotec). Flow cytometry was performed on a FACSCanto, a FACSCanto II, or a FACS Fortessa (BD).

Quantitative RT-PCR

Lymphocyte subsets stained with the appropriate antibodies were sorted using a FACSAria cell sorter (BD). RNA was extracted using TRIzol reagent. We used High capacity RNA-to-cDNA kit (Applied Biosystems) to generate cDNA for RT-PCR. PCR was performed with a SybrGreen-based kit (Fast-Start Universal SYBR Green Master; Roche) on a StepOne plus instrument (Applied Biosystems). Primers were designed using the Roche software. The following primers were used: T-bet forward, 5′-CAACCAGCACCAGACAGAGA-3′; T-bet reverse, 5′-ACAAACATCCTGTAATGGCTTG-3′.

Cell culture and stimulation

For assessing di□erentiation of Eomes-NK cells. We sorted Eomes-GFP$^-$NKp46$^+$ and Eomes$^+$NKp46$^+$ from the liver of Eomes-GFP mice using flow cytometry. Cells were then cultured in complete medium in 24-well plates in the presence of 100 ng/ml IL-15 with or without 20 ng/ml IL-12. Cytokines were from R&D Systems.

For NK cell cytokine assays. Hepatic lymphocytes were prepared and cultured with cytokines, coated antibodies (29A1.4, anti-NKp46; PK136, anti-NK1.1; CX5, anti-NKG2D), or 10 ng/ml PMA and 1 µg/ml ionomycin (Sigma-Aldrich) and Golgi-stop (BD). 25 ng/ml IL-12 and 5 ng/ml IL-18 (R&D Systems) were used. When NK cells were stimulated in vivo, hepatic lymphocytes were cultured in complete medium supplemented only with Golgi-stop. After stimulation/culture, intracellular stainings for cytokines were performed using the Cytofix/Cytoperm kit (BD) and cytokine production was measured by flow cytometry.

For cytotoxic assays. Hepatic lymphocytes were prepared and cultured with 25 ng/ml IL-15 overnight. The next day, NK cells were sorted by flow cytometry and cultured 4 h with YAC1 cells previously labeled with CFSE at different E/T ratio. The percentage of TOPRO3$^+$ YAC1 cells was then measured by flow cytometry.

Microarrays

Eomes-GFP$^+$NK1.1$^+$CD3$^-$, Eomes-GFP$^-$NK1.1$^+$CD3$^-$, and NK1.1$^+$CD3$^+$ (NKT) hepatic cells were sorted by flow cytometry. Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Quality and absence of genomic DNA contamination were assessed with a Bioanalyzer (Agilent Technologies). Total RNA from each sample was amplified, labeled, and hybridized to a mouse GeneChip HT MG-430 PM 16-Array Plate (Affymetrix). Affymetrix CEL files were analyzed in R using the Bioconductor suite of packages. Raw probe signals were background corrected using the maximum likelihood estimation of the normal-exponential mixture model (Silver et al., 2009) and normalized using the variance stabilization normalization (Huber et al., 2002), followed by a quantile normalization

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(Bolstad et al., 2003). Summarization was performed using the median-polish (Irizarry et al., 2003) using version 17.1 of the Entrez-Gene based reannotated chip description file (Dai et al., 2005). Non-informative genes were filtered using the I/NI algorithm (Talloen et al., 2007). Linear models were applied using the limma package to compute the mean expression level for each cell type corrected for the animal sex. Statistical contrasts were then applied to compute differential expression between the different cell types. The empirical Bayes method was used to compute moderated p-values that were then corrected for multiple comparisons using the Benjamini and Hochberg's false discovery rate (FDR) controlling procedure. Microarray data were deposited into the Gene Expression Omnibus (accession no. GSE53486).

Statistical analyses

Statistical analyses were performed using two-tailed Student's *t* tests or non-parametric tests when appropriate. These tests were run on the Prism (GraphPad Software). Levels of significance are expressed as p-values.

Online supplemental material

Fig. S1 shows the gating strategy used to identify pre-pro NK cells and NKp. Table S1 shows genes differentially expressed between $Eomes^+$ and $Eomes^-$ NK cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131560/DC1.

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4. **Article 5 : The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells**

nature immunology

The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells

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Interleukin 15 (IL-15) controls both the homeostasis and the peripheral activation of natural killer (NK) cells. The molecular basis for this duality of action remains unknown. Here we found that the metabolic checkpoint kinase mTOR was activated and boosted bioenergetic metabolism after exposure of NK cells to high concentrations of IL-15, whereas low doses of IL-15 triggered only phosphorylation of the transcription factor STAT5. mTOR stimulated the growth and nutrient uptake of NK cells and positively fed back on the receptor for IL-15. This process was essential for sustaining NK cell proliferation during development and the acquisition of cytolytic potential during inflammation or viral infection. The mTORC1 inhibitor rapamycin inhibited NK cell cytotoxicity both in mice and humans; this probably contributes to the immunosuppressive activity of this drug in different clinical settings.

Natural killer (NK) cells are innate lymphoid cells (ILCs) involved in the immunosurveillance of cancers and in the early control of infection by intracellular pathogens[1]. They can kill cells recognized as targets through a battery of surface receptors and produce large amounts of interferon-γ (IFN-γ) upon activation[1]. The growing ILC family has been reclassified into three groups according to the pattern of cytokines they secrete. In this classification, NK cells are part of the group 1 ILC subset (ILC1)[2]. They express the activating NK cell receptor NKp46 (ref. 3), a characteristic they share with the interleukin 22 (IL-22)-producing subset ILC3, which is involved in gut innate immunity[4–6]. In mice, NK cells develop mainly in the bone marrow (BM). Sequential developmental intermediates, from immature to mature, can be defined on the basis of surface expression of the tumor-necrosis factor superfamily member CD27 and the integrin CD11b: $CD11b^{lo}CD27^{hi}$ NK cells (called '$CD11b^{lo}$' cells here), $CD11b^{hi}CD27^{hi}$ NK cells and $CD11b^{hi}CD27^{lo}$ NK cells (called '$CD27^{lo}$' cells here)[7,8]. Upon disruption of IL-15 signaling, the survival of NK cells is considerably reduced and development of the remaining cells is arrested at the $CD11b^{lo}$ immature stage, which demonstrates a nonredundant role for this cytokine in the homeostasis and differentiation of NK cells[9–12]. The 'trans-presentation' of IL-15 by dendritic cells activated by ligands of Toll-like receptors also controls the acquisition of effector functions by NK cells[13]. How a single cytokine can have homeostatic effects as well as inflammatory effects remains a challenging question. It has been suggested that different quantities of IL-15 signaling induce graded responses by NK cells; this could explain its functional duality[14–16]. In terms of signal transduction, ligation of the receptor for IL-15 is known to induce phosphorylation and activation of the transcription factor STAT5. Deletion of STAT5 compromises the survival of NK cells[17]. Whether STAT5 is sufficient to induce the proliferation of NK cells and upregulate their cytotoxic potential, however, remains unknown.

The link between metabolic regulation and activation of cells of the immune system has received considerable attention[18]. Following antigenic challenge, T cells upregulate their metabolism to face the biosynthetic demands, which results in a change from a quiescent state to a proliferative state. Conversely, the resolution of the response is accompanied by a shift of the T cells back to a quiescent state. Metabolic regulation is also coupled to the acquisition of effector functions[19] and a migratory pattern of effector cells[20]. A central molecule that integrates various metabolic, antigenic and inflammatory cues is the evolutionarily conserved serine-threonine kinase mTOR ('mechanistic target of rapamycin')[21]. mTOR is part of two distinct complexes, mTORC1 and mTORC2. mTORC1 controls translation mainly through phosphorylation of the translation-initiation factor eIF4E–binding protein 4EBP1 and the S6 ribosomal kinase (S6K). S6K then phosphorylates the ribosomal protein S6 and mTOR itself (on Ser2448)[22]. Moreover, mTORC1 also takes part in the control of glycolysis by promoting expression of the transcription factors HIF-1α and c-Myc, as well as by upregulating the expression of

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nutrient transporters, and also takes part in the control of lipid synthesis by activating the transcription factor SREBP, and in the control of autophagy. mTORC2 phosphorylates the kinase Akt at Ser473, which completes activation initiated by phosphorylation of Akt at Thr308 by the phosphoinositide-dependent kinase PDK1 and allows export of members of the Foxo family of transcription factors from the nucleus. mTORC2 also controls cytoskeletal organization. Moreover, mTOR phosphorylates itself at Ser2481. Many studies have explored the role of mTOR and downstream effector molecules in T cell differentiation[23]. In contrast, there is a dearth of information on the metabolic regulation of NK cells and the role of mTOR in their physiology.

We thus set out to characterize the basic metabolic needs of NK cells and how they are linked to differentiation and priming following stimulation with IL-15. We found that as NK cells mature, they progressed to quiescence. That state was reversed upon stimulation with viruses or cytokines. These changes were controlled by mTOR; deletion of mTOR revealed its critical nonredundant role in the regulation of two key checkpoints of NK cell biology: proliferation in the BM and activation in the periphery. Moreover, mTOR was an essential component of signaling via IL-15 and was activated upon exposure of NK cells to high concentrations of IL-15.

RESULTS

Development and activation regulate NK cell metabolism

We monitored metabolic changes during the differentiation and activation of NK cells. We detected substantial contraction of cell size and granularity as the cells terminally differentiated (**Supplementary Fig. 1a**). Conversely, when NK cells were activated *in vitro* with IL-15 (**Supplementary Fig. 1b**) or were activated *in vivo* in mice given injection of the synthetic RNA duplex poly(I:C) (the ligand of Toll-like receptor3 and RIG-I) (**Supplementary Fig. 1c**) or in mice infected with influenza virus strain A/WSN/33 H1N1 (**Supplementary Fig. 1d**), the size and granularity of NK cells increased substantially. In metazoans, a cell's ability to access nutrients depends on the expression of dedicated transporters[18]. Expression of the transferrin receptor CD71 and amino-acid transporter CD98 (the heavy chain of the system L transporter), as well as glucose uptake (estimated by measurement of uptake of the fluorescent glucose analog 2-NBDG), decreased by two- to threefold following the transition from $CD11b^{hi}CD27^{hi}$ to $CD27^{lo}$ in the BM (**Fig. 1a**). There was lower expression of those markers (CD71 and CD98) in the spleen, but their expression also decreased upon differentiation (**Fig. 1a**). *In vivo* activation in mice following injection of poly(I:C) resulted in higher expression of CD98 and CD71, as well as in more uptake of glucose, in splenic NK cells than that in resting NK cells from uninjected mice (**Fig. 1b**).

To gain a more precise view of the metabolic changes that occur during differentiation, we searched for unique gene-expression signatures that were different in $CD11b^{lo}$ NK cells and $CD27^{lo}$ NK cells by using previously generated microarrays of these subsets[7] and software for gene-set enrichment analysis (**Supplementary Table 1**). Genes encoding molecules in pathways associated with cell growth ('cell cycle' and 'ribosome') were downregulated in the $CD27^{lo}$ subset. In contrast, genes encoding molecules in pathways associated with the development of a quiescent state ('aerobic sugar or fatty acid catabolism' and 'autophagy') were upregulated in the $CD27^{lo}$ subset. Notably, the $CD27^{lo}$ subset showed higher expression of genes encoding several negative regulators of the mTOR signaling pathway (data not shown).

We undertook a similar analysis to investigate the metabolic regulation associated with the activation of NK cells. We used data sets generated with NK cells activated at 1.5 d after infection with mouse cytomegalovirus (MCMV)[24] or after 24 h of activation *in vitro* with IL-15 (ref. 25). The MCMV-elicited genes encoding molecules identified by metabolic terms were all also induced by treatment with IL-15 (**Supplementary Table 2**). Activated NK cells had higher expression of genes encoding molecules identified by terms associated with the cell cycle, protein, lipid biosynthesis and carbohydrate catabolism, consistent with the observed enhanced cell growth and proliferation (**Supplementary Fig. 1b–d** and data not shown).

To further characterize the metabolic activity of NK cells, we took advantage of Seahorse technology to analyze the oxygen-consumption rate (OCR) and extracellular acidification rate (ECAR), which are proportional to oxidative phosphorylation and aerobic glycolysis, respectively. The basal metabolism of splenic NK cells was very low for both parameters (**Fig. 1c,d**). However, stimulation with IL-15 enhanced the metabolism of splenic NK cells, substantially increasing the basal oxidative phosphorylation and aerobic glycolysis (as revealed by the addition of glucose). Addition of the mitochondrial uncoupler FCCP to assess potential spare respiratory capacity revealed that stimulated NK cells were already developing their maximal respiratory activity. Similarly, the glycolytic reserve (the difference between the ECAR after the injection of glucose and the ECAR after the injection of oligomycin) was nearly zero, which indicated that the maximal glycolytic

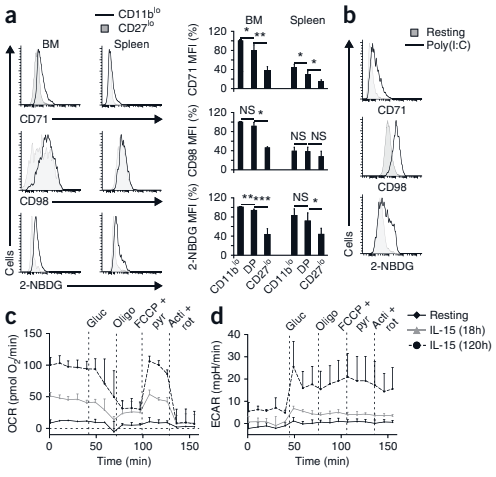

Figure 1 NK cell metabolism is regulated developmentally and after activation. (**a**) Expression of CD71 and CD98 and incorporation of 2-NBDG by $CD11b^{lo}$ and $CD27^{lo}$ NK cell subsets from the BM or spleen, assessed by flow cytometry. Right, results for $CD11b^{hi}CD27^{hi}$ (DP) and $CD27^{lo}$ NK cells, presented as mean fluorescent intensity (MFI) relative to that of the $CD11b^{lo}$ subset in the BM, set as 100%. NS, not significant; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (*t*-test). (**b**) Expression of CD71 and CD98 and incorporation of 2-NBDG by splenic NK cells obtained from mice 18 h after injection of saline (Resting) or poly(I:C). (**c**,**d**) Real-time analysis of oxidative phosphorylation (OCR) (**c**) and aerobic glycolysis (ECAR) (**d**) in primary NK cells left unstimulated (Resting) or stimulated for 18 h or 120 h with IL-15, followed by injection of (dashed vertical line) glucose (Gluc; 25 mM), oligomycin (Oligo; 1 μM), FCCP (1.5 M) plus pyruvate (1 mM) (FCCP + pyr) and antimycin A (1 μM) plus rotenone (0.1 μM) (Acti + rot). Data are from four independent experiments (**a**; average and s.d. of four mice), one experiment representative of three experiments (**b**) or two independent experiments (**c**,**d**; average and s.d. of three mice assessed in triplicate).

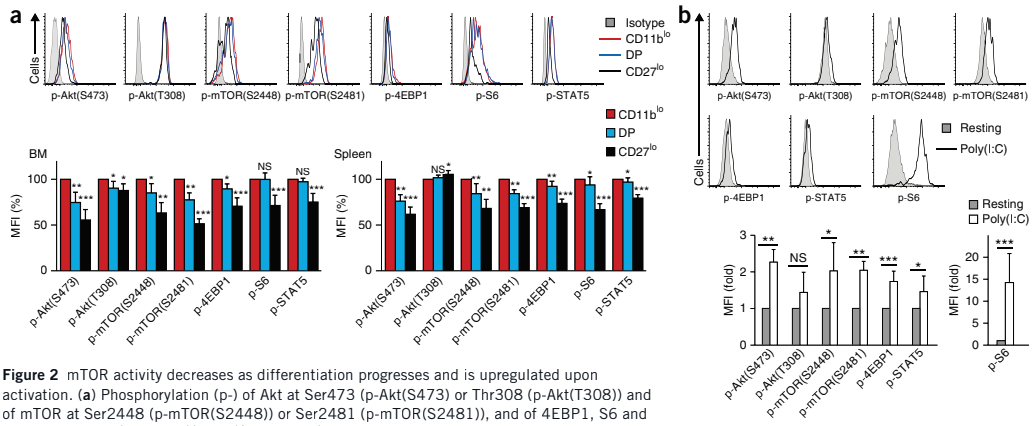

Figure 2 mTOR activity decreases as differentiation progresses and is upregulated upon activation. (**a**) Phosphorylation (p-) of Akt at Ser473 (p-Akt(S473) or Thr308 (p-Akt(T308)) and of mTOR at Ser2448 (p-mTOR(S2448)) or Ser2481 (p-mTOR(S2481)), and of 4EBP1, S6 and STAT5, in CD11b^{lo}, CD11b^{hi}CD27^{hi} and CD27^{lo} BM NK cell subsets (top); Isotype, isotype-matched control antibody. Below, cumulative results (presented as in **Fig. 1a**). (**b**) Phosphorylation (as in **a**) in splenic NK cells obtained from mice 18 h after injection of saline or poly(I:C) (top). Below, cumulative results, presented relative to those of the resting population, set as 1. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (*t*-test). Data are from one experiment representative of four (**a**, top) or three (**b**, top) experiments or are from four (**a**, bottom) or three (**b**, bottom) independent experiments (average and s.d. of four (**a**) mice or three (**b**) mice).

activity was already reached. Together these data showed that the basal metabolism of splenic NK cells was very low but was inducible by stimulation with IL-15.

mTOR regulation upon differentiation and activation

The observation of metabolic changes during NK cell differentiation prompted us to assess the phosphorylation status of mTOR targets. We analyzed the phosphorylation status of mTOR itself (at Ser2448 and Ser2481), of downstream targets of mTORC1 (4EBP1, at Thr37 and Thr46; and S6, at Ser235 and Ser236) and of a target of mTORC2 (Akt, at Ser473). In parallel, we assessed the phosphorylation of STAT5 at Tyr694 by the kinase Jak3, downstream of the receptor for IL-15, and the phosphorylation of Akt at Thr308 by the kinase PDK1. We first measured the phosphorylation status of these proteins in BM and splenic NK cells at steady state and correlated it to the expression of CD11b and CD27. Phosphorylation decreased in a coordinated fashion as the cells matured (**Fig. 2a**), indicative of a progressive shutdown in mTOR activity both in the BM and in the spleen. We observed a similar pattern for the phosphorylation of STAT5 (**Fig. 2a**). In contrast, the phosphorylation of Akt at Thr308 decreased only in the BM (**Fig. 2a**). Direct comparison of phosphorylation in BM and splenic NK cells showed significantly greater phosphorylation of Akt at Ser473 and of mTOR at Ser2448 and Ser2481 on BM NK cells, especially in the CD11b^{lo} subset (**Supplementary Fig. 2**). We then set out to measure phosphorylation events induced *in vivo* after stimulation with poly(I:C). The extent of the various phosphorylation events was upregulated in a coordinated fashion (**Fig. 2b**), which indicated an overall increase in the activity of the pathway. As expected, since injection of poly(I:C) increases the availability of IL-15, phosphorylation of STAT5 was significantly upregulated (**Fig. 2b**). In contrast, phosphorylation of Akt at Thr308, which is dependent on the activity of phosphatidylinositol-3-OH kinase (PI(3)K) via PDK1, was not significantly changed (**Fig. 2b**). Together these results demonstrated that mTOR activity was under the control of developmental and inflammatory signals in NK cells. Moreover, there was a close parallel between the metabolic status of NK cells and mTOR activity.

mTOR activity is mainly under the control of IL-15

To identify the signals able to regulate mTOR activity in NK cells, we treated splenic NK cells for 1 h *in vitro* with a wide range of stimuli. Among all the signals tested, IL-15 was the only one to elicit a substantial increase in phosphorylated S6 (**Fig. 3a**). In particular, we observed no effect on phosphorylated S6 after triggering of activating or inhibitory receptors on NK cells (**Fig. 3a**). We also noted slight but reproducible effects after exposure of cells to IL-18, alone or in combination with IL-12 (**Fig. 3a**). To better characterize the response to IL-15, we exposed splenocytes for 1 h to increasing concentrations of this cytokine. We measured in parallel the phosphorylation of S6 and that of STAT5. Larger amounts of IL-15 were needed to activate mTOR (median effective dose of 1.5 ng/ml or 5.3 ng/ml for phosphorylated STAT5 or phosphorylated S6, respectively; **Fig. 3b**).

To determine whether IL-15 signaling is necessary *in vivo* to maintain physiological mTOR activity, we treated wild-type mice with an F(ab′)$_2$ fragment that blocks signaling via CD122 (the β-chain of the receptor for IL-2). Treatment with antibody to CD122 (anti-CD122) led to a rapid decrease in the steady-state phosphorylation of S6 in BM NK cells (**Fig. 3c**, left). We confirmed an overall decrease in the activity of this pathway by analysis of the phosphorylation of other molecules (**Fig. 3c**, right). As expected, we obtained similar results for phosphorylated STAT5 (**Fig. 3c**).

To determine if the increased bioavailability of IL-15 consequent to the injection of poly(I:C) was responsible for the increased mTOR activity, we injected blocking antibody to CD122 together with poly(I:C). We measured phosphorylation in splenic NK cells 4 h later. The increase in phosphorylated STAT5 was completely abrogated (**Fig. 3d**), which indicated complete inhibition of signaling via IL-15. The increase in phosphorylated Akt and phosphorylated S6 was also significantly dampened by the antibody treatment (**Fig. 3d**). However, phosphorylation of 4EBP1 was not affected (**Fig. 3d**), which suggested that other signals *in vivo* might be able to compensate for the absence of IL-15.

Overall, these results showed that IL-15 was sufficient to activate mTOR in NK cells. It was also necessary for the maintenance of steady-state activity during the development of NK cells. Finally, this

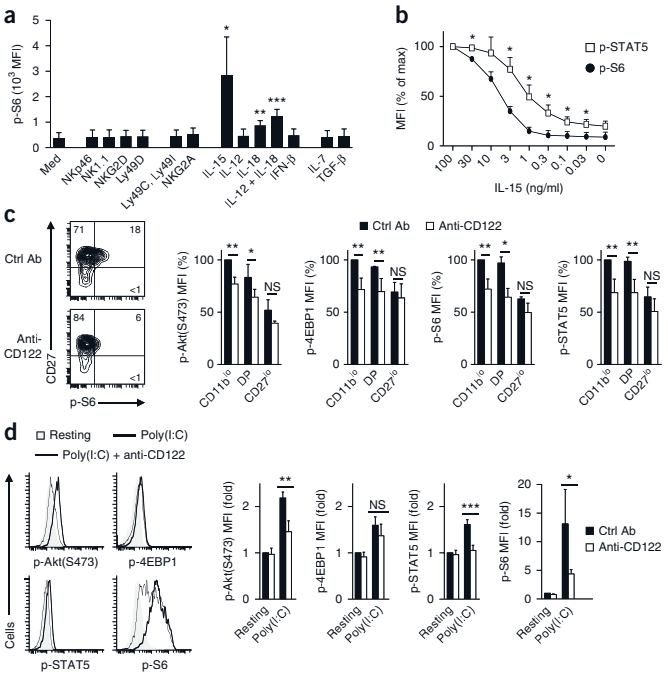

Figure 3 mTOR activity is mainly under the control of IL-15. (**a**) Intracellular phosphorylated S6 in NK cells among splenocytes cultured for 1 h on plates in medium alone (Med) or plates coated with crosslinking antibodies to various receptors (left group) or cytokines (right group). (**b**) Intracellular phosphorylated S6 and STAT5 in NK cells among splenocytes cultured for 1 h with 'graded' concentrations of IL-15 (horizontal axis); results are presented relative to the maximal response (100%). (**c**) CD27 expression versus abundance of phosphorylated S6 in BM cells freshly isolated from mice given injection of isotype-matched control antibody (Ctrl Ab) or anti-CD122, analyzed by flow cytometry (left); numbers in quadrants indicate percent cells in each. Right, phosphorylated proteins in BM NK cells from mice treated as at left (presented as in **Fig. 1a**). (**d**) Phosphorylation of proteins in splenic NK cells obtained from mice 4 h after injection of saline or poly(I:C) with or without anti-CD122 (left). Right, phosphorylated proteins in splenic NK cells obtained from mice after injection of saline or poly(I:C) along with isotype-matched control antibody or anti-CD122 (key); results are presented relative to those of the resting population treated with control antibody, set as 1. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (*t*-test). Data are from four (**a**) or three (**b**) independent experiments with one mouse in each (average and s.d.) or are from one experiment representative of three experiments (**c**,**d**, left) or are from three independent experiments (**c**,**d**, right; average and s.d. of three mice).

cytokine was a chief but not exclusive source of extracellular signaling that led to hyperactivation of mTOR in an inflammatory context.

mTOR controls the maturation and number of NK cells

To establish the physiological relevance of mTOR signaling in NK cells, we deleted mTOR in NK cells by crossing mice with *lox*P-flanked alleles encoding mTOR (*Mtor*$^{lox/lox}$)[26] with mice expressing Cre recombinase from the gene encoding the NK cell–specific activating receptor NKp46 (*Ncr1*; called '*Nkp46*-iCre' here)[27]. This cross resulted in *Mtor*$^{lox/lox}$*Nkp46*-iCre mice (called 'NK-*Mtor*$^{-/-}$' mice here). We analyzed the consequences of this deletion in NK cells in various organs (**Supplementary Fig. 3**). We confirmed that in NK-*Mtor*$^{-/-}$ mice, mTOR was indeed absent from NK cells but was present in normal amounts in the surrounding T cells (**Supplementary Fig. 4a**). In addition, NK cells were also smaller, and phosphorylation of mTOR targets was reproducibly diminished (**Supplementary Fig. 4b**,**c**), which demonstrated that mTOR was functionally absent. Despite that finding, the frequency and number of BM NK cells were normal in NK-*Mtor*$^{-/-}$ mice (**Fig. 4a**). In contrast, NK cells almost completely disappeared from the peripheral organs of NK-*Mtor*$^{-/-}$ mice (**Fig. 4a** and **Supplementary Fig. 4d**). Phenotyping of the remaining NK cells in NK-*Mtor*$^{-/-}$ mice revealed a substantial block in differentiation in the BM at the CD11b^{lo}-to-CD11b^{hi}CD27^{hi} stage (**Fig. 4b** and **Supplementary Fig. 5a**). This resulted in a shift in the distribution among the various NK cell subsets in the spleen (**Fig. 4b** and **Supplementary Fig. 5a**). Consistent with that observation, expression of the maturation and senescence marker KLRG1 on NK-*Mtor*$^{-/-}$ NK cells almost completely disappeared (**Fig. 4c**).

Next we conducted a broad phenotypic analysis of splenic *Mtor*$^{+/+}$ *Nkp46*-iCre (called 'NK-*Mtor*$^{WT/WT}$' here) NK cells and NK-*Mtor*$^{-/-}$ NK cells (**Fig. 4d**). To compensate for the differentiation bias that occurred in the absence of mTOR, we focused our analysis on the CD11b^{lo} subset. The expression of markers such as CXCR4, CD127 and NK1.1 was upregulated on splenic NK-*Mtor*$^{-/-}$ NK cells relative to their expression on NK-*Mtor*$^{WT/WT}$ NK cells (**Fig. 4d**). In contrast, NK-*Mtor*$^{-/-}$ NK cells had lower expression than did NK-*Mtor*$^{WT/WT}$ NK cells of activating receptors acquired during maturation, such as 2B4, NKG2D, Ly49D, Ly49G2 and Ly49H (**Fig. 4d**). The frequency of cells expressing receptors of the Ly49 family, however, was similar in the presence or absence of mTOR, with the exception of Ly49H (**Supplementary Fig. 5b**). Expression of the T-box transcription factors Eomes and T-bet, which are responsible for the maturation of NK cells[28], was also downregulated in NK-*Mtor*$^{-/-}$ NK cells relative to such expression in NK-*Mtor*$^{WT/WT}$ NK cells (**Fig. 4d**). As expected, expression of the nutrient receptor CD71 and glucose uptake were downregulated in the absence of mTOR (**Fig. 4d**). Expression of CD122 and CD132, molecules that constitute the IL-15Rβγc heterodimer of the receptor for IL-15, was halved in the absence of mTOR (**Fig. 4d**). The functional relevance of that last result is explored below. Expression of the other markers analyzed was not altered (**Fig. 4d**), which indicated that deletion of mTOR selectively affected part of the NK cell gene-expression program. We observed a similar pattern for the BM (**Supplementary Fig. 5c**).

The *Nkp46* promoter also drives Cre expression in IL-22-producing NCR$^+$ ILC3 cells in the gut. We thus investigated whether deletion of mTOR affected this cell subset. Indeed, NKp46$^+$ ILC3 cells were absent from the gut of NK-*Mtor*$^{-/-}$ mice (**Supplementary Fig. 4d**), which indicated a nonredundant role for mTOR in the generation of this population. Overall, these results demonstrated that functional mTOR was required for the presence of mature NK cells in peripheral organs and selectively affected part of the NK cell gene-expression program.

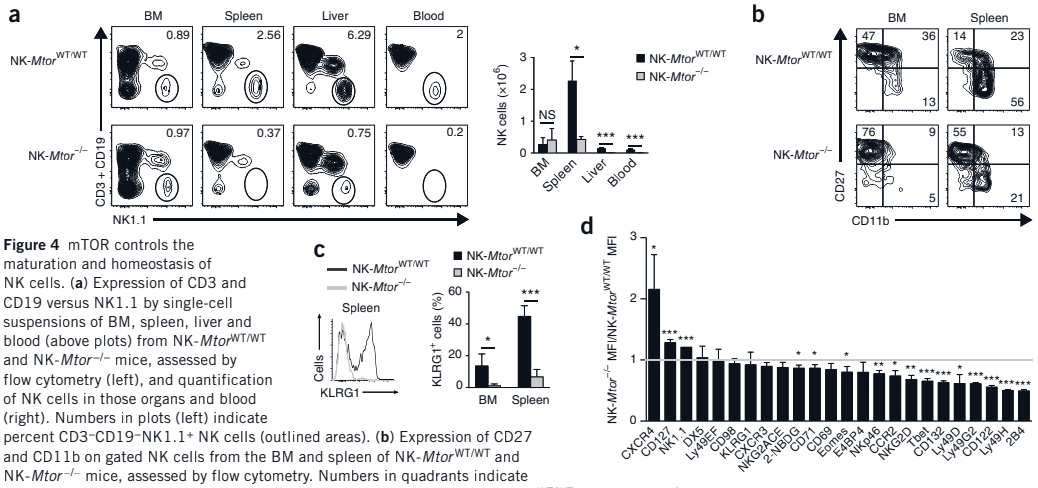

Figure 4 mTOR controls the maturation and homeostasis of NK cells. (**a**) Expression of CD3 and CD19 versus NK1.1 by single-cell suspensions of BM, spleen, liver and blood (above plots) from NK-*Mtor*$^{WT/WT}$ and NK-*Mtor*$^{-/-}$ mice, assessed by flow cytometry (left), and quantification of NK cells in those organs and blood (right). Numbers in plots (left) indicate percent CD3$^-$CD19$^-$NK1.1$^+$ NK cells (outlined areas). (**b**) Expression of CD27 and CD11b on gated NK cells from the BM and spleen of NK-*Mtor*$^{WT/WT}$ and NK-*Mtor*$^{-/-}$ mice, assessed by flow cytometry. Numbers in quadrants indicate percent cells in each. (**c**) KLRG1 expression on splenic NK cells from NK-*Mtor*$^{WT/WT}$ and NK-*Mtor*$^{-/-}$ mice (left), and frequency of KLRG1$^+$ cells among NK cells from the BM and spleen (right). (**d**) Abundance of various markers (horizontal axis) in splenic CD11b$^-$ NK cells, presented as the ratio of MFI for NK-*Mtor*$^{-/-}$ NK cells to that of NK-*Mtor*$^{WT/WT}$ NK cells (NK-*Mtor*$^{-/-}$ MFI/NK-*Mtor*$^{WT/WT}$ MFI); gray horizontal line indicates a ratio of 1. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (*t*-test). Data are from one of three experiments (**a**, left) or are from three independent experiments (**a**, right; average and s.d. of three mice), are from one experiment of more than ten experiments with similar results (**b**) or are from three independent experiments (**c**,**d**; average and s.d. of four (**c**) or three (**d**) mice).

Optimal response to IL-15 depends on mTOR

We next investigated whether the absence of mature NK cells in the periphery of NK-*Mtor*$^{-/-}$ mice was a result of diminished survival, decreased generation of NK cells or both. The viability of NK-*Mtor*$^{-/-}$ splenic NK cells was identical to that of their NK-*Mtor*$^{WT/WT}$ counterparts in the CD11b^{lo} subset and CD11b^{hi}CD27^{hi} subset and was only slightly lower than that of NK-*Mtor*$^{WT/WT}$ cells in the mature CD27^{lo} subset (**Fig. 5a**). Moreover, 'acute' deletion of mTOR in *Mtor*$^{lox/lox}$ NK cells treated *in vitro* with the cell-permeable Cre recombinase fusion protein TATCre and then transferred back into wild-type mice *in vivo* did not impair their grafting capacity compared with that *Mtor*$^{+/+}$ cells treated with TATCre and transferred together into the mice (**Supplementary Fig. 6a**). Thus, diminished viability probably had only a minor role in the defect observed. The CD11b^{lo}-to-CD11b^{hi}CD27^{hi} transition is preceded in the BM by a proliferation phase[29]. Deletion of mTOR resulted in a threefold decrease in the frequency of proliferating cells, as determined by incorporation of the thymidine analog BrdU (**Fig. 5b**) or staining with the proliferation marker Ki67 (**Supplementary Fig. 6b**). This probably profoundly affected the output from the BM and

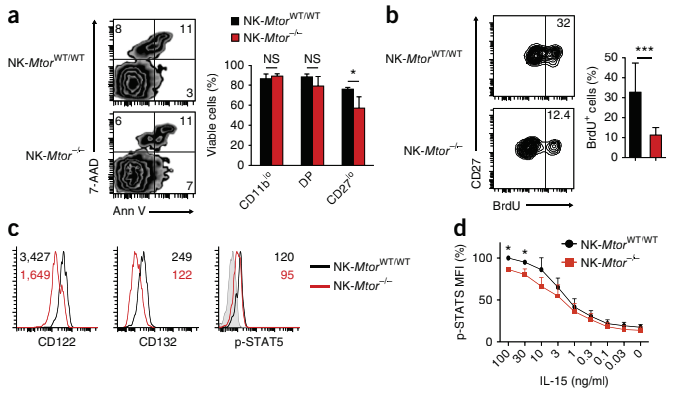

Figure 5 mTOR is necessary for the optimal fitness of NK cells, their proliferation in the BM and their maximal response to IL-15. (**a**) Staining of NK-*Mtor*$^{WT/WT}$ and NK-*Mtor*$^{-/-}$ splenic NK cells with annexin V (Ann V) and the DNA-intercalating dye 7-AAD (left), and frequency of viable cells (negative for annexin V and 7-AAD) in CD11b^{lo}, CD11b^{hi}CD27^{hi} and CD27^{lo} NK cell subsets from NK-*Mtor*$^{WT/WT}$ and NK-*Mtor*$^{-/-}$ mice (right). Numbers in quadrants (left) indicate percent cells in each. (**b**) BrdU staining of the CD11b^{lo} subset of BM NK cells from NK-*Mtor*$^{WT/WT}$ and NK-*Mtor*$^{-/-}$ mice, assessed by flow cytometry (left), and frequency of BrdU$^+$ cells among those cells (right). Numbers in plots (left) indicate percent BrdU$^+$ cells. (**c**) Expression of CD122 and CD132 and abundance of phosphorylated STAT5 in CD11b^{lo} BM NK cells from NK-*Mtor*$^{WT/WT}$ and NK-*Mtor*$^{-/-}$ mice; numbers in plots indicate mean fluorescence intensity. (**d**) Abundance of phosphorylated STAT5 in NK cells among NK-*Mtor*$^{WT/WT}$ and NK-*Mtor*$^{-/-}$ splenocytes cultured for 1 h with graded concentrations of IL-15, presented relative to the maximal response (100%). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (*t*-test). Data are from one experiment representative of three experiments (**a**,**b**, left) or are from three independent experiments (**a**,**b**, right; average and s.d. of three (**a**) or six (**b**) mice), are from one experiment representative of four experiments with one mouse in each (**c**) or are from three independent experiments with one mouse in each (**c**; average and s.d.).

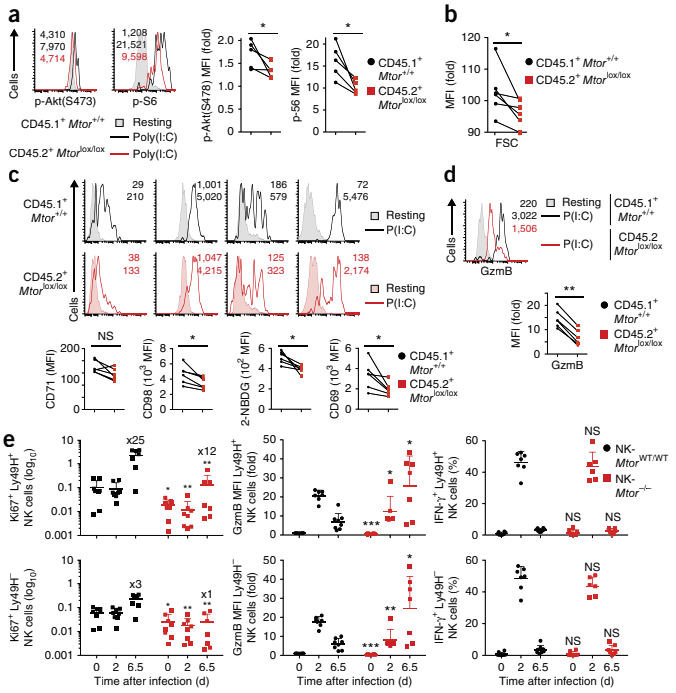

Figure 6 Defective activation of NK cells in the absence of mTOR. (**a**) Phosphorylation of Akt at Ser473 and of S6 in splenic cells from CD45.1⁺CD45.2⁺ host mice given CD45.1⁺ *Mtor*$^{+/+}$ and CD45.2⁺ *Mtor*$^{lox/lox}$ splenocytes pretreated for 2 d *in vitro* with TATCre, analyzed by flow cytometry 18 h after injection of saline or poly(I:C) (left), and quantification of results of cells from mice given injection of poly(I:C) as at left, presented relative to those of the resting population, with lines linking the *Mtor*$^{+/+}$ and *Mtor*$^{-/-}$ NK cells transferred together into the same host. Numbers in plots (left) indicate mean fluorescence intensity. (**b**) Forward scatter (FSC) of NK cells from mice given injection of poly(I:C) as in **a** (presented as in **a**, right). (**c**) Expression of CD71, CD98 and CD69 and staining of 2-NBDG in splenic NK cells from mice treated as in **a** (top; presented as in **a**, left), and quantification of results above (bottom; presented as in **a**, right). (**d**) Intracellular expression of granzyme B (GzmB) in splenic NK cells from mice treated as in **a** (top), and quantification of results above (below; presented as in **a**, right). (**e**) Expression of intracellular Ki67, granzyme B and IFN-γ in Ly49H⁺ and Ly49H⁻ splenic NK cell subsets from NK-*Mtor*$^{WT/WT}$ and NK-*Mtor*$^{-/-}$ mice at day 0, 2 or 6.5 after infection with MCMV, presented as quantification of Ki67⁺ NK cells, abundance of granzyme B (relative to that of uninfected mice) and frequency of IFN-γ⁺ NK cells. Numbers above columns (left) indicate the population expansion of Ki67⁺ NK cells after infection relative to that of NK cells in uninfected mice. Each symbol represents an individual mouse; small horizontal lines indicate the average (± s.d.). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (*t*-test). Data are from one experiment representative of three independent experiments with one mouse in each (**a**, left) or from three independent experiments with five mice (**a**, right), are from three independent experiments with six mice (**b**), are from one experiment representative of three experiments (**c**, left) or from three independent experiments with six mice (**c**, right), are from one experiment representative of three experiments (**d**, top) or from three independent experiments with five mice (**d**, bottom) or are from four independent experiments with at least six mice (**e**).

blocked subsequent differentiation of NK cells. NK cell proliferation is controlled by IL-15. As noted above, NK-*Mtor*$^{-/-}$ NK cells had around half the surface expression of the β-chain (CD122) and γ-chain (CD132) of the receptor for IL-15 that NK-*Mtor*$^{WT/WT}$ cells had (**Fig. 5c** and **Supplementary Fig. 6c**). That 'translated' into a lower steady-state amount of phosphorylated STAT5 in the BM of NK-*Mtor*$^{-/-}$ mice than in that of NK-*Mtor*$^{WT/WT}$ mice (**Fig. 5c** and **Supplementary Fig. 6c**). In support of the proposal of a role for mTOR in the maintenance of optimal IL-15R expression, 'acute' deletion of mTOR induced by treatment with TATCre led to a reproducible decrease in the surface expression of CD122 (**Supplementary Fig. 6d**). Quantification of the phosphorylation of STAT5 in response to *in vitro* treatment with increasing concentrations of IL-15 led to the finding that NK-*Mtor*$^{-/-}$ cells properly sensed low concentrations of IL-15 and become defective only at concentrations able to induce proliferation (**Fig. 5d** and data not shown). This suggested that IL-15R was limiting when NK-*Mtor*$^{-/-}$ NK cells were exposed to an IL-15-rich environment that dictated NK cell proliferation. Overall, these results showed that mTOR deficiency had a substantial effect on NK cell proliferation in the BM due to direct and indirect effects through the regulation of IL-15R expression. The maturation block observed in NK-*Mtor*$^{-/-}$ NK cells was probably a consequence of this proliferation defect.

Defective activation of NK cells in the absence of mTOR

To assess the role of mTOR in NK cell activation, we treated CD45.2⁺ *Mtor*$^{lox/lox}$ splenocytes and CD45.1⁺ *Mtor*$^{+/+}$ splenocytes *in vitro* with TATCre and transferred them *in vivo* into CD45.1⁺CD45.2⁺ host mice. Then 2 d later, we gave the host mice an injection of poly(I:C) or saline and collected spleens 18 h later. Flow cytometry with phosphorylation-specific antibodies demonstrated a consequent decrease in the catalytic activity of mTOR resulting from deletion of mTOR (**Fig. 6a**). The increase in cell size that results from activation of NK cells was suppressed in mTOR-deficient cells (**Fig. 6b**), which suggested these cells were unable to upregulate their metabolism. Consistent with that idea, upregulation of expression of the nutrient receptors CD71 and CD98, as well as uptake of 2-NBDG, was blunted, as was upregulation of expression of the activation marker CD69 (**Fig. 6c**). This was consistent with the known role of mTOR in metabolic regulation and nutrient uptake.

As for effector functions, poly(I:C)-induced expression of granzyme B was halved in NK cells rendered deficient in mTOR (**Fig. 6d**). We obtained similar results when we compared the responses of NK-*Mtor*$^{WT/WT}$ mice and NK-*Mtor*$^{-/-}$ mice to poly(I:C) (**Supplementary Fig. 7a–c**). We also monitored the degranulation of CD11b^{lo} NK cells and secretion of IFN-γ from NK cells following stimulation *in vitro* with a combination of IL-12 and IL-18 or plate-bound agonistic antibodies specific for the activating NK cell receptors NKp46, NK1.1 and

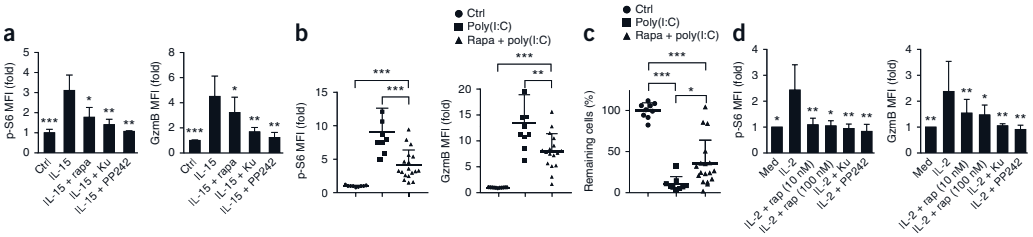

Figure 7 Acute inhibition of mTOR abrogates inflammation-induced priming. (**a**) Phosphorylated S6 and abundance of granzyme B in gated NK cells among splenocytes cultured for 14 h in medium alone (control (Ctrl)) or with IL-15 (100 ng/ml) alone or IL-15 plus the mTOR inhibitors rapamycin (IL-15 + rapa), Ku-0063794 (IL-15 + Ku) or PP242 (IL-15 + PP242), analyzed by flow cytometry. (**b**) Phosphorylated S6 and abundance of granzyme B in gated NK cells among splenocytes from untreated control mice (Ctrl) or from untreated mice (Poly(I:C)) or rapamycin-treated mice (Rap + poly(I:C)) given injection of poly(I:C), analyzed 18 h later by flow cytometry; results are presented relative to those of control mice. (**c**) *In vivo* cytotoxicity of NK cells, from mice treated as in **b**, toward missing-self target cells, presented as percentage of remaining target cells. (**d**) Phosphorylated S6 and granzyme B in gated NK cells among human peripheral blood mononuclear cells cultured for 36 h in medium alone (Med) or with IL-2 alone or IL-2 plus 10 or 100 nM rapamycin, or Ku-0063794 or PP242, analyzed by flow cytometry. Each symbol (**b**,**c**) represents an individual mouse ($n = 9$ (control), $n = 9$ (poly(I:C) or $n = 18$ (rapamycin plus poly(I:C))); small horizontal lines indicate the average (± s.d.). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (paired *t*-test). Data are from five independent experiments with one mouse in each (**a**; average and s.d.), four independent experiments (**b**,**c**) or three independent experiments (**d**; average and s.d. of nine individual donors).

Ly49D. For these analyses, we used resting splenic NK cells or splenic NK cells preactivated with poly(I:C). As expected, preactivation with poly(I:C) led to a threefold increase in the proportion of cells that degranulated in response to cytokines or antibodies (**Supplementary Fig. 7d**). In contrast, although basal degranulation was fairly normal in the absence of mTOR, stimulation with poly(I:C) was unable to enhance it (**Supplementary Fig. 7d**). IFN-γ production followed a similar pattern when we assessed the response to stimulation of activating receptors on NK cells (**Supplementary Fig. 7d**). However, stimulation with IL-12 plus IL-18 drove normal IFN-γ responses, in terms of the frequency of IFN-γ^{+} cells (**Supplementary Fig. 7d**) and quantity of IFN-γ per cell (data not shown). The last finding demonstrated that NK-*Mtor*$^{-/-}$ cells were reactive to IL-12 plus IL-18 and were not generally insensitive to stimuli.

To assess the effect of mTOR deficiency on a more physiological NK cell response, we infected NK-*Mtor*$^{WT/WT}$ mice and NK-*Mtor*$^{-/-}$ mice with MCMV. During MCMV infection, it is well described that early activation of NK cells is mediated by a combination of various cytokines[30], while later during the infection, the interaction between Ly49H and the microbe-encoded protein m157 drives the proliferation and sustains the activation of Ly49H^{+} NK cells[31]. We measured the proliferation of Ly49H^{+} and Ly49H^{-} NK cell subsets and the expression of granzyme B, IFN-γ, KLRG1 and the chemokine CCL3 by these cells, as well as degranulation of these cells, during the cytokine-driven (early) and m157- and Ly49H-dependent (late) phases of the response. The cytokine-driven phase of the response was severely impaired in terms of expression of granzyme B (**Fig. 6e**, middle) and CCL3 (**Supplementary Fig. 7e**, middle) as well as degranulation (**Supplementary Fig. 7e**, right), in agreement with data reported above (**Fig. 6d** and **Supplementary Fig. 7c**,**d**). In contrast, we confirmed that IFN-γ secretion was unaffected by mTOR deficiency (**Fig. 6e**, right). Unexpectedly, at day 6.5, the mTOR-deficient Ly49H^{+} NK cell subset expanded 12-fold (**Fig. 6e**). However, that proliferation was less than the proliferation of mTOR-sufficient NK cells (25-fold), and the number of mTOR-deficient Ly49H^{+} NK cells never reached that of mTOR-sufficient Ly49H^{+} NK cells (data not shown). Notably, the induction of KLRG1^{+} NK cells following infection with MCMV was impaired in the absence of mTOR in both the Ly49H^{+} subset and the Ly49H^{-} subset (**Supplementary Fig. 7e**, left).

Overall these results demonstrated that mTOR deficiency profoundly impaired the early cytokine-driven activation of NK cells at multiple levels. In contrast, proliferation induced by m157 and Ly49H was partly independent of mTOR. The last result correlated with the reported independence of IL-15 of Ly49H-mediated NK cell proliferation during MCMV infection[32].

Inhibition of mTOR abrogates inflammation-induced priming

The results reported above suggested that mTOR deficiency might affect the cytotoxicity of NK cells. To test this hypothesis further and to eliminate confounding developmental effects, we first stimulated wild-type NK cells with IL-15 *in vitro* and 'acutely' inhibited mTOR with three pharmacological inhibitors: rapamycin, an inhibitor of mTORC1 that is used clinically[33]; and two competitors of ATP, Ku-0063794 and PP242, that inhibit mTORC1 and mTORC2 (these inhibitors have been tested 'preclinically')[34,35]. To evaluate inhibitor efficacy, we measured phosphorylation of S6. All three inhibitors consistently impaired the increase in the phosphorylation of S6 and granzyme B in response to IL-15, with Ku-0063794 and PP242 showing higher potency (**Fig. 7a**). As rapamycin is an inhibitor that is already in use clinically, we fed mice an orally administered form of rapamycin for 2 d before treatment with poly(I:C) to test whether *in vivo* inhibition could be achieved. Indeed, treatment with rapamycin resulted in a decrease in the abundance of phosphorylated S6 and granzyme B (**Fig. 7b**). Treatment with rapamycin did not seem to affect the 'trans-presentation' of IL-15 to NK cells, since the abundance of Akt phosphorylated at Ser473 and upregulation of CD69 expression were normal after such treatment (**Supplementary Fig. 8a**,**b**).

We then directly investigated whether treatment with rapamycin impaired the reactivity of NK cells to 'missing-self' targets. For this we transferred a mixture of wild-type target cells and target cells sensitive to NK cells and lacking major histocompatibility complex class I (deficient in the gene encoding β_2-microglobulin) into untreated (control) mice or mice given injection of poly(I:C), previously fed rapamycin orally or not given rapamycin. The killing of β_2-microglobulin deficient target cells was significantly less efficient in rapamycin-treated mice than in their control counterparts (**Fig. 7c**), which emphasized the importance of mTOR activity in NK cell function.

Rapamycin is used in various therapeutic settings. We thus thought it important to investigate whether the priming of human NK cells was sensitive to inhibitors of mTOR. To address this, we stimulated human NK cells *in vitro* with IL-2 in the presence of mTOR inhibitors and measured phosphorylation of S6 and the abundance of granzyme B. IL-2 induced an increase in phosphorylated S6, which, as expected, was suppressed by pharmacological inhibitors of mTOR (**Fig. 7d**). The inhibitors of mTOR also prevented IL-2-induced expression of granzyme B (**Fig. 7d**), with little effect on the expression of perforin (**Supplementary Fig. 8c**). These results showed that, as in the mouse, the mTOR pathway controlled the cytotoxicity of human NK cells downstream of IL-15R. Overall, these results showed that mTOR activity in NK cells was inhibited *in vitro* and *in vivo* by pharmacological inhibitors and that this inhibition resulted in diminished priming and cytolytic functionality of NK cells.

DISCUSSION

mTOR is an integrator of various extracellular cues[21]. Among all the signals we tested *in vitro*, we found that IL-15 and IL-18, with or without IL-12, were the only inducers of mTORC1 and mTORC2 activity in NK cells. In contrast, none of the other homeostatic cytokines or agonistic antibodies known to stimulate activating receptors on NK cells had any effect on mTOR activity. This was unexpected, since ligation of the T cell antigen receptor plus the coreceptor CD28 is a potent inducer of mTOR activity[36–38], and the pathways induced by ligation of activating receptors on NK cells or ligation of the T cell antigen receptor plus CD28 are similar[39]. Our *in vitro* findings were confirmed by the finding that following infection with MCMV, Ly49H-dependent responses were affected less than were cytokine-dependent responses of the Ly49H$^-$ NK cell subset. It thus seems that the Ly49H-dependent signal is able to bypass the need for mTOR signaling. Proliferation in particular was still important in the absence of mTOR. Ly49H-mediated proliferation can be independent of IL-15 (ref. 32); this may explain the nonessential role of mTOR in this context. We hypothesize that Ly49H-dependent signals compensate in part for the lack of IL-15 responsiveness in the induction of NK cell proliferation. In CD8$^+$ T cells, several pathways converge at the level of the phosphorylation of S6 to control metabolic signaling[40]. In particular, pathways involving the mitogen-activated protein kinases MEK and Erk contribute to the phosphorylation of S6, and this might compensate for mTOR deficiency in NK cells[40].

How mTOR is activated downstream of IL-15 receptor in NK cells remains also unsolved. So far, mTORC2's mode of activation remains elusive but involves PI(3)K[41]. As for mTORC1, the exact sequence of events that lead to its activation in T cells and in particular the involvement of PI(3)K signal are still debated[42]. However, a relevant fact is the observation that NK cells doubly deficient in both the p110γ and p110δ catalytic subunits of PI(3)K[43] or deficient in p110δ alone[44,45] have a phenotype partially convergent with that of NK-*Mtor*$^{-/-}$ NK cells, albeit milder. Indeed, a defect in PI(3)K signaling prevents the final maturation of NK cells[43] and impairs their response to activating receptors on NK cells[43–45]. The importance of PI(3)K in the responsiveness of NK cells to IL-15 was not tested in those studies[43–45]. This suggests that mTOR activity is controlled partly but not exclusively by PI(3)K in NK cells.

IL-15 is a pivotal cytokine that controls various aspects of NK cell biology. We found that IL-15 controlled both steady-state mTOR activity and activation-induced mTOR activity in NK cells. Several groups have reported that different degrees of bioavailability of IL-15 trigger distinct effects on NK cells, ranging from the induction of cell survival at low concentrations to the induction of activation and proliferation at higher concentrations[14,16,25]. The molecular basis of this phenomenon, however, remains unclear. Here we found that the Jak-STAT5 and mTOR pathways were activated by different doses of IL-15. We thus hypothesize that the different outcomes resulting from varying strengths of IL-15 signaling are due to the relative involvement of these two pathways. When triggered alone, STAT5 would be expected to control cell viability[17,46], while activation of both pathways together would be responsible for proliferation and activation. This model is supported by the finding that deletion of mTOR marginally affected the viability of NK cells but impaired their proliferation and activation.

The phenotype of NK-*Mtor*$^{-/-}$ NK cells correlates well with the metabolic requirements of NK cells during development and activation. Indeed, NK cell differentiation was associated with the onset of cell quiescence, as shown by the decrease in cell size and uptake of glucose concomitant with loss of the transmembrane nutrient receptors CD71 and CD98. These changes were regulated transcriptionally, since we observed higher expression of genes encoding molecules in categories of metabolic terms associated with decreased cell cycling and increased catabolism along differentiation. We propose that deletion of mTOR blocks subsequent differentiation by blocking early steps of NK cell development associated with proliferation. In this context, the developmental defect observed in NK cells obtained from mice under caloric restriction might be due to partial inhibition of mTOR[47]. Moreover, it has become evident that metabolic regulators control effector T cell function[18]. We made a similar observation with NK cells, since deletion of mTOR greatly diminished their ability to be activated in response to treatment with poly(I:C). Indeed, despite their normal upregulation of the expression of early markers such as CD69, NK-*Mtor*$^{-/-}$ NK cells had lower expression of granzyme A and granzyme B. Moreover, the responses of mTOR-deficient NK cells to the triggering of activating receptors on NK cells and in particular degranulation were completely insensitive to injection of poly(I:C). Therefore, mTOR controls a key checkpoint for the activation of NK cells. How mTOR mediates these effects remains a field for future investigation. We found that IL-15-mediated activation of NK cells induced a substantial increase in glycolysis and respiration. These essential bioenergetic pathways probably support the enhanced energetic demands associated with cellular activation and proliferation. Moreover, mTOR is known to increase mRNA translation both qualitatively and quantitatively through inactivation of 4EBP1 (ref. 48). This could be particularly relevant for NK cells that contain large amounts of untranslated mRNA encoding effector molecules such as granzyme B or perforin[25]. Finally, mTOR also regulates rearrangements of the cytoskeleton through the kinase PKC-α (ref. 49), which could be important for cell-cell interactions required for proper activation of NK cells. For example, the role of the 'trans-presentation' of IL-15 by dendritic cells to NK cells during inflammation is well described in the literature[50].

Rapamycin is a clinically approved inhibitor of mTORC1 that is used mainly in renal transplantation, in the treatment of renal and breast cancers and in the treatment of afflictions such as tuberous sclerosis[33]. Several derivatives of rapamycin and ATP-competitive inhibitors of the active site of mTOR have also been developed and tested clinically. mTOR is also viewed as a potential target for anti-aging therapy, and some of the aforementioned inhibitors could be used on healthy patients[33]. Given our results obtained with mice, we tested the effects of some of these inhibitors on the cytotoxicity of NK cells. Such treatment resulted in inhibition of the induction of granzyme B following stimulation with IL-15 *in vitro*. *In vivo* treatment with Sirolimus (a clinical form of rapamycin) noticeably

decreased the cytotoxicity of NK cells toward missing-self target cells. Notably, these results could be 'translated' to humans, since activation of NK cells from healthy donors was inhibited by inhibitors of mTOR; this emphasizes the evolutionarily conserved role of this kinase in the control of NK cell cytotoxicity. These results could have broad implications in the design of therapies that target mTOR.

In summary, our findings have revealed mTOR as an essential part of the IL-15 signaling pathway in NK cells that controls two key checkpoints of their biology: development in the bone marrow and activation in the periphery. Moreover, we have expanded to NK cells the well-documented role of rapamycin as an immunosuppressant of adaptive immunity. Given the frequent therapeutic use of inhibitors of mTOR, our findings might have direct clinical applications.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.M., J.C.-V., C.V., S.D., S.V., A.F., J.R., K.M. and A.T. did experiments; T.W. designed the study with the help of A.M., J.B., Y.-G.G., E.G. and E.V.; and A.M. and T.W. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. This study was carried out in strict 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. Wild-type C57BL/6 mice were from Charles River Laboratories. *Nkp46*-iCre mice[27] were crossed with *Mtor*$^{lox/lox}$ mice[26] and bred in animal facility of the T.W. laboratory. Female mice 8–24 weeks of age were used unless specified otherwise. For some experiments, mice were given intraperitoneal injection of 150 µg poly(I:C) (polyinosinic-polycytidylic acid; Invivogen) and were killed 4 or 18 h later. For infection with MCMV, mice were infected intraperitoneally with 50,000 plaque-forming units of MCMV, Smith strain.

Flow cytometry. Single-cell suspensions of BM, blood, spleen and liver were obtained and stained. Foxp3 Fixation/Permeabilization Concentrate and Diluent (BD) were used for intracellular staining of E4BP4, T-bet, Eomes, Ki67, granzyme A and granzyme B. Cytofix/Cytoperm (BD Biosciences) was used for intracellular staining of mTOR. Lyse/Fix and PermIII buffers (BD Biosciences) were used for intracellular staining of phosphorylated proteins. For analysis of the incorporation of BrdU, BrdU (2 mg per mouse) was injected intraperitoneally on days 0 and 1, mice were killed on day 2 and incorporation was measured with a kit (Becton-Dickinson). Cell viability was measured by staining with annexin V and 7-AAD (7-amino-actinomycin D; BD Biosciences). A FACSCanto, LSR II or FACSFortessa (all from Becton-Dickinson) or a Navios 5 (Beckman Coulter) was used for flow cytometry. Data were analyzed with FlowJo software (Treestar).

Cell culture and stimulation. Splenic lymphocytes were prepared and then were cultured for 4 h with cytokines (recombinant mouse IL-15 (100 ng/ml), recombinant mouse IL-12 (25 ng/ml), recombinant mouse IL-18 (5 ng/ml), human TGF-β1 (5 ng/ml) or recombinant mouse IL-7 (20 ng/ml; all from R&D Systems) or recombinant mouse IFN-β, 100 U/ml (PBL)) or on antibody-coated plates (anti-NKp46 (29A1; BD Biosciences), anti-NK1.1 (PK136; BD Biosciences), anti-Ly49D (4E5; BD Biosciences), anti-NKG2D (CX5; BD Biosciences), anti-Ly49C/I (5E6; BD Biosciences) or anti-NKG2A (20D5; all from BD Biosciences; all at a concentration of 10 µg/ml) and GolgiStop (BD Biosciences) in the presence of anti-CD107a (1D4B; BD Biosciences)). Cell surfaces were stained with anti-NK1.1 (PK136; BD Biosciences) and anti-CD3 (2C11; BD Biosciences), followed by intracellular staining with anti-IFN-γ (XMG1.2; BD Biosciences) before analysis by flow cytometry.

Whole blood samples were collected from healthy human donors by venipuncture into heparin-containing vials. Peripheral blood mononuclear cells were then isolated by Ficoll gradient centrifugation and were stimulated for 36 h at 37 °C in 1,000 UI/ml recombinant human IL-2 in the presence or absence of mTOR inhibitors. The FoxP3 Fixation/Permeabilization Concentrate and Diluent (eBioscience) was used for intracellular staining to assess phosphorylated S6, granzyme B and perforin.

Rapamycin (10–100 nM; Sigma-Aldrich), PP242 (1 µM; Sigma-Aldrich) and Ku-0063794 (3 µM; Stemgent) were used.

Treatment with TatCre and adoptive transfer. A mixture of spleen cells from CD45.1$^+$ C57BL/6 and CD45.2$^+$ *Mtor*$^{lox/lox}$*Nkp46*-iCre (NK-*Mtor*$^{-/-}$) mice (1:1) was treated for 45 min at 37 °C *in vitro* with TatCre (50 µg/ml; Excellgene) in medium without serum. The cells were then washed and were injected retro-orbitally into recipient CD45.1$^+$CD45.2$^+$ C57BL/6 mice (1×10^7 to 2×10^7 cells per mouse). 2 d later, some recipient mice were given injection of poly(I:C), while other were left untreated, as controls. All mice were killed on the next day, and transferred cells present in the spleen were analyzed.

Antibodies. The following monoclonal antibodies were used: anti-CD19 (ebio1D3), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CXCR4 (2B11), anti-Ly49G2 (4D11), anti-CD69 (H1.2F3), anti-IFN-γ (XMG1) and anti-CD107a (1D4B; all from BD Biosciences); anti-NK1.1 (PK136) and anti-CD27 (LG.7F9; both from BioLegend); anti-CD3 (145-2C11), anti NKp46 (29A1.4), anti-CD11b (M1/70), anti-CD122 (5H4 or TMb1), anti-CD132 (TUGh4), anti-CD127 (A7R34), anti-CXCR3 (CXCR3-173), anti-Ly49EF (CM4), anti-Ly49D (4e5), anti-Ly49H (3D10), anti-NKG2ACE (20d5), anti-NKG2D (CX5), anti-CD71 (C2), anti-CD98 (RL388), anti-KLRG1 (2F1), anti-CD49b (DX5), anti-CD244 (ebio244f4), anti-E4BP4 (S2M-E19), anti-T-bet (ebio4B10), anti-Eomes (Dan11mag), anti-granzyme B (NGZB) and anti-Ki67 (SolA15; all from eBioscience); anti-CCR2 (475301; R&D Systems); and anti–granzyme A (3G8.5; Santa Cruz Biotechnology); and the relevant isotype-matched control antibodies rat IgG1 (eBRG1), rat IgM (eBRM), rat IgG2a (eBR2a), rat IgG2b (eB149) and hamster IgG (ebio229; all from eBioscience). Anti-mTOR (2983) was from Cell Signaling Technologies. For human cells, the following monoclonal antibodies were used: anti-CD3 (UCHT1; BD Biosciences), anti-CD14 (RMO52; BD Biosciences), anti-CD19 (HD237; Beckman-Coulter), anti-CD56 (HLDA6; eBioscience), anti–granzyme B (GB11; Life Technologies) and anti-perforin (ΔG9; eBioscience). Antibodies to phosphorylated proteins were as follows: antibody to Akt phosphorylated at Ser473 (M89-61; BD Biosciences), antibody to STAT5 phosphorylated at Tyr694 (47/Stat5(pY694); BD Biosciences), antibody to phosphorylated S6 (5316; Cell Signaling Technologies), antibody to phosphorylated 4EBP1 (7547; Cell Signaling Technologies), antibody to Akt phosphorylated at Thr308 (2965; Cell Signaling Technologies), antibody to mTOR phosphorylated at Ser2448 (5536) and antibody to mTOR phosphorylated at Ser2481 (2974; Cell Signaling Technologies).

IL-15 activity was neutralized *in vivo* by blockade of the IL-2–IL-15Rβ subunit with monoclonal anti-IL-2Rβ (TM-b1; Bio X Cell) and control monoclonal antibody (clone LTF-2; Bio X Cell). F(ab)′ was prepared with a Pierce F(ab)′2 Micro Preparation Kit (Thermo Scientific). Mice were given injection of 0.5 mg of F(ab)′ 4 h before analysis. In some experiments, mice were fed Sirolimus (Rapamune; Wyeth Europa) by oral gavage at a dose of 100 µg per day for the appropriate number of days. Gut cells were prepared as described[4].

NK cell purification. NK cells were purified with biotinylated anti-CD3 (2C11), anti-CD19 (ebio1D3), anti-CD5 (53-7.3), anti-CD24 (M1/69), anti-F4/80 (BM8) and anti-Ly6G (1A8; all from eBioscience), which were then recognized by anti-biotin beads (Miltenyi) before passage onto an AutoMACS (Miltenyi) with the DepleteS program.

Assessment of glucose uptake. Glucose uptake was measured with 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose; Invitrogen). Freshly isolated cells were resuspended in RPMI-1640 medium (Life Technologies) in the presence of 100 µM 2-NBDG and were cultured for 10 min at 37 °C, then surface markers (CD3, NK1.1, CD11b and CD27) were stained (antibodies identified above).

Seahorse analysis. The OCR and ECAR were measured in XF medium (unbuffered DMEM containing 2 mM glutamine, pH7.4) under basal conditions and in response to glucose (25 mM), oligomycin (1 µM), FCCP (1.5 M) plus pyruvate (1 mM) and antimycin A (1 µM) plus rotenone (0.1 µM) with an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). NK cells activated with IL-15 (100 ng/ml) or resting NK cells were purified and plated (350,000 cells per well) in Seahorse plates coated with CellTak (Corning). After adhesion, the OCR and ECAR were analyzed in real time during 155 min.

***In vivo* cytotoxicity assay.** Splenocytes from C57BL/6 or β_2-microglobulin-deficient mice were labeled with CellTraceViolet (1 µM; Invitrogen) or CFSE (carboxyfluorescein diacetate succinimidyl ester; 5 µM; Invitrogen), respectively, and 5×10^6 to 10×10^6 cells were transferred into recipient mice by intravenous injection. 14 h after transfer, splenocytes were isolated and analyzed by flow cytometry. The abundance of the remaining β_2-microglobulin-deficient cells was calculated by the following formula: % remaining cells = 100 × (β_2-microglobulin-deficient cells/wild-type cells) at 14 h/(β_2-microglobulin-deficient cells/wild-type cells) in input mix.

Gene-set enrichment analysis. Various sets of public expression data were used for the identification of genes whose expression was modulated upon differentiation or activation of NK cells. For statistical analysis of whether gene sets were enriched in specific conditions, we made pairwise comparisons of conditions by the gene-set enrichment analysis method (with software from the GSEA website of the Broad Institute. Enrichment with an false-discovery rate of <0.1 of was considered significant.

Statistical analysis. Two-tailed *t*-tests or nonparametric tests, where appropriate, were used for statistical analyses. These tests were run on Prism software (GraphPad).

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5. Article 6 : Natural Killer cells from Multiple Myeloma patients display an exhausted phenotype and their functions are not improved by lenalidomide treatment.

Natural Killer cells from Multiple Myeloma patients display an exhausted phenotype and their functions are not improved by lenalidomide treatment.

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Abstract

Multiple myeloma (MM) is a proliferation of tumoral plasma B cells that is still incurable. Natural Killer (NK) cells can recognize and kill MM cells *in vitro*, and can limit MM growth *in vivo*. Previous reports have suggested that NK cell function is impaired during MM progression but that treatments with immunomodulatory drugs (IMIDs) such as lenalidomide (LEN) could enhance it. However, available data remain fragmentary and incomplete and the effects of IMIDs have been tested mostly *in vitro*. Here, we set up a standardized flow cytometry procedure to monitor NK cell activity in fresh blood samples from patients during MM progression, after frontline induction chemotherapy (CTX) and longitudinally, after maintenance therapy with LEN. We found that peripheral NK cells from MM patients at diagnosis have an exhausted phenotype characterized by the up regulation of activation markers and by a decreased reactivity and polyfunctionality, associated with a decreased expression of NKp30 and 2B4. Following frontline chemotherapy, peripheral NK cells displayed a very immature phenotype and retained poor reactivity towards target cells *ex vivo*, but activation markers and NKp30 and 2B4 were normalized. Finally, upon maintenance treatment with LEN, we observed a progressive normalization of NK cell maturation, likely caused by discontinuation of chemotherapy. However, LEN treatment neither activated NK cells nor improved their effector functions. These results caution against the use of LEN as single therapy to improve NK cell activity in patients with cancer and provide a rationale to combine LEN with other drugs such as cytokines for this purpose.

Introduction

Multiple Myeloma (MM) is a genetically heterogeneous disease characterized by a proliferation of tumoral plasma B cells[1]. MM is among the most frequent hematologic malignancies and induces anemia, bone lesions, amyloidosis and renal dysfunction. Despite the advent of several novel dedicated therapies, MM remains of poor prognosis, because most patients become refractory to treatments[2]. MM frequently progresses from a premalignant stage called monoclonal gammopathy of undetermined significance (MGUS). Upon diagnosis, patients present either smoldering (or indolent) asymptomatic MM (hereafter referred as MM1) or symptomatic MM (MM2) with organ damages that require immediate treatment. Efforts are currently made to understand genetic and other factors that influence progression of indolent to active MM, and to find treatments that could prevent or delay this progression[1].

Cytotoxic lymphocytes such as Natural Killer (NK), NKT and CD8 T cells are important players of tumor immune surveillance. NK cells mediate various anti-tumor functions, including granule-dependent cytotoxicity, secretion of cytokines like TNF-α and IFN-γ as well as secretion of CCL3-5 chemokines, that have distinct roles during immune responses[3]. NK cells recognize tumor cells through an arsenal of activating or inhibitory receptors. They can also recognize and kill antibody-coated target cells through CD16, the low affinity activating receptor for the Fc fragment of immunoglobulins G (IgG), a process called antibody-dependent cellular cytotoxicity (ADCC)[4]. The efficacy of many therapeutic antibodies is supposed to be dependent on ADCC[5]. NK cells have long been shown to recognize and kill MM cells *in vitro*. HLA, and activating receptors NKG2D, DNAM1 and NKp46 are important for the recognition of MM cells by NK cells[6,7]. A recent study using a novel mouse model of MM[8] has demonstrated the existence of a robust anti-myeloma immune response mediated both by NK and CD8 T cells. In this study, the authors found a prominent role of DNAM1 in this immune response[9]. However, as for many tumors, other articles have suggested a gradual alteration of peripheral NK cell activity during MM progression. Reported defects include decrease in NKG2D expression[10,11], decrease in CD16 and 2B4 expression[12] and decreased *ex vivo* cytotoxicity against K562 cells[13]. Peripheral NK cell counts have also been shown to increase, including at

MGUS stages[13,14]. Yet, data on NK cell status in MM patients are scattered, several articles show conflictive results and multiparametric analyses of NK cell phenotype and function during MM progression are missing[15]. A better understanding of NK cell activity at different MM stages could provide rationale for starting immunotherapies or novel antibody treatments such as elotuzumab (anti-SLAMF7)[16] which anti-myeloma function is presumably dependent on ADCC activity of NK cells.

Lenalidomide (LEN) is part of the immunomodulatory (iMIDs) drugs and is a synthetic derivative of thalidomide currently approved by regulatory agencies for the treatment of MM, in combination with dexamethasone. LEN presumably targets the ubiquitin ligase Cereblon (CRBN), and CRBN is required for LEN anti-tumor function[17]. The mechanisms of LEN actions in MM include direct anti-tumor effects, effects on tumor stroma and angiogenesis and a positive effect on the anti-tumor immune response[18]. LEN affects many cell types and in particular enhances the anti-tumor functions of NK and NKT cells[19]. This could be due to several mechanisms. First LEN could suppress the production of inhibitory cytokines, and promote the production of IL-2, an NK-cell growth factor, by T cells[20]. The latter effect could be the result of induced degradation of Ikaros and Aiolos, two transcription factors repressing IL-2 production in T cells[21]. LEN could also operate directly as a recent article demonstrated that LEN enhanced cytotoxicity and IFN-γ production by purified NK cells stimulated through various receptors, in the presence of stimulatory concentrations of IL-2[22]. The proposed mechanism involves nanometer-scale rearrangement of the actin cytoskeleton at the immune synapse even though LEN targets were not identified in this context. Importantly, in this study, LEN alone had limited activity[22], thus showing that indirect effects on IL-2 production are mandatory for improvement of NK cell cytotoxicity. Despite this accumulating evidence of the stimulatory activity of LEN on immune cells *in vitro* or in mouse preclinical models, very few studies have addressed the effect of LEN on immune cells in LEN-treated MM patients. One longitudinal study did not report any effect of LEN on NKT cells in a small number of patients[23]. Another one reported weak signs of NK cell activation one month after the start of LEN as maintenance therapy, but the interpretation of the results was complicated by the prior allogenic stem cell transplantation of all patients and the discontinuation of immunosuppressive therapy used to reduce GVHD at the time of LEN treatment[24]. Thus, a stimulatory effect of LEN on NK cell activity in human *in vivo* remains to be formally proven.

Here we monitored NK cell phenotype and function in patients during MM progression, after frontline induction chemotherapy (CTX) and longitudinally, after maintenance therapy with LEN in the absence of any other treatment. We report that MM progression is significantly associated with an activated status of NK cells. However NK cells from MM patients display reduced expression of activating receptors such as NKp30 and 2B4 and a reduced *ex vivo* reactivity to K562 or rituximab-coated B cell lines. Induction CTX had a profound negative effect on NK cell maturation and ADCC but restored some of the defects observed during MM progression. Finally, LEN treatment had no objective effect on NK cell activation or anti-tumor function in this maintenance therapy regimen. These data thus suggest that LEN alone is not sufficient to enhance anti-tumor NK cell function in patients.

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Results

Progression to multiple myeloma correlates with phenotypic NK cell activation but reduced *ex vivo* effector functions

To better understand the impact of MM on NK cells, we monitored the immune phenotype and effector functions of peripheral NK cells in patients at different stages of multiple myeloma using flow cytometry. To maximize the relevance of our analysis, we only used fresh blood samples. We therefore used a standardization procedure to minimize variability caused by staining procedure and instrument measures between experiments (see methods). We measured the expression of a wide range of activating or inhibitory NK cell receptors ("surface receptors" category Figure 1A), other surface receptors or intracellular proteins that mark NK cell activation or cytotoxic potential ("activation markers" category, Figure 1B). We also performed a functional analysis, again optimized to reduce variability between experiments. Degranulation as measured by CD107a exposure and IFN-γ, TNF-α and MIP1-β secretion were measured upon a 4-hour *ex vivo* culture without stimulus (spontaneous response) or in the presence of K562 cells or Granta B cells coated with rituximab anti-CD20 antibody, to measure natural cytotoxicity and antibody-dependent cell cytotoxicity, ADCC respectively. Two types of measurements were performed: frequency of individual responses (degranulation or cytokine secretion) in the K562, Granta or medium condition (Figure 1C) and frequency of polyfunctional NK cells (two or three functions simultaneously, only for K562 and Granta culture conditions). Functional parameters were grouped in the "anti-tumor function" category except for the spontaneous production of cytokines and spontaneous degranulation that were considered as "activation markers". Finally, we also measured the frequency of various immune subsets, including NK cells in the blood (Figure 1D).

Three groups of patients were analyzed, of increasing disease severity: monoclonal gammapathy of undetermined significance (MGUS), smoldering MM (MM1), symptomatic MM at diagnosis (MM2). Patient characteristics are shown in Table 1. MM1 and MM2 patients differed by the degree of bone marrow involvement. Importantly, none of these patients had any anti-tumor treatment at the time of NK cell analysis. Healthy volunteers (HV) matched in sex and ages were also analyzed.

Figure 2A-B shows mean values of each parameter calculated for each group of patients, and presented as heatmaps. Each group of patients was compared to the HV group with a logistic regression model in order to determine significant parameter changes induced by MM progression. Figure S1 shows graphs of individual data for parameters that showed significant variation between groups. Figure 2C shows actual FACS plots for typical individuals in each group, for functional parameters and for CD69 and NKp30. Overall, our data show a gradual and general decrease in NK cell "anti-tumor function" during myeloma progression well visualized in the heatmap figure 2A. The frequency of polyfunctional NK cells was particularly altered in MM2 patients, especially when stimulated with Granta cells, even though the level of CD16 was not significantly altered in NK cells from MM2 patients. The response to K562 cells was also decreased, which correlated with a decrease in NKp30, a receptor previously found to be important for the recognition of K562 cells[25]. When analyzing "activation markers", we observed a wide up regulation of those in the MM2 group compared to HV, which was statistically significant for the percentage of CD69 or perforin positive NK cells, the percentage of NK cells spontaneously producing TNFα, and the MFI of GZMB and NKG2C. The percentage of NKG7 expressing NK cells was also reduced. In terms of "surface receptors", besides the above mentioned change in NKp30 expression, we also observed a significant decrease in the percentage of NK cells expressing 2B4. Of note, NKG2D and DNAM1, whose expression were previously shown to be down regulated in MM patients[7,10,11] or important for NK cells anti-MM response[9], were not changed in our study. Regarding the numeration of main subsets within PBMC (Figure 2B), the most noticeable observation was a significant decrease in B cell frequency and numbers in MM1 patients. The $CD56^{bright}$ to $CD56^{dim}$ NK cells ratio was not significantly different between the groups, neither were the percentage of $CD57^{+}$ or $CD94^{+}$ NK cells, showing that myeloma progression does not impact on NK cell maturation, which could have explained the functional results. Interestingly, when looking at the parameters that significantly correlate with NK cell poly-functionality within HV (Table 2), none of them was decreased during myeloma progression, showing that the mechanism decreasing NK cell function in MM2 patient is different from the one explaining natural variation in HV.

Next, we sought to test the effect of tumor mass on the different NK cell parameters. We used the measured amount of monoclonal Ig at diagnosis in the

MM2 group as correlate of tumor mass. Figure 3 shows that the highest correlation was observed for the percentage of NKp30 expressing cells that decreased during MM progression and the percentage of CD57 that increased during progression. Significant correlations were also observed for %NKG2C, MFI GzmB and %NKG2D that increased during MM progression, confirming the activated status of NK cells in advanced stages of MM, while %NKp46 also decreased during progression.

Altogether, these data show that progression from indolent to symptomatic MM is associated with an increased NK cell activation status, but a decreased *ex vivo* reactivity to K562 or Granta cells and an alteration of their phenotype with the most prominent effect on NKp30, suggesting that NK cells from MM patients become exhausted as a result of continuous triggering by MM cells, perhaps through NKp30.

Induction CTX partly restores NK cell activity but reduces NK cell maturation

Next, we investigated the effect of anti-myeloma treatments on NK cell status. We first focused on the effect of induction/consolidation CTX. Our patients were enrolled in a clinical trial conducted by the Intergroupe Francophone du Myélome (IFM group, protocol IFM/DFCI 2009, http://www.af3m.org/ressources/10185/43/1.pdf). The induction/consolidation CTX consisted in either 8 cycles of bortezomib, dexamethasone and LEN (VRD for Velcade / Revlimid / Dexamethasone) or in autologous stem cell transplantation (SCT), followed by 2 VRD cycles (Table 3 and methods)[26]. Blood samples were obtained from patients one month after the end of the last VRD cycle ("post-VRD group") and NK cell parameters were compared to those of HD and MM2 patients. The induction/consolidation CTX had a profound impact on NK cell maturation, as assessed by the percentage of NK cells expressing CD94 and CD57[27] (Figure 4A), which probably reflects the elimination of most mature NK cells during CTX and the progressive reappearance of neo-developed NK cells. The CTX treatment normalized some activation parameters (CD69 and NKG2C) but not GZMB and perforin that were even increased after induction CTX (Figure 4B). The expression of NCRs NKp30 and NKp46 was also normalized, which could reflect the fact that more immature NK cells express higher levels of both receptors. The skewed NK cell maturation in the CTX group could also explain the reduced expression of various other receptors associated with NK cell maturation such as KIR, DNAM1, and CX3CR1. NK cell reactivity to K562 cells, which is known to be dependent on NKp30[25] was restored by CTX but NK cell reactivity to Granta cells

was not. The latter effect could be due to the reduced percentage of CD16 expressing cells among neo-developed immature NK cells.

Thus, induction/consolidation CTX has a profound effect on the peripheral NK cell compartment, overcoming the initial effect induced by MM cells, but altering overall maturation and consequently narrowing the repertoire of NK cell receptors expressed.

Longitudinal study of the impact of LEN on NK cell phenotype and function

LEN is often cited as an NK cell-stimulating agent, based on in vitro data or on experiments in mouse models. However, evidence that LEN stimulates NK cell activity in patients is still lacking. To test this point, we took advantage of the protocol IFM/DFCI 2009. In both arms of the study, induction and consolidation CTX were indeed followed by maintenance treatment with LEN as single agent for one year, starting one month after the last VRD cycle (Table 3). We monitored NK cell phenotype and function before and during treatment with LEN, on a monthly basis over the one-year treatment. We also monitored NK cell phenotype and function after the end of the last LEN cycle (on average 6 months after the last LEN cycle). The most striking effect we observed over the treatment was a gradual increase in NK cell maturation status, as shown by a decrease in CD94 or NKp30 positive cells (markers of immature NK cells) and an increase in CD57 or CD16 positive cells (markers of mature NK cells, Figure 5A-B). Changes in KIR2DL1 and NKp46 could also fit within this scheme. This effect was however likely not due to LEN treatment but rather to the progressive return to immune homeostasis following induction/consolidation CTX or SCT (blue dots in figure 5A). Indeed, the effect was not immediate following the start of LEN treatment and persisted after LEN discontinuation. Regarding activation markers, we noted a progressive increase in the percentage and MFI of NKG2C expression during LEN treatment. This effect was however not associated with an increase in any other activation marker, and persisted after LEN discontinuation, suggesting that it was not induced by LEN. We didn't monitor human cytomegalovirus (HCMV) replication but we cannot exclude that HCMV reactivation could explain these results, as HCMV is known to drive the expansion of NKG2C positive NK cells[28]. Spontaneous production of TNF-α significantly decreased following the start of LEN therapy. However, this production was anyway very low at all time points. When looking at anti-tumor function, we noted a significant decrease

in NK cell reactivity to K562 cells upon LEN treatment, irrespective of the parameter analyzed. This decrease correlated with a progressive decrease in NKp30 expression, itself associated with the gradual return to NK cell homeostasis. ADCC, which was already low before LEN therapy, was not improved by LEN treatment, despite a progressive increase in the percentage of CD16 expressing cells. Finally, not a single parameter showed clear-cut changes in expression upon discontinuation of LEN treatment, which clearly indicates the lack of LEN effect on peripheral NK cells, at least in this clinical setting.

Discussion

Here, using a multiparametric approach, we demonstrated that peripheral NK cells gradually acquired an activated phenotype during MM progression with the acquisition of CD69, the upregulation of GzmB and NKG2C and spontaneous degranulation when cultured *ex vivo*. This activated phenotype was however associated with a decreased reactivity to K562 and rituximab-coated B cells, and a decreased expression of several activating receptors including NKp30 and 2B4. Moreover, CD57 expression increased as tumor mass increased, as measured by the serum level of monoclonal component. Some of these findings had been previously reported, such as decreases in 2B4[12] and decrease in NK cell function[13]. Our results also confirm previous findings that NKG2D expression on NK cells does not change during MM progression[12] even though other authors have reported a decreased NKG2D in MM patients [10,11]. The reason for these discrepancies is unclear but differences in the methods of measurement may provide an explanation. Regarding NK cell function, our study is the first to show the gradual impairment of multiple effector functions and polyfunctionality in response to different stimuli. Polyfunctionality is considered to be extremely important for anti-tumor responses. While limited information is available in the NK cell field, multiple studies have proven the importance of polyfunctional T cells for the rejection of tumors[29–31]. Importantly, our analysis shows that NK cells retain a quasi-normal function at the MM1 (smoldering MM) stage, in particular in terms of ADCC. These data therefore provide a rationale for treating the high-risk MM1 patients with therapeutic antibodies such as elotuzumab or others[16]. The decrease in polyfunctionality in NK cells from MM2 patients contrasts with the presence of multiple activation markers. The occurrence of early activation markers such as CD69 or GZMB suggest that NK cells from MM2

patients are chronically activated, presumably by tumor cells, which could lead to a form of exhaustion, as demonstrated for T cells[32]. The normal NK cell maturation status in MM2 patients also argues against senescence as a cause of the decreased reactivity[33]. The molecular mechanisms of T cell exhaustion have been extensively studied. Continuous stimulation through the TCR, engagement of inhibitory receptors such as PD-1, dysregulated balance between other immune subsets and environmental factors such as tumor-secreted molecules could all contribute to this hyporeactive state[33]. NK cell exhaustion is by contrast, poorly defined but several reports have shown that chronic infections[34,35] or tumor progression[36–40] can lead to gradual impairment of NK cell function in different contexts, despite signs of activation, a situation that is reminiscent of T cell exhaustion. The blockade of inhibitory receptors could be a valid strategy to harness NK cell potential in patients, as recently demonstrated in mouse models[41]. This also suggests that T and NK cell exhaustion are driven by similar mechanisms.

Our data show a profound effect of induction therapy on NK cell maturation and activity. This was expected given the broad myeloablative effects of CTX. The long delay -about a year- before NK cells reached a quasi-normal maturation status was however unexpected. Indeed, data in mouse models suggested a much faster kinetics following NK cell depletion[42]. This suggests that even though NK cells repopulate the periphery much faster than T cells following myelo-ablation, their maturation and functions are restored much more slowly, similar to T cells.

Importantly, our data also exclude a major stimulatory effect of LEN on NK cells when given alone. Indeed, we could not detect any signs of NK cell activation or any improvement of their functional capacity in patients undergoing several cycles of LEN treatment. This finding goes against a large number of articles describing a direct or indirect impact of LEN on NK cell activation. In particular several articles reported a positive effect of LEN on NK cell-mediated anti-tumor activity in vivo in mouse models of transplanted tumors[43–45]. However, our study is the first one to directly test the impact of LEN on multiple parameters of NK cell activation and function, longitudinally in human patients undergoing maintenance therapy with LEN alone. The absence of LEN effects on NK cells in our study correlates with the reported absence of protection conferred by LEN against infections by herpesviruses or against second primary cancers in MM patients[46,47]. As NK cells are major players of immune responses against cancer and infection by herpes viruses, a stimulatory

effect of LEN should have led to some degree of protection. It is possible that NK cells from patients that underwent previous induction CTX are not responsive to LEN because of excessive damage on immune cells or on their environment. Induction cycles include also dexamethasone that has been shown to counter LEN effects on NK cells[48]. However, all patients in this study started their LEN maintenance therapy at least one month after the end of induction CTX, which left them some time to recover from it and also to be cleared of dexamethasone. We therefore propose that LEN is not by itself sufficient to stimulate NK cell activity in patients and that it should be combined with more potent and directly stimulating drugs. Among potential candidates, ALT-803, an IL-15 derivative or the H9 IL-2 derivative show very interesting results in different mouse models[49–51]. The synergy between LEN and IL-2 on NK cell activity *in vitro*[22] also supports this combination. Immumodulatory antibodies targeting immune checkpoints[52] and anti-KIR antibodies could also improve the LEN effects on NK cells. A Phase I Trial of the Anti-KIR Antibody IPH2101 and LEN was recently reported in Patients with Relapsed/Refractory MM[53]. This was based on preclinical data in a mouse models showing the efficacy of a LEN/anti-Ly49C combination[54].

In conclusion, we have shown that MM progression induces gradual exhaustion of peripheral NK cells, characterized by up regulation of activation markers but impairment of natural killing, ADCC and polyfunctionality associated with a decreased expression of several activating receptors. Moreover, we performed the first exhaustive monitoring of NK cell activity in patients taking LEN as single therapy. Our results unambiguously show a lack of stimulatory effect of LEN on peripheral NK cell activity. These results caution against the use of LEN as single immunomodulatory therapy to improve NK cell activity in patients with cancer and provide a rationale to combine LEN with other drugs such as cytokines or monoclonal antibodies for this purpose.

Material and methods

Patients and samples

MGUS, MM1, MM2 patients treated at the Lyon-Sud Hospital were prospectively recruited on diagnosis between 2011 and 2013. Blood was sampled before administration of any treatment related to tumor progression. Blood samples were also collected following ethical approval and informed consent. HV were from EFS Lyon and were over 50 years old. For the treatment part, patients were recruited in the context of the IFM/DFCI 2009 trial. This trial compares the combination of 8 VRD cycles versus ASCT + 2 VRD cycles in the treatment of MM. In both arms of the trial, patients received LEN as single maintenance therapy (12 cycles during one year). Our study was not interventional and blood samples were collected at the same time as those required for the trial, with an additional informed consent.

All blood samples were received and processed within 4 hours after collection. Whole blood was stained for phenotypic markers and PBMC were obtained using ficoll. PBMC were stored at 4°C and stimulated the next day. In most cases, 1-3 blood samples from patients were received and processed per day. To avoid any bias in subsequent comparisons, blood samples from healthy donors were also processed by groups of 3 samples maximum.

Phenotypic analysis of NK cells and absolute counts

We stained fresh whole blood with four different antibody panels covering the whole range of markers analyzed (100 μL per staining, Table S1). All antibodies have been previously titrated and whenever possible, the same antibody lots have been used for staining all patients and controls. Intracellular stainings were performed after staining for cell surface markers using Cytofix/Cytoperm from BD biosciences. Tubes were run on a Beckman-Coulter Navios instrument. To allow comparisons between experiments, we always used the same FACS settings. We performed routine testing of the instrument using Rainbow calibration particles (Spherotech) using these FACS settings, to check for laser parameters, and if appropriate, PMT were slightly modulated to compensate for laser variations. Whenever possible, a minimum of 3000 NK cells were acquired for each sample. Data were analyzed using Kaluza (BeckmanCoulter) software. NK cells were defined $CD3^-$ $CD4^-$ $CD19^-$ $CD56^+$ cells.

Absolute lymphocyte subsets counts were determined by multiplying the percentage of the subset among lymphocytes by the absolute lymphocyte counts determined using the hematological analyzer SYSMEX (Roche).

Functional analysis of NK cells

PBMC were co-cultured for 4 h at a 1:1 ratio with K562 cells or Granta B lymphoma cells (ATCC) or cultured alone ("no stim" condition). Granta cells were incubated with anti-CD20 antibody (Rituximab, Hoffman Laroche) for 30 minutes at 4°C prior to stimulation. Golgi-stop (BD-biosciences) was added after one hour of culture. Cells were then stained for surface markers before staining for intracellular cytokines (panel 4 in Table S1). During the setup phase of this study, we noticed a significant drop in NK cell responses when stimulating PBMC with overgrown K562 or Granta cells, or over successive passages. For this reason, we made a stock of frozen K562 and Granta aliquots at the beginning of the project and never kept them in culture for more than two months. Moreover, cell stimulations were always performed during the next two days after target cell passage. To analyze polyfunctionality, we used FlowJo (Treestar) and the "boolean gates" function. As TNF-a production was negligible, we focused on IFN-γ, CD107a and MIP1-β. The percentage of NK cells with only one function was obtained by adding percentages of IFN-γ^+ CD107a$^-$ MIP1-β^-, IFN-γ^- CD107a$^+$ MIP1-β^- and IFN-γ^- CD107a$^-$ MIP1-β^+, the percentage of NK cells with two functions was obtained by adding percentages of IFN-γ^+ CD107a$^+$ MIP1-β^-, IFN-γ^- CD107a$^+$ MIP1-β^+ and IFN-γ^+ CD107a$^-$ MIP1-β^+. The percentage of NK cells with 3 functions was obtained by gating on cells positive for all three markers.

Statistical analyses

Statistical analysis was performed using the R computing environment, R version 3.2.3[55]. Heatmap figures were produced with the gplots package using the "heatmap.2" function. See the figure legends for details about when logistic regression, linear mixed-effects models and correlation tests were used.

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Figure legends

Figure 1: Multi-parameter flow cytometry analysis of peripheral NK cell phenotype and functions.

Whole blood from patients or healthy donors was analyzed by flow cytometry for the expression of various surface receptors (A-B) or intracellular molecules (B) by gated NK cells. Representative stainings are shown for each antibody (red) or for the FMO control (blue). We determined the percentage of positive cells for each marker by setting gates on the basis of the FMO staining. (C) PBMC were cultured for 4 hours in the presence of K562 cells or rituximab-coated Granta cells at a 1:1 ratio. Intracellular staining for IFN-γ, MIP1-β, TNF-α and surface staining for CD107a was then performed. Representative FACS plots are shown for each condition, using a concatenation plot. (D) The frequency of different lymphocyte populations was measured using the indicated antibody panels and electronic gates.

Figure 2: NK cells from MM2 patients display an exhausted phenotype

Flow cytometry analysis of the indicated parameters in peripheral NK cells from HV, MGUS, MM1 and MM2 patients. Data were obtained and analyzed as indicated in figure 1. (A-B) Parameters were clustered in functional categories “anti-tumor function”, “activation markers”, “cell surface receptors” and “cell counts and frequencies”, as labeled and displayed in heatmaps. Stars summarize significance level of the p-values adjusted for multiple testing by the Benjamini-Hochberg method. These p-values were obtained using logistic regression models which compare each patient group with the HV group. Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’1. (C) Flow cytometry data are shown for one representative patient in HV, MGUS, MM1 and MM2 groups. Top panels show CD107/IFN-γ/MIP1-β/TNF-α response in the different culture conditions and lower panels show expression of CD69 and NKp30.

Figure 3: Tumor progression further amplifies NK cell exhaustion

The correlation between each flow cytometry measurement and the concentration of the myeloma-derived monoclonal Ig, measured at diagnosis was analyzed in the MM2 patients using the Pearson correlation test, since the required hypotheses of linearity and homoscedasticity were fulfilled. Graphs show data for significant

correlations, each dot corresponds to one MM2 patient. The correlation coefficient (R) and the p-value are indicated on each graph.

Figure 4: Induction CTX impairs NK cell maturation but normalizes anti-K562 functions.

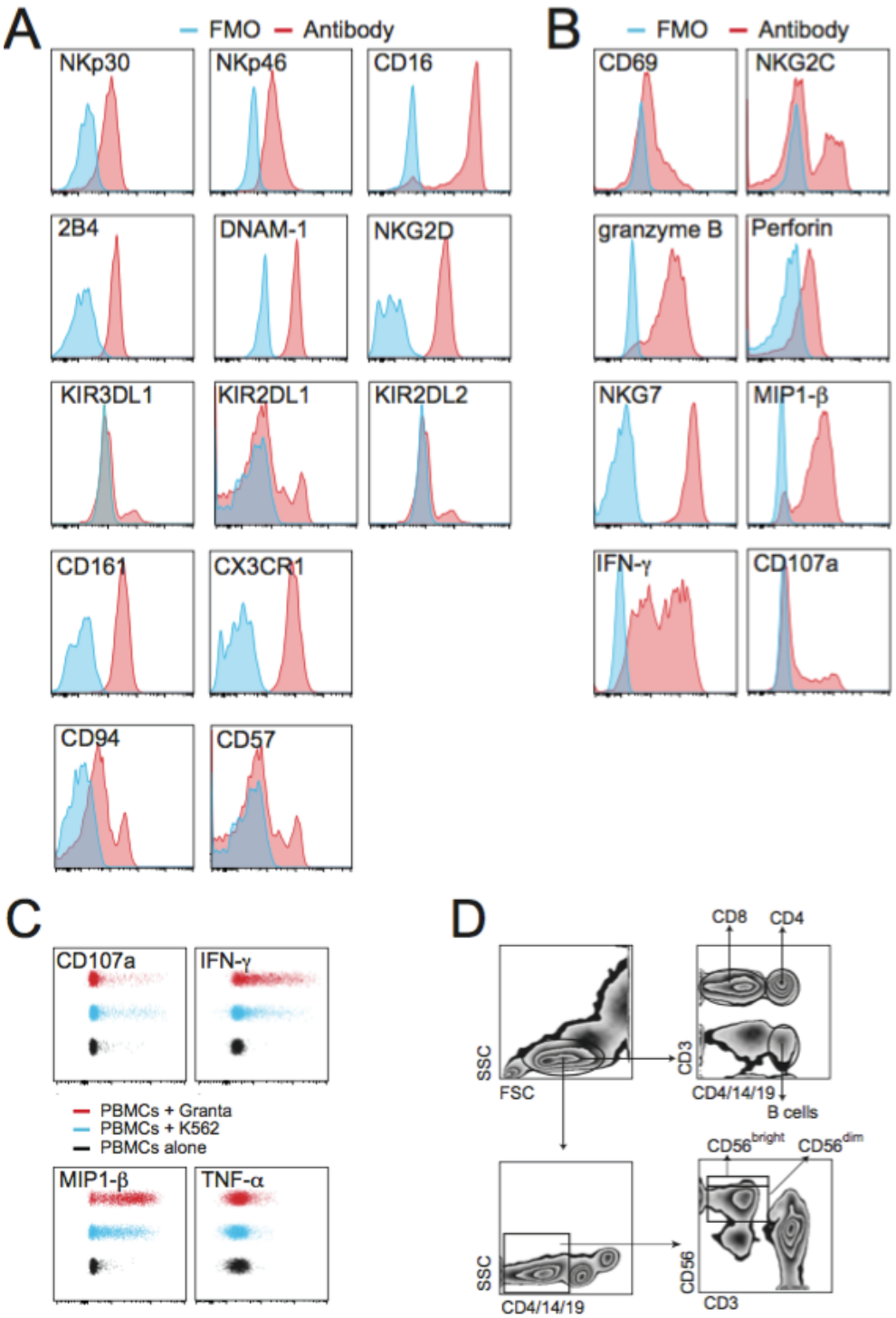
Flow cytometry analysis of the indicated parameters in peripheral NK cells from HV, MM1 and MM2 patients after induction/consolidation CTX (post-VRD). Data were obtained and analyzed as indicated in figure 1. (A-B) Parameters were clustered in functional categories “anti-tumor function”, “activation markers”, “cell surface receptors” and “cell counts and frequencies”, as labeled and displayed as heatmaps. Stars summarize significance level of the p-values adjusted for multiple testing by the Benjamini-Hochberg method. These p-values were obtained using logistic regression models, which compare each patient group with the HD group. Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’1.

Figure 5: LEN treatment neither activates NK cells nor improves their effector functions.

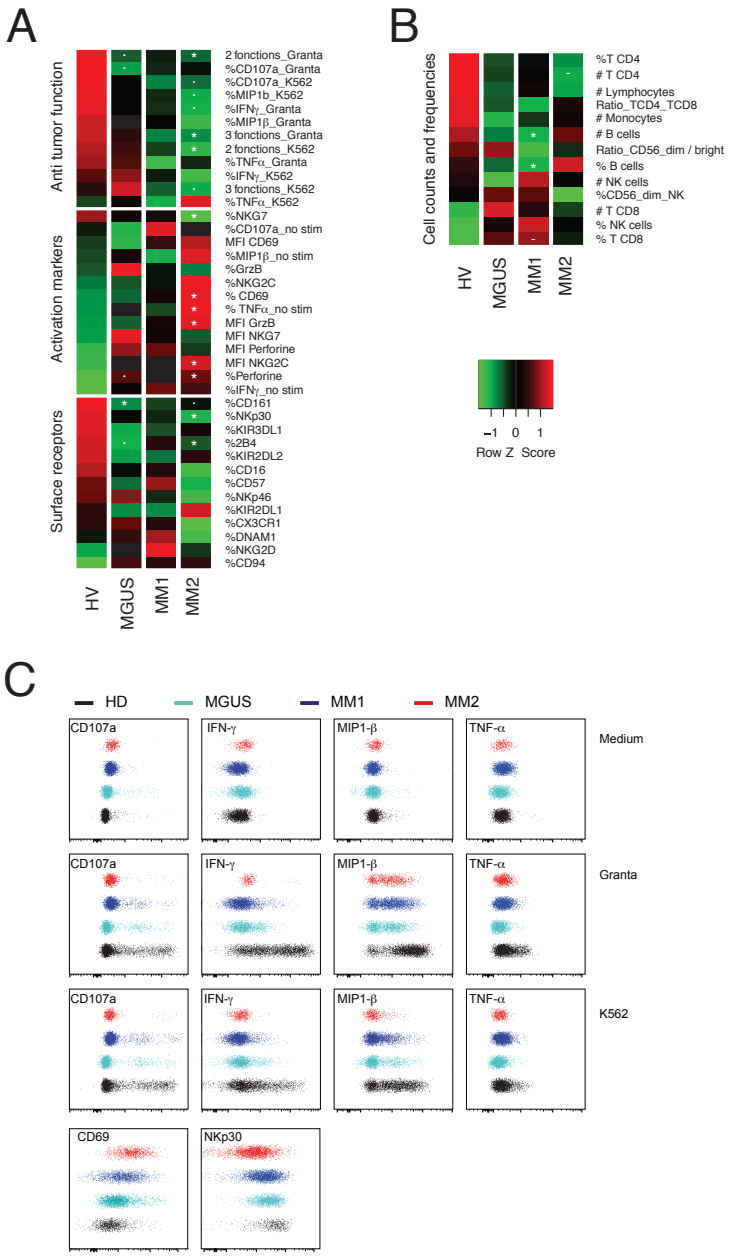
Flow cytometry analysis of the indicated parameters in NK cells from patients monitored at different time-points before, during or after LEN therapy. Data were obtained and analyzed as indicated in figure 1. (A) Charts of the percentages of CD57 and CD94 positive cells within gated NK cells. Each line corresponds to one patient. (B) Parameters were clustered in functional categories “anti-tumor function”, “activation markers”, “cell surface receptors” and “cell counts and frequencies”, as labeled and displayed in heatmaps. Stars summarize significance level of the p-values obtained using linear mixed effect models analyzing the effect of LEN on each parameter, compared to the pre-treatment time-point (i.e T0) Our analysis takes into account the fact that data are paired over time points. Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’1.

Table 1: Clinical characteristics of MGUS, MM1 and MM2 untreated patients

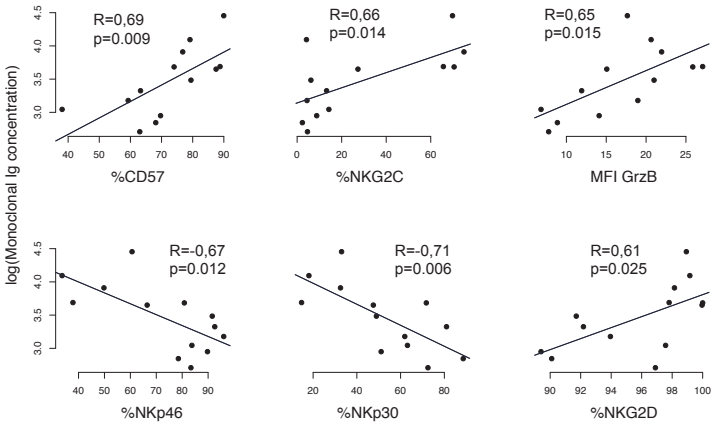
Table 2: NK cell surface or intracellular parameters correlating with polyfunctionality in HV



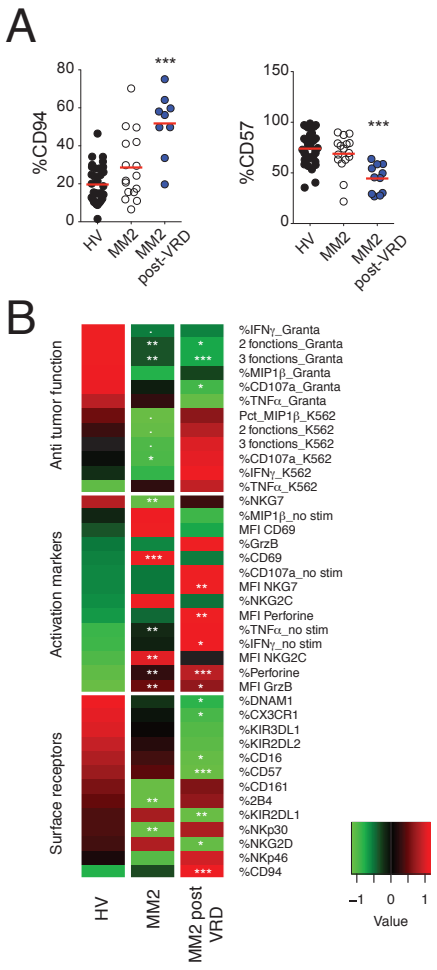
Viel, Charrier et al. Figure 1



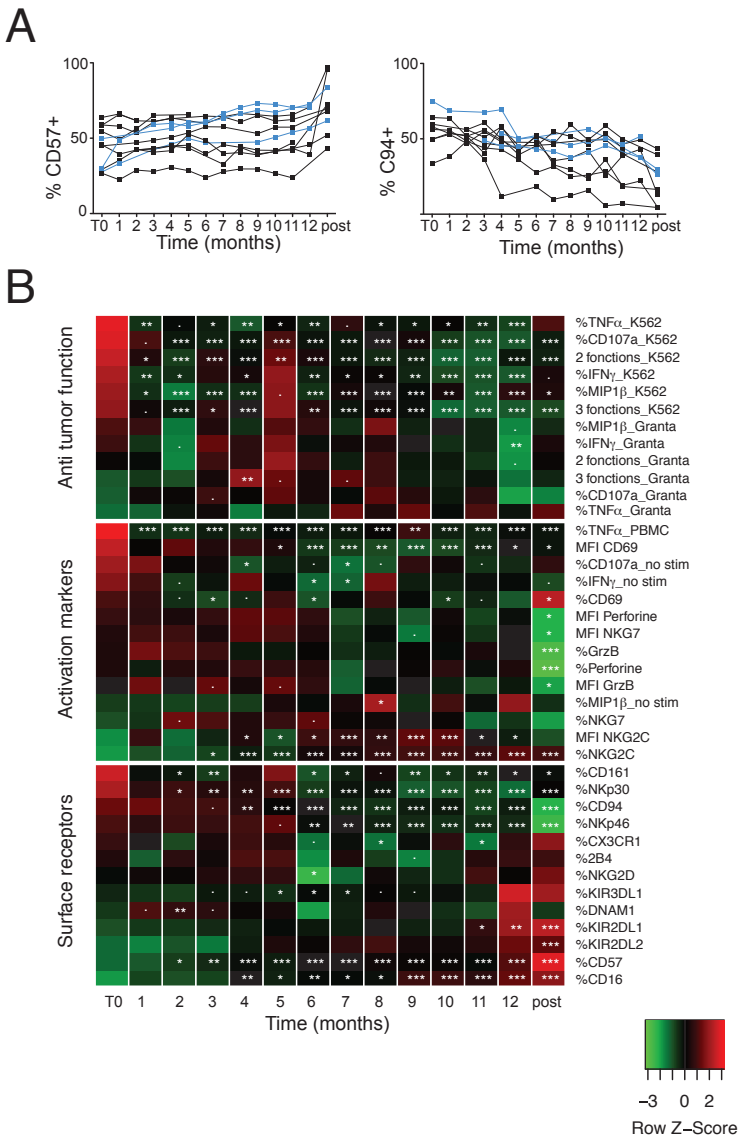
Viel, Charrier et al. Figure 2



Viel, Charrier et al. Figure 3

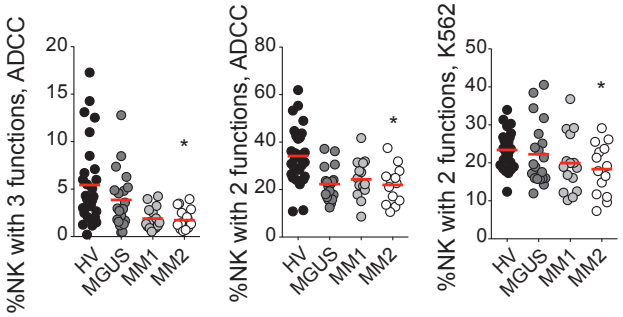


Viel, Charrier et al. Figure 4

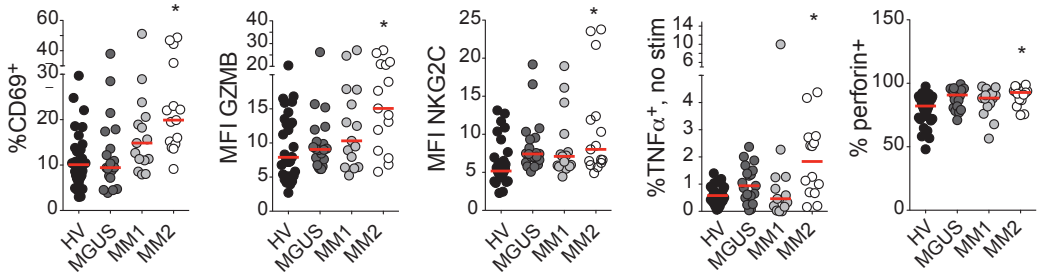


Viel, Charrier et al. Figure 5

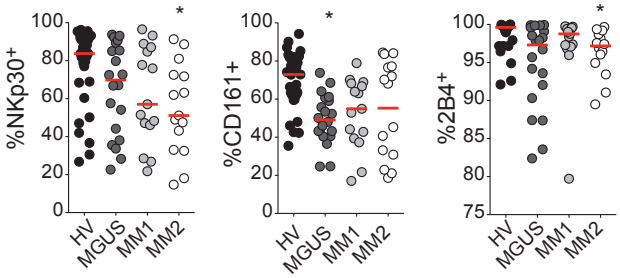
Antitumor functions



Activation markers



Surface receptors



Viel, Charrier et al. Figure S1

Table 1: Clinical, metabolic and immunological characteristics of the population

	MGUS (n=20)	MM1 (n=15)	MM2 (n=15)
Male/Female	11/9	6/9	5/10
Age (range)	68 (48-84)	67 (48-86)	66(54-78)
Monoclonal Ig :			
IgG kappa	11	6	7
IgG lambda	6	2	2
IgA kappa	2	4	3
IgA lambda	0	2	1
IgM kappa	1	1	0
FLC kappa	0	0	2
Peak (g/L)	8.9	15.7	36.2
b2-microglobulin (mg/L)	NA	2.7	6
Albumin (g/L)	NA	37.5	34.1
ISS :			
I	NA	2	3
II	NA	1	5
III	NA	NA	6
NA	20	12	1
% of BM plasma cells	NA	17.2	38

FLC: Free Light Chains, ISS: International Staging System

Table 2: Parameters associated with polyfunctionality in HD

ADCC	parameter	correlation coefficient
	%KIR2DL2	0,67
	%DNAM1	0,56
	%KIR3DL1	0,48
	%CD16	0,47
	%CX3CR1	0,46
	%NKp30	-0,52

K562	parameter	correlation coefficient
	%KIR3DL1	0,71
	%CX3CR1	0,47
	%KIR2DL2	0,41
	%CD16	0,37

Table 3: Clinical, metabolic and immunological characteristics of the lenalidomide treated population

	Group A (VRD) (n=10)	Group B (SCT) (n=4)
Male/Female	7/3	3/1
Age (range)	59 (50-67)	60 (48-65)
Monoclonal Ig :		
IgG kappa	4	1
IgG lambda	2	1
IgA kappa	2	1
IgA lambda	1	1
FLC kappa	1	0

VRD: Velcade Revlimid Dexamethason, SCT: Stem Cells Transplantation, FLC: Free Light Chain

Table S1: antibody panels

Panels	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10
1	KIR2DL2-FITC	NKG2A-PE	CD4/14/19-ECD	CD94-Percp-Cy5.5	KIR2DL1-PE-cy7	CD56-APC	KIR3DL1-AF700	CD3-APC-AF750	CD57-EF450	Live-dead Aqua
2	NKG2C-AF488	CD69-PE	CD4/14/19-ECD	CD161-Percp-Cy5.5	CX3CR1-PE-cy7	CD56-APC		CD3-APC-AF750	CD16-PacBlue	Live-dead Aqua
3	DNAM1-FITC	NKp30-PE	CD4/14/19-ECD	2B4-Percp-Cy5.5	NKG2D-PE-cy7	CD56-APC		CD3-APC-AF750	NKp46-BV421	Live-dead Aqua
4	CD107a-FITC	IFN-G-PE	CD4/14/19-ECD		TNF-PE-cy7	CD56-APC		CD3-APC-AF750	MIP1b-V450	Live-dead Aqua
5	GZMB-FITC	NKG7-PE	CD4/14/19-ECD	Perforine--Percp-EF710		CD56-APC		CD3-APC-AF750	CD16-PacBlue	Live-dead Aqua

6. Article 7 : Alteration of Natural Killer cell phenotype and function in obese individuals

Alteration of Natural Killer cell phenotype and function in obese individuals

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ABSTRACT

Obesity is associated with increased cancer rates and higher susceptibility to infections. The adipose tissue of obese individuals is inflammatory and may negatively impact on innate and adaptive immunity in a systemic way. Here, we explored the phenotype and function of peripheral Natural Killer (NK) cells of patients in correlation with their body mass index (BMI). We found that high BMI was associated with an increased activation status of peripheral NK cells, as measured by surface levels of CD69 and levels of granzyme-B. However, these activated NK cells had an impaired capacity to degranulate or to produce cytokines/chemokines when exposed to tumor cell lines deficient in MHC-I expression or coated with antibodies. This suggests that chronic stimulation of NK cells during obesity may lead to their incapacity to respond normally and eliminate target cells, which could contribute to the greater susceptibility of obese individuals to develop cancers or infectious diseases.

Key Words

Natural Killer cells, Obesity, Body Mass Index, multiparameter FACS, degranulation, cytotoxicity

Abbreviations

ADCC: Antibody Dependent Cell-mediated Cytotoxicity

BM: Bone Marrow

BMI: Body Mass Index

IFN: Interferon

MHC: Major Histocompatibility Complex

MIP: Macrophage Inflammatory Protein

NK: Natural Killer

TNF: Tumor Necrosis Factor

VAT: Visceral Adipose Tissue

1. Introduction

Obesity is a major health issue in Western countries. Obesity is defined as a Body Mass index (BMI) greater than or equal to 30. Besides altering the quality of life, this condition is associated with many co-morbidities including type 2 diabetes, cardiovascular diseases and fatty liver disease. Obese individuals are also at increased risk of developing cancers both in humans [1–3] and in mouse models [4,5]. Moreover, obese patients are more susceptible to infectious diseases [6] while diet-induced obese mice have increased mortality when infected with viruses [7]. How obesity impairs immunity remains unclear. The pro-inflammatory environment in the adipose tissue of obese patients, characterized by increased concentrations of Interferon(IFN)-γ, IL-6 and Tumor Necrosis Factor (TNF)-α [8], has an important role in the pathophysiology of obesity-related disorders. IFN-γ secreted by Natural Killer (NK) cells and Th1 CD4 T cells initiates early accumulation of T and B lymphocytes in the adipose tissue, activates local macrophage recruitment and promotes their M1 differentiation [9].

NK cells are major players in the defense against intracellular pathogens [10] and against tumors. They have the ability to kill other cells recognized as targets through an arsenal of receptors recognizing Major Histocompatibility Complex (MHC) class I molecules, various surface ligands associated with cellular stress [11,12] or membrane-bound IgG via CD16 / FcγR3, an activating receptor which drives Antibody Dependent Cell-mediated Cytotoxicity (ADCC). The main cytotoxic pathway involves the release of cytotoxic proteins such as perforin, granzymes and granulysin [13]. NK cells also secrete large amounts of IFN-γ and other cytokines in response to stimulation through NK cell or cytokine receptors. NK cells develop mainly in the

bone marrow (BM) in response to IL-15. IL-15 is pivotal to instruct the NK cell lineage and also to maintain NK cell survival at the periphery by inducing STAT5 phosphorylation [14]. High IL-15 concentrations also induce the activation of the mTOR kinase in NK cells, boosting both their metabolism and their cytotoxic arsenal [15]. The cytokine environment regulates NK cell phenotype and function and a recent study has described NK cell impairment in obese patients [16]. In mouse, it has been shown that, in the absence of T and B lymphocytes, NK cells were able to produce IFN-γ and TNF-α driving macrophage recruitment in the adipose tissue during obesity [17]. Moreover, NK cell ablation reduces macrophage infiltration of adipose tissue [18]. Finally, a high-fat diet increases NK cell IFN-γ production in the adipose tissue [9]. In humans, adipose tissue NK cells of obese individuals display an activated phenotype [19] and peripheral NK cells from obese individuals have impaired capacities to kill tumor cells *in vitro* [20]. This phenomenon seems to be reversible after weight loss [21–23]. Moreover, in mouse, the obesity-related NK cell phenotype was normalized after adoptive transfer into lean animals [24]. Altogether, these data suggest that obesity may induce the production of factors leading to an alteration of both NK cell phenotype and function.

Here, using an extensive flow cytometry analysis of the phenotypic and functional properties of NK cells, we observed that peripheral blood NK cells from obese individuals displayed an activated phenotype and were hypo-responsive to stimulation *in vitro*. These alterations were highly correlated with the severity of obesity (as evaluated by the BMI) and seemed to be corrected by weight loss.

2. Methods

2.1. Study subjects

47 subjects were included in this study after having provided informed consent, including 11 obese individuals (Mean BMI = 40), 10 pre-obese individuals (Mean BMI = 27), 12 ex-obese (Mean BMI = 29) and 14 age-matched healthy volunteers (HV) (Mean BMI = 22). Clinical, metabolic and immunological characteristics of obese individuals are listed in Table 1.

2.2. Cell lines

The K562 cell line, a human erythroleukemic cell line which does not express MHC-I molecules and the Granta-519 cell line, a B cell lymphoma, were provided by the American Type Culture Collection (ATCC) and maintained in RPMI 1640 containing L-glutamine (Gibco), 10% FBS (PanBiotech), 100 IU/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco).

2.3. Antibodies

The following antibodies were used: anti–KIR2DL2 FITC (Biolegend), anti-KIR3DL1 AF700 (Biolegend), anti-CX3CR1 PE-Cy7 (Biolegend), anti-CD16 Pacific Blue (Biolegend), anti-NKp30 PE (Biolegend), anti-2B4 PerCP-Cy5.5 (Biolegend), anti-NKG2D PE-Cy7 (Biolegend), anti-NKp46 BV421 (Biolegend), anti-granzyme B FITC (Biolegend), anti-NKG2A PE (R&D systems), 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-CD69 PE (Beckman Coulter), anti-NKG7 PE (Beckman Coulter), anti-CD94 PerCP-Cy5.5 (BD Biosciences), anti-DNAM-1 FITC (BD

Biosciences), anti-KIR2DL1 PE-Cy7 (eBiosciences), anti-CD57 eF450 (eBiosciences), anti-CD161 PerCP-Cy5.5 (eBiosciences) and anti-perforin PerCP-eF710 (eBiosciences).

2.4. Phenotypic analysis of NK cells and NK cell frequency

100uL of fresh blood were lyzed and stained for 15 minutes for extracellular markers. For the measure of intracellular proteins, cells were permeabilized with Cytofix/Cytoperm kit (BD biosciences) and stained for granzyme B, perforin and NKG7. Absolute NK cell count was obtained by multiplying the percentage of CD3-CD56+ cells among lymphocytes by the absolute lymphocyte count determined using the hematological analyzer SYSMEX (Roche).

2.5. Functional analysis of NK cells

Peripheral mononuclear cells (PBMC) were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation. Flow cytometric analysis of intracellular cytokine production and CD107a were evaluated after co-culture of PBMC with two types of target cell lines: K562 cells (ATCC) or Granta cell lines (ATCC) previously coated with anti-CD20 (Rituximab, Hoffman Laroche, 10 □g/mL). Briefly, PBMC and target cell lines were resuspended at 2 x 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. Golgi-Stop (BD Biosciences) was added one hour after the start of culture and cells were incubated for a total of 4 hours at 37 °C in 5% CO2. Next, cells were stained for surface markers with anti-CD107a FITC (eBiosciences), anti-CD19 ECD (Beckman Coulter), anti-CD4 ECD (Beckman Coulter), anti-CD14

ECD (Beckman Coulter), anti-CD56 APC (Beckman Coulter) and anti-CD3 APC-AF750 (Beckman Coulter). Samples were then fixed and permeabilized with Cytofix/Cytoperm kit (BD biosciences) according to the manufacturer's instructions, and stained for intracellular IFN-γ using a PE specific antibody (eBiosciences), TNF-α using a Pe-Cy7 specific antibody (Biolegend) and macrophage inflammatory protein (MIP)-1 β using a v450 specific antibody (BD biosciences). After washing, cells were resuspended in PBS and activated cell sorting analysis was performed on a Navios flow cytometer (Beckman Coulter). Flow cytometry data were analyzed using FlowJo (Treestar Inc.)

2.6. Statistics

All statistical analyses were performed using the Prism 4 software (GraphPad Software, San Diego, CA, USA). Intergroup comparisons were assessed using unpaired T-tests or Mann–Whitney U-test when appropriate. Levels of significance are expressed as p-values (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Linear regression tests were also performed to analyze parameters depending on BMI.

3. RESULTS AND DISCUSSION

3.1. NK cells from obese individuals have an impaired phenotype associated with signs of activation

To analyze the phenotype of blood NK cells, we measured using flow cytometry their expression of activating and inhibitory receptors, activation markers, and molecules associated with NK cell maturation and cytotoxicity (Table 2). In this analysis, NK cells were defined as $CD4^-$ $CD14^-$ $CD19^-$ $CD3^-$ $CD56^+$ cells (for gating strategy see Fig 1A). This workflow was applied to blood samples from normal, pre-obese, obese and ex-obese people (as defined in the methods section) that can also be ranked according to their BMI. We observed that peripheral blood NK cell counts increased with BMI (Fig 1B). This increase was rather due to an increase of the number of total lymphocytes than a change in NK cell percentage (Fig 1B). Interestingly, NK cells from obese individuals had a phenotype characterized by a reduction of expression of the natural cytotoxicity receptor NKp46 and the NKG2A/CD94 complex, which was correlated with BMI (Fig 1C). NK cells of obese individuals also showed signs of activation (Fig 1D) characterized by an elevation of the expression of CD69 and granzyme B and a reduction of the expression of CD16, which is known to be down-regulated during NK cell activation [25]. Activation parameters like CD69 and granzyme B were found to be normal in ex-obese people. All other parameters listed in Table 2 were found to be not significantly different between patient categories or not significantly correlated with BMI score (data not shown). Interestingly, obese individuals with the highest BMI values displayed the most marked phenotypes in terms of both absolute NK numbers, and expression of CD94 and granzyme B.

Thus, NK cells from obese individuals have an altered phenotype with notably decreased NKp46 and CD94 expression and display signs of recent activation.

3.2. Defects of NK cell function are correlated with the BMI score

Next, we assessed the function of NK cells by measuring their degranulation and their secretion of cytokines IFN-γ and chemokine MIP1-β following a 4-hour incubation with either K562 cells to explore "natural cytotoxicity" or Granta cells coated with anti-CD20 antibody, to explore ADCC.

Results presented in Figure 2 show that the BMI was inversely correlated with the capacity of blood NK cells to degranulate or to produce MIP1-β in response to stimulation with K562 cells. On the other hand, NK cell production of IFN-γ induced by K562 cells was not significantly associated with the BMI. Moreover the BMI was also inversely correlated with the capacity of blood NK cells to secrete IFN-γ and MIP1-β in response to stimulation with antibody-coated cells, while NK cell degranulation was not significantly associated with the BMI but tended to decrease in obese individuals. There was also a tendency for functional parameters to normalize in ex-obese patients. As it was observed for phenotypic markers, NK cell functional hypo-responsiveness was more evident in obese individuals with the highest BMI. Altogether, these results demonstrate a general impairment of NK cell function associated with BMI increase, affecting all effector functions and in response to both types of stimuli tested.

3.3. Discussion

Several groups have described an association between obesity and the occurrence of tumors or infections [1–3,6]. Moreover, one of the immunological features of obesity is the development of a Th1 cytokine environment within the adipose tissue. Taking into account the roles of NK cells in tumor immunosurveillance, in the defense against intracellular pathogens and also their capacity to secrete high amounts of

Th1 cytokines such as IFN-γ, we decided to perform an extended analysis of NK cell phenotype and function in obese individuals. In terms of number of markers analyzed (26), our work is the most complete addressing NK cell phenotype and function in obese individuals. As already described in the adipose tissue [19], we showed that peripheral NK cells from obese individuals displayed an activated phenotype, characterized by an elevation of the expression of granzyme B, CD69 and a downregulation of CD16. This was associated with other alterations of NK cell phenotype, such as decreased expressions of NKG2A/CD94 and NKp46. Interestingly, visceral adipose tissue (VAT) from obese mice is described to express Nkp46 ligands [9]. Since peripheral blood and VAT NK cells displayed similar phenotypes, we hypothesize that the alterations observed at the periphery are caused by the inflammation in the adipose tissue. As supporting evidence of this model, a recent study reported that the suppression of adipocytes as a source of IL-15 has local but also systemic consequences on NK cell development and homeostasis [26].

We have also observed that the absolute number of peripheral NK cells tended to increase with BMI, even if there was no statistically significant difference between HV and obese groups. This suggests that only individuals with the highest BMI display an elevation of NK cells in the periphery. This result was not in accordance with a recent study [16] nor with a previous one which reported a decrease of NK cells percentage in the blood of obese individuals [20]. However, the increase of NK cell number we observed in high BMI individuals has also been described in animal models [24]. Several hypotheses could explain the increase of NK cells in obese patients. First, the hyperplasia of adipose tissue could lead to an increased production of tissue resident NK cells that could reach the periphery. Another

possibility could be that chronic inflammation may lead to an increased production of NK cells in the bone marrow. Finally, one may speculate that the altered NK cell phenotype described here could be associated with defects in the trafficking machinery, that could impede NK cell recirculation to lymphoid organs. Here, the fact that total lymphocytes number also increases with BMI suggests that the mechanism leading to the increase of NK cell number is not specific to NK cells.

We have also demonstrated that NK cells from obese people were less responsive to stimulation by MHC-I deficient tumor cells or by B lymphoma cells coated with rituximab, even though they displayed signs of recent activation. Interestingly, such an exhausted phenotype was described in other contexts such as virus infections [27] or cancer [28].

Interestingly, in the group of obese individuals we have noticed that people with highest BMI values differ from those with lowest BMI in terms of NK cells number, expression of markers like granzyme B or CD94, but also in terms of NK cell function. In obesity, the mechanisms that lead to these alterations are unknown. Adipokines such as leptin or adiponectin are known to control the activity of NK cells [29–32] and are altered in obesity [33]. A recent study has shown that the phosphorylation of JAK-2 downstream of the leptin receptor was altered in obese individuals [16]. At a molecular level, leptin has recently been described to inhibit mTOR [34], an IL-15 dependent kinase which controls NK cell metabolism and function. As adipocytes are an important source of IL-15 [26], we could hypothesize that leptin impairment in obesity could lead to a reduction of mTOR activity in NK cells. Moreover IL-15 has beneficial effects on global metabolic activity and obesity, when given systematically [35–37] and IL-15 plasma levels are negatively associated with fat mass [38] also suggesting a defect of this pathway in obesity. Finally, different other mechanisms

were proposed to explain NK cell alterations in obesity like impairment of DNA methylation in NK lymphocytes [39] or inhibition of NK cell-mediated ADCC by adipose cells [40].

Due to the limited cohort size, we have not been able to compare males with females but the eventual impact of the hormonal environment is worth discussing and should be investigated.

To conclude, we have observed an exhausted phenotype of NK cells highly correlated with BMI. This phenomenon could explain the enhanced susceptibility of obese individuals to develop immune-related diseases such as cancers and infections. It could also be involved in the resistance of obese patients to treatments whose efficacy relies on ADCC, such as monoclonal antibodies used against cancer.

AUTHORSHIP

S.V analyzed results, drew figures and wrote the manuscript. L. B. analyzed results and performed flow cytometry experiments. E.C. performed flow cytometry experiments. E.D. included patients. A.M and J.B wrote the manuscript. TW and CD performed the experimental design, analyzed the results and wrote the manuscript.

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Figure legends

Figure 1: NK cells from obese individuals have an impaired phenotype associated with signs of activation

(A) Representative gating strategy for the identification of peripheral blood NK cells. After exclusion of doublets and $CD4^+$, $CD14^+$ and $CD19^+$ cells, NK cells were defined as $CD3^-$ $CD56^+$ lymphocytes. (B) Linear regression of NK cell blood counts on BMI value. Absolute lymphocyte subset counts were determined by multiplying the percentage of subset among lymphocyte by the absolute lymphocyte counts determined using the hematological analyzer SYSMEX (Roche) (p = 0.0035). (C) Representative FACS histogram of the expression of NKp46 in NK cells and linear regression of NKp46 MFI (p = 0.002) and CD94 (p = 0.0005) on BMI value. (D) Intergroup comparison of the expression of CD69 and granzyme B and linear regression of CD16 MFI (p = 0.049) on BMI value. *P < 0.05, **P<0.01,***P<0.001 (T-test and Mann-Whitney test). Healthy volunteers: n = 14, Pre-obese individuals: n=10, Obese individuals: n = 11, Ex-obese individuals: n = 12)

Figure 2: Defects of NK cell function are correlated with BMI score

Representative flow cytometry scatter-plots of intracellular expression of MIP1-β and IFN-γ after stimulation with Granta cells in a healthy volunteer and an obese patient. Linear regression between the percentage of $CD107a^+$ (p=0.043), IFN-γ□ (p=0.25) and MIP1-β□ (p=0.009) NK cells after stimulation with K562 and BMI values and $CD107a^+$ (p=0.075), IFN-γ□□p=0.027) and MIP1-β□ (p=0.007) NK Cells after stimulation with Granta cells coated with anti-CD20 and BMI values. Healthy

volunteers: n = 14, Pre-obese individuals: n=10, Obese individuals: n = 11, Ex-obese individuals: n = 12)

Table 1

Table 1: Clinical, metabolic and immunological characteristics of the obese population

	Healthy volunteers (n=14)	Pre-obese individuals (n=10)	Obese individuals (n=11)	Ex-obese individuals (n=12)
Male/Female	5/9	4/6	4/7	4/8
Mean age (years)	47	69	46	51
Age range (years)	21-69	53-79	21-70	24-71
Mean weight (kg)	65	76	110	85
Mean BMI (kg/m2)	22	27	40	29
HbA1c (%)	5.1	NA	6.1	NA
Cholesterol (mmol/L)	4.3	NA	5	NA
Triglycerides (mmol/L)	1.1	NA	2	NA
LDL cholesterol (mmol/L)	3	NA	3	NA
HDL cholesterol (mmol/L)	1.55	NA	1.05	NA
Leptin (ng/mL)	4.5	NA	47.9	NA
Absolute lymphocytes (G/L)	2.4	2	2.8	2
Absolute Monocytes (G/L)	0.63	0.64	0.79	0.50
Absolute CD8 T Cells (G/L)	0.55	0.39	0.48	0.38
Absolute CD4 T Cells (G/L)	0.92	0.74	1.30	0.90
Absolute Treg (G/L)	0.01	0.01	0.01	0.01
Absolute NK Cells (G/L)	0.25	0.34	0.30	0.23
Absolute B Cells (G/L)	0.23	0.16	0.28	0.24
Absolute $CD3^{+}CD56^{+}$ Cells (G/L)	0.17	0.14	0.13	0.08

BMI: Body Mass Index, NA: Not Available

Table 2

Table 2: List by category of the NK cell markers analyzed by flow cytometry

Activating Receptors
CD16
DNAM-1
2B4
NKG2C
NKG2D
NKp46
NKp30
Inhibitory Receptors
NKG2A
KIR2DL1
KIR2DL2
KIR3DL1
CD161
Activation Markers
CD69
GranzymeB
Maturation Markers
CD57
NKG2C
CD94
CD56
Chemokine Receptor
CX3CR1

Cytotoxicity Markers	
	GranzymeB
	Perforin
	NKG7
Functional Markers	
	CD107a
	TNF-α
	IFN-γ
	MIP1-β

Figure 1

Figure 1

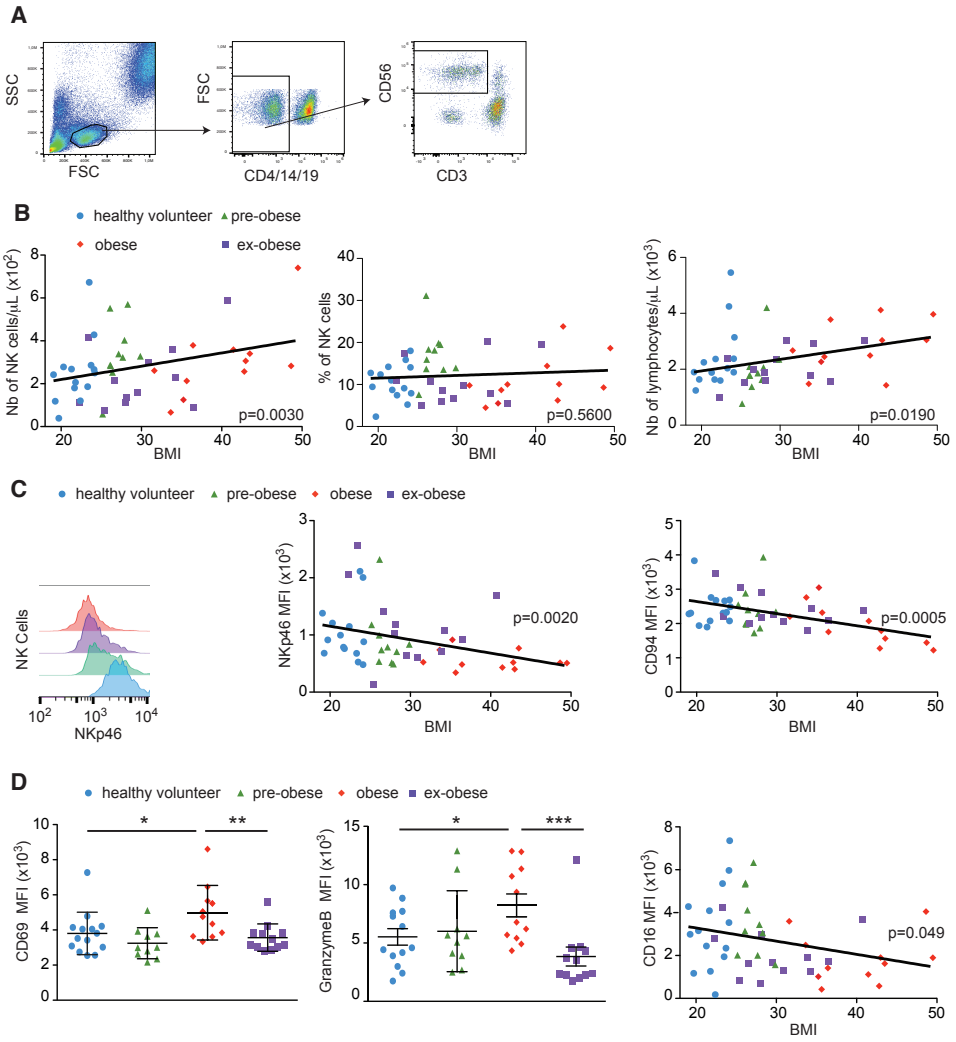
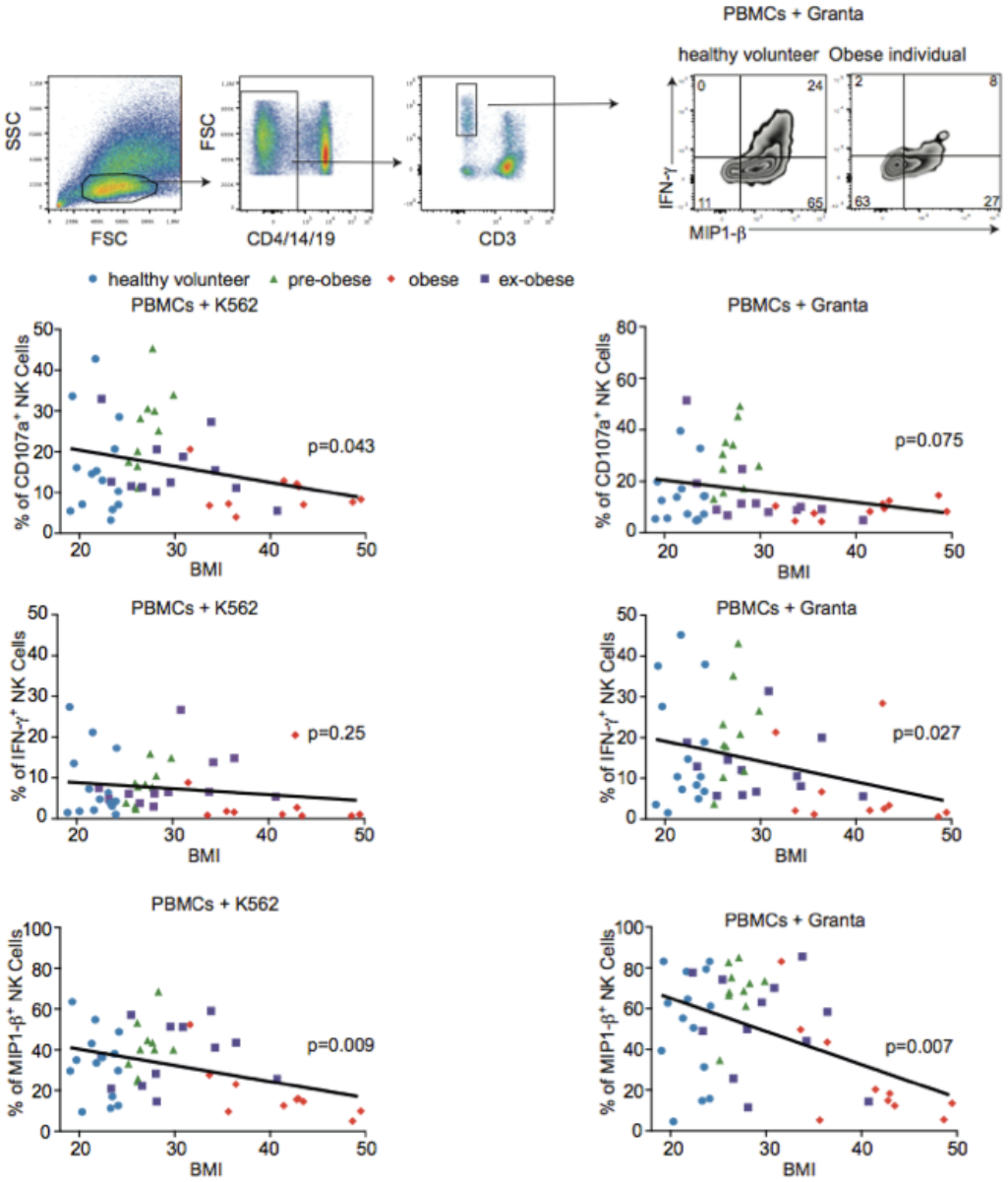


Figure 2

Figure 2



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