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Regulation of the mTOR pathway by interleukin 15 and 18 in Natural Killer cells

Lucie Fallone

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THESE

en vue de l'obtention du grade de Docteur, délivré par
l'ECOLE NORMALE SUPERIEURE DE LYON

Ecole Doctorale N° 340
Biologie Moléculaire, Intégrative et Cellulaire

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Lucie FALLONE

Regulation of the mTOR pathway by interleukin 15 and 18 in Natural Killer cells

**Étude de la régulation de la voie mTOR par l'interleukine 15
et 18 dans les cellules Natural Killer**

Devant le jury composé de :

Adelheid CERWENKA	Professeur, Heidelberg University	Rapporteuse
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Title: Regulation of the mTOR pathway by interleukin 15 and 18 in Natural Killer cells.

Abstract:

Natural Killer (NK) cells are cytotoxic innate lymphocytes important in the response against intracellular pathogens and tumor cells. Their activity depends on the integration of a large set of intracellular and environmental cues, including antigenic signals, cytokine stimulation and nutrient availability. This integration is achieved by signaling hubs, such as the metabolic checkpoint kinase mechanistic target of rapamycin (mTOR). mTOR is a conserved protein kinase that controls cellular growth and metabolism in eukaryotic cells and, therefore, is essential for NK cells development and function. The objective of this PhD was to decipher the pathway regulating mTOR in primary NK cells. We show that interleukin 15 (IL-15) and IL-18, two cytokines that are key for NK cells activation, activate the mTOR complex 1 (mTORC1) in a synergic manner. This activation is essential for NK cell proliferation and metabolic reprogramming *in vitro* and *in vivo*. Through genetic and pharmacological approaches, we show that IL-15 and IL-18 activate mTORC1 in a non-canonical manner, independently of the molecular inhibitor TSC. Instead, mTORC1 activation relies on the concomitant activation of the PI3K-AKT and ERK pathways by IL-15 and a non-canonical p38-MK2/3 pathway by IL-18 in primary NK cells. These findings shed a new light on the activation of NK cells by cytokines, a knowledge that could be exploited to boost NK cell potential in therapeutic settings.

Key words: NK cell, mTOR, cytokine, cell signaling.

Titre en français : Étude de la régulation de la voie mTOR par l'interleukine 15 et 18 dans les cellules Natural Killer.

Résumé en français :

Les cellules Natural Killer (NK) sont des lymphocytes innés cytotoxiques importants dans la réponse aux pathogènes intracellulaires et aux cellules tumorales. Leur activité repose sur l'intégration de nombreux signaux intracellulaires et environnementaux, comme les signaux antigéniques, les cytokines ou encore la disponibilité en nutriment. Ces signaux sont perçus au niveau de centre intégrateur comme la protéine mechanistic target of rapamycin (mTOR). mTOR est une protéine kinase qui contrôle la croissance et le métabolisme cellulaire dans les cellules eucaryotes et qui par conséquent est essentiel au développement et aux fonctions des cellules NK. L'objectifs de ces travaux de thèse était d'étudier la régulation de la voie mTOR dans les cellules NK primaires. Ainsi, nous avons montré que l'interleukine 15 (IL-15) et l'IL-18, deux cytokines essentielles à l'activation des cellules NK, activent le mTOR complex 1 (mTORC1) de manière synergique. Cette activation est essentielle pour la prolifération et l'activation métabolique des cellules NK, à la fois in vitro et in vivo. Par des approches génétique et pharmacologique, nous avons montré que l'IL-15 et l'IL-18 activent mTORC1 de manière non-canonique, indépendamment de la protéine TSC. Ainsi, l'activation de mTORC1 repose sur l'engagement concomitant de la voie PI3K-AKT et ERK par l'IL-15 et la voie p38-MK2/3 par l'IL-18 dans les cellules NK primaire. Ces résultats permettent une meilleure compréhension de l'activation des cellules NK par les cytokines, une connaissance importante pour le développement d'immunothérapies utilisant les cellules NK.

Mots clés : cellule NK, mTOR, cytokine, signalisation cellulaire.

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LIST OF ABBREVIATIONS

ADCC: Antibody-Dependent Cell-mediated Cytotoxicity

AICD: Activation Induced Cell Death

AML: Acute Myeloid Leukemia

BCR: B Cell Receptor

BiKE: Bispecific Killer Engager

CAR: Chimeric Antigen Receptor

cDC: conventional Dendritic Cell

CIML: Cytokine-Induced Memory-Like

CLP: Common Lymphoid Progenitor

cNK: conventional Natural Killer

DAMP: Damage Associated Molecular Pattern

DC: Dendritic cell

FDA: Food and Drug Administration

GAP : GTPase-Activating Protein

GEF : Guanine nucleotide Exchange Factor

HBV: Hepatitis B Virus

HSC: Hematopoietic Stem Cells

ICP: Immune Checkpoint

IL: Interleukin

ILC: Innate Lymphoid Cell

iNK: immature Natural Killer

ITAM: Immunoreceptor Tyrosine-based Activation Motif

ITIM: Immunoreceptor Tyrosine-based Inhibitory Motif

KO: Knock-out

LAK: Lymphokine-Activated Killer cell

LAP: Latency-Associated Protein

LLC: Large Latent Complex

LPS: Lipopolysaccharide

LTBP: Latent TGF β Binding Protein

MCMV: Mouse Cytomegalovirus

MHC: Major Histocompatibility Complex

mNK: mature Natural Killer

mTOR: mechanistic Target of Rapamycin

NK: Natural Killer

NKP: Natural Killer Precursors

PAMP: Pathogen Associated Molecular Pattern

RMA-KR: RMA MHC-I KO, Rae1- β^+

SLC: Small Latent Complex

SMAC: Supramolecular Adhesion Cluster

SMIC: Supramolecular Inhibitory Cluster

TCR: T Cell Receptor

Th: helper T cell

TLR: Toll Like Receptor

Treg: regulatory T cell

TriKE: Trispecific Killer ENGager

trNK: tissue resident Natural Killer

PART I : INTRODUCTION

I. Biology of Natural Killer (NK) cell

1. NK cell identification and classification

In 1975, lymphoid cells with natural, rapid and spontaneous cytotoxicity against tumor cells *in vitro* were identified (Kiessling, Klein, et Wigzell 1975). These cells, henceforth called Natural Killer (NK) cells, do not express receptors produced from somatic recombination like B or T lymphocytes, and therefore were classified in a distinct lineage. Since NK cells, other innate lymphocytes have been discovered, forming the group of innate lymphoid cells (ILC) which is divided today into three subgroups based on developmental, phenotypic and functional similarities. Group 1 is composed of NK cells and ILC1 which share, among other things, the expression of the transcription factor T-bet and the capacity to produce IFN- γ (Vivier et al. 2018)(**Figure 1**).


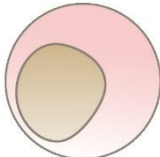
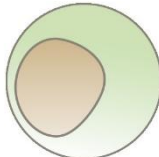
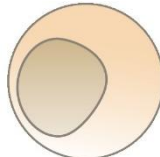
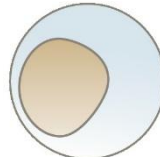
	Group 1		Group 2	Group 3	
	NK	ILC1	ILC2	ILC3	LTi
					
Transcription factors	T-bet EOMES	T-bet	GATA-3	ROR γ T	ROR γ T
Activating factors	IL-15 IL-12 IL-18	IL-15 IL-12 IL-18	IL-25 IL-33 TSLP	IL-1 β IL-6 IL-23	IL-1 β IL-6 IL-23
Effector factors	IFN- γ Perforin Granzyme	IFN- γ	IL-4 IL-5 IL-13	IL-7 IL-22 GM-CSF	IL-17 IL-22
Functions	Immunity to virus and tumors	Mucosal immunity	Immunity to parasites Allergy	Immunity to bacteria and fungi	Lymphoid organs development
Localisations	Lymphoid organs Blood Tissues	Tissue resident	Tissue resident	Tissue resident	Lymphoid organs

Figure 1: Innate Lymphoid Cells (ILC) classification. Innate Lymphoid Cells (ILC) are lymphocytes that do not express receptors resulting from recombination. Since 2013, they are divided in 3 sub-groups, mainly based on the cytokines they produce and the transcription factor they depend on for their development and function.

NK cells can be identified by the absence of CD3 expression and the expression of the NK1.1 receptor (CD161, NKR-P1), NKp46 (CD335, NCR1) and CD49b (integrin alpha 2) in

C57BL/6J mice. In humans, other markers such as the expression of CD56 (NCAM) are used for NK cell identification. NK cell identification is further complicated by their similarities with ILC1 cells with recent work even demonstrating the possible conversion of NK cells into ILC1-like cells in certain tumor context (Gao et al. 2017). Therefore, additional criteria may be used for NK/ILC1 distinction. NK and ILC1 differ in localization, with ILC1 cells being more tissue resident and NK cells rather circulating. More recently, a transcriptional signature, valid regardless of the organ and species, has also been proposed, considering EOMES, GZMA, IRF8, JAK1, NKG7, PLEK, PRF1 and ZEB2 as specific determinants of NK cells (Lopes et al. 2022).

2. NK cell development and distribution

2.1. NK cell development

As other immune cells, NK cells develop from hematopoietic stem cells (HSC) primarily in the bone marrow (Haller et Wigzell 1977) (**Figure 2**). Like T and B lymphocytes, NK cells derive from the common lymphoid progenitor (CLP) which is characterized by the expression of IL-7R α (CD127), c-Kit (CD117), Sca-1 and FLT3(CD135) in mice (Kondo, Weissman, et Akashi 1997). CD244 (2B4) expression, followed by IL-15 receptor beta chain (CD122) expression, marks the differentiation into pre-NK cell precursors (pre NKP) (Fathman et al. 2011; Carotta et al. 2011) and NK precursors (NKP) (Rosmaraki et al. 2001; Fathman et al. 2011) respectively. These precursors then become immature NK cells (iNK), characterized by the loss of IL-7R α , and the expression of the NK cell markers NK1.1 and Nkp46 (S. Kim et al. 2002; Geiger et Sun 2016). Indeed, unlike B cells, T cells or other ILCs, the differentiation of ILCs from group 1 to which NK cells belong, depends on IL-15 and not on IL-7, and therefore is associated with the loss of the IL-7 receptor in favor to the IL-15 receptor. NK cells finally reach the periphery. Peripheral NK cells can be divided according to their expression of CD27 and CD11b. CD27⁺CD11b⁻ (CD11b^{lo}) are the most immature cells, followed by double positive CD27⁺CD11b⁺ (DP) and CD27⁻CD11b⁺ (CD27^{lo}), which are the most differentiated cells (Y. Hayakawa et Smyth 2006). Maturation is associated with an increase in cytotoxic capacity and cytokine production (Y. Hayakawa et Smyth 2006) and the expression of CD49b and combinations of Ly49 receptors. Maturation is also under the control of different transcription factors including Eomes and T-bet. Eomes is dominantly expressed in immature cells and controls early lineage differentiation, whereas T-bet is more expressed by mature NK cells (Townsend et al. 2004; Gordon et al. 2012; Geiger et Sun 2016; J. Zhang et al. 2021).

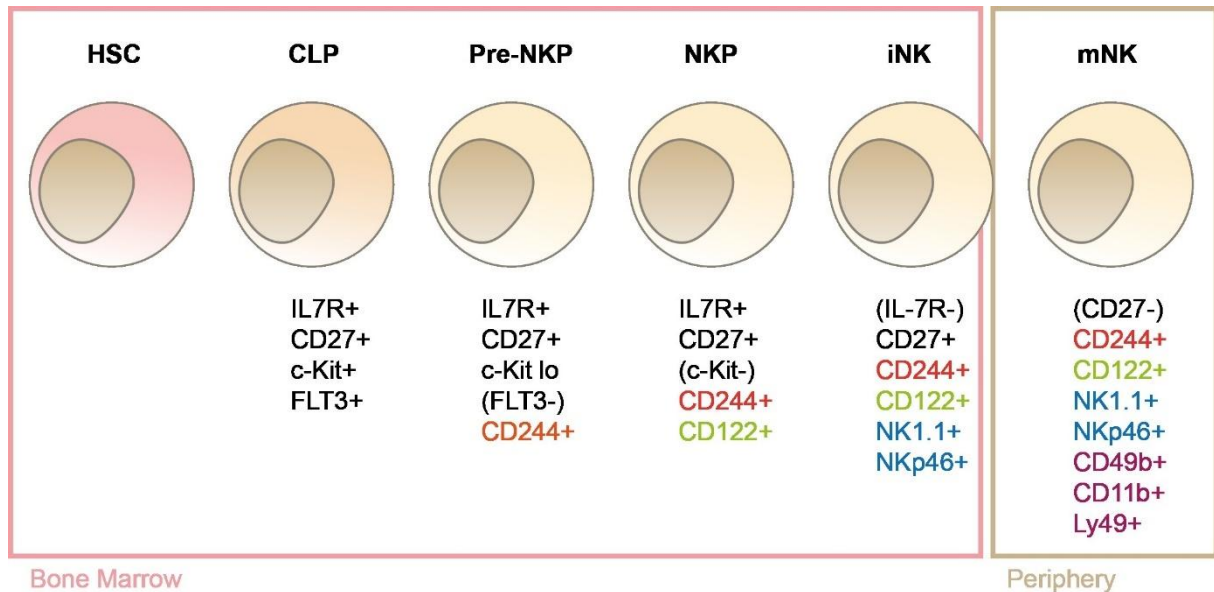


Figure 2: Mouse NK cell maturation. NK cells develop from Hematopoietic Stem Cells (HSC) in the bone marrow, which differentiate into Common Lymphoid progenitors (CLP). Expression of CD244 defined pre-NK precursors (pre-NKP) followed by the expression of CD122 which defined NK precursor (NKP) stage. Immature NK (iNK) and mature NK (mNK) cell finally reach the periphery (see text for details). Adapted from (Geiger et Sun 2016).

In human, NK cell development is classically divided into 5 stages from the CLP (review in (Freud et Caligiuri 2006; J. Yu, Freud, et Caligiuri 2013)). Stage 1 or NK progenitors (pro-NK) are characterized by the expression, among others, of the stem cell marker CD34 and the markers CD45RA and CD10 (stage 1: CD34⁺CD45RA⁺CD10⁺). The expression of c-Kit (CD117) and the IL-15 receptor beta chain, CD122, marks the transition to stage 2 or NK precursors (pre-NK) (stage 2: CD34⁺CD45RA⁺CD10⁻CD117⁺CD122⁺). These NK precursors then differentiate into immature NK cells expressing LFA-1 (stage 3: CD34⁻CD117⁺LFA-1⁺). As in mice, immature NK cells then reach the periphery. Stages 4 and 5 are characterized by the progressive loss of c-Kit expression and the increase in CD94 expression. Stage 4 and 5 are both considered as mature NK cells in human (Huntington, Vosshenrich, et Di Santo 2007). These two stages are differentiated by the level of CD56 and CD16 expression. Stage 4 NK cells (CD56^{bright}, CD16⁻) represent the minority of blood NK cells and are classically described as abundant cytokine producer while they are weakly cytotoxic. In contrast, stage 5 NK cells (CD56^{dim}, CD16⁺) represents more than 90% of blood NK cells and are highly cytotoxic (M. A. Cooper, Fehniger, Turner, et al. 2001; Poli et al. 2009). Beyond these markers, expression of CD57 further indicate terminal NK cell maturation. CD57 is expressed by 30 to 60% of CD56^{dim}, CD16⁺, and less than 1% of the CD56^{bright}CD16⁻ and its expression increase after NK cell activation. CD57⁺ cells display are less proliferative and highly cytotoxic, features of terminally differentiated NK cell (Lopez-Vergès et al. 2010).

An attempt of classification that reconcile human and mouse mature NK cell features was recently proposed by the group of Eric Vivier. Using unsupervised classification algorithm

analysis of single-cell RNA-sequencing dataset, they identified two major NK cell population. Type 1 NK (NK1) correspond to CD56^{dim}CD16⁺ in human and CD27^{lo} in mouse and strongly express cytotoxicity related molecules, whereas type 2 NK (NK2) correspond to CD56^{bright}CD16⁻ in human and CD11b^{lo} in mouse and shared the expression of certain chemokine such as XCL1 (Crinier et al. 2018; Vivier et al. 2024).

2.2. NK cell distribution

In relation with their role in immunosurveillance, NK cells are present in various lymphoid and non-lymphoid organs. In mice, immature NK cells are mainly located in the bone marrow and lymph nodes while mature NK cells migrate to the periphery and are found in large numbers in the blood, liver, spleen or lungs (Grégoire et al. 2007). Similarly, in humans, CD56^{bright} NK cells are preferentially located in secondary lymphoid organs such as lymph nodes, whereas CD56^{dim} constitute the majority of circulating peripheral blood NK cells (Ferlazzo et al. 2004; Dogra et al. 2020). NK cell maturation is associated with changes in chemokine receptor expression, driving migration towards periphery of mature cells. Chemokine receptor CXCR4 expression decrease through NK cell development whereas SIP5 receptor and CX3CR1 increase (Mayol et al. 2011; Walzer et Vivier 2011). Experiments in mice show that SIP5 deficiency leads to NK cell accumulation in the bone marrow at the expense of the blood, the spleen, the lung and even the liver (Walzer et al. 2007). In contrast, inhibition of CXCR4 leads to the recruitment of NK cells in the blood (Bernardini et al. 2008). The expression of these receptors is associated with differential chemokine gradients. For example, S1P, the ligand for S1P5, is more concentrated in the blood, promoting mature cell migration.

The relationships between circulating NK cells, sometimes referred as conventional NK cells (cNK) and tissue resident NK cells (trNK) are still not completely understood. At steady states, circulating NK cell can established tissue residency in peripheric organs, as shown by the study of parabiotic mice. (Gasteiger et al. 2015). Compared to ILC which self-renew locally, NK cells present in the tissue are replenished by the circulation, with some exception such as the salivary gland. Other studies indicate that ILC-like trNK can arise from ILC progenitors during ontogeny, although the lack of unique NK cell markers and the similarities between trNK and ILC render their identification difficult (Cortez et al. 2016; Nixon et al. 2022; Friedrich et al. 2021; Sojka et al. 2014). Nevertheless, is becoming increasingly clear that tissue location imprints NK cell phenotype and functions in line with NK cell role in tissue immunosurveillance (Dogra et al. 2020).

In response to inflammation, circulating NK cells also migrate to tissues. In particular, NK cells express CXCR3, whose ligands CXCL9 and CXCL10, are expressed in response to IFN- γ . They also express CCR5 whose ligand CCL5 is produced in large quantities by activated dendritic cells (Walzer et Vivier 2011; Ran et al. 2022). Understanding the mechanisms of NK cell recruitment to inflamed sites constitute an important challenge in particular for oncology research, one of the current limitations of cell therapies being the difficulty to infiltrate the tumor microenvironment, particularly in the case of solid tumors.

3. NK cell effector functions

NK cells have two major effector roles: they trigger the lysis of infected or tumor cells (cytotoxicity) and they also produce large quantity of signaling molecules (secreting function) (**Figure 3**).

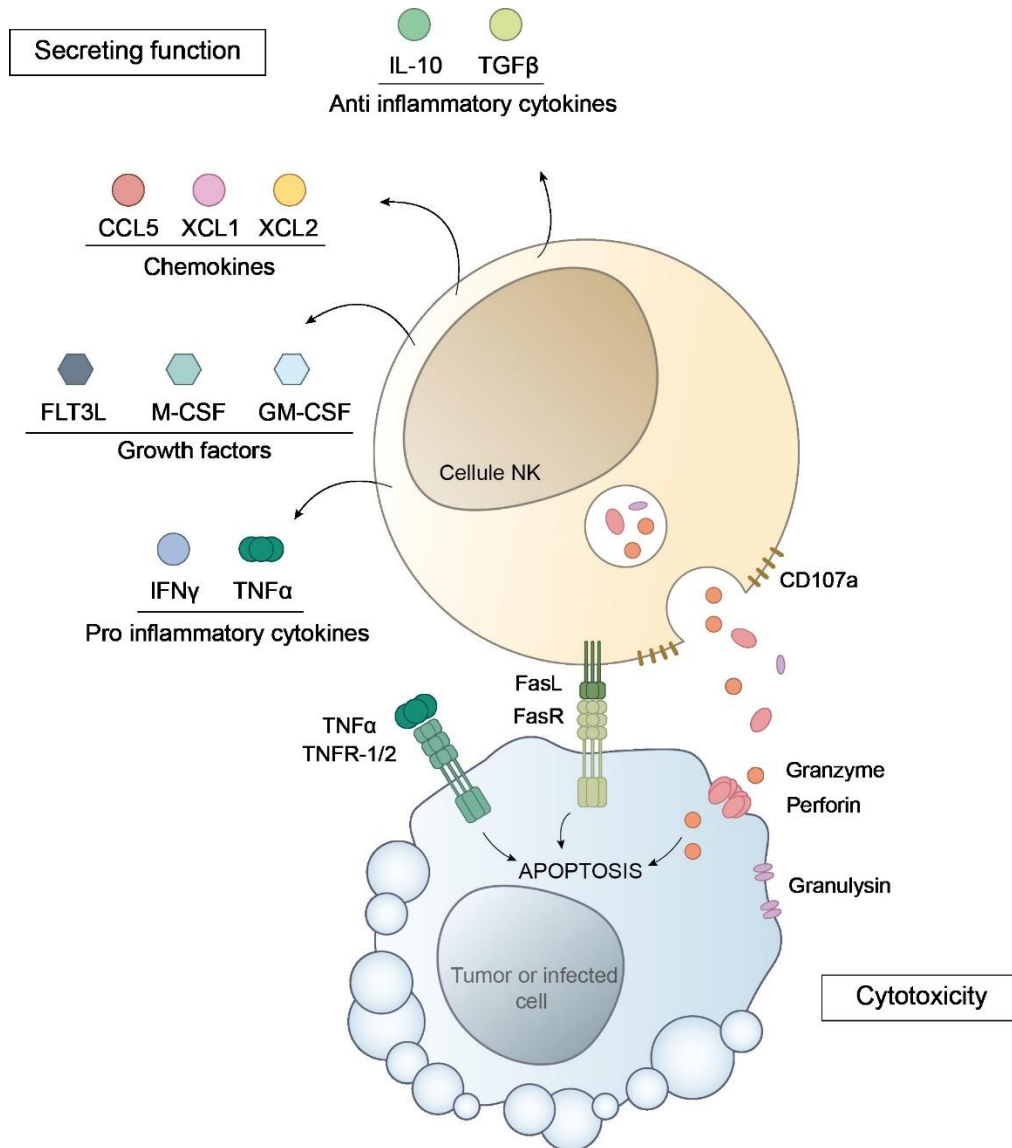


Figure 3 : NK cell effector functions. NK cells are cytotoxic lymphocytes which can induce target cell lysis through direct cytotoxicity mediated by the secretion of cytotoxic granules or by engagement of target cell death receptors. NK cells are also secretory cells and produced large amount of IFN- γ , growth factors, chemokines but also anti-inflammatory cytokines.

3.1. Cytotoxicity

NK cells can directly kill their target cells by two mechanisms; secretion of lytic granules or engagement of death receptors (review (Prager et Watzl 2019)).

Like cytotoxic CD8⁺ T lymphocytes, NK cells express the proteins granzymes, perforins and granulysins in human, which are stored in an inactive form in specific organelles derived from lysosomes, the lytic granules (Lieberman 2003). Target cell recognition by

the NK cell leads to the sequential assembly of a highly organized structure at the interface between the two cells, the immunological synapse, also referred to as the supramolecular adhesion cluster (SMAC). Integrins such as LFA1 (CD11a/CD18) and MAC1 (CD11b/CD18), activating receptors (presented later), and cytoskeleton all participate in this cellular adhesion complex, which has both a physical role in cellular adhesion and a signaling role, promoting the effector phase of cytotoxicity (Barber, Faure, et Long 2004; Orange 2008; Bryceson et al. 2005; Bryceson, Ljunggren, et Long 2009). Lytic granules are then transported via the cytoskeleton to the immunological synapse and fuse with the membrane of the NK cell, permitting a polarized release of their content. Perforin assemble into multimeric pores on the surface of the target cell and allow the entry of granzymes (Law et al. 2010; Lopez et al. 2013). Granzymes constitute a family of 5 proteases in human and 10 proteases in mouse, among which granzyme A and B are the major and most-studied one in both species (Lieberman 2003). Granzyme B is a serine protease which induce target cell apoptosis by direct cleavage of caspase 3 (Darmon, Nicholson, et Bleackley 1995). Granzyme A also induce another type of cell death independently of the caspases, through the cleavage of different factors associated with DNA such as histones or laminins, resulting in DNA fragmentation (Beresford et al. 1999; D. Zhang, Pasternack, et al. 2001; D. Zhang, Beresford, et al. 2001). Finally, human NK cell express granulysin, which belongs to the saponin-like protein family and can also form pores on the surface of targets (Krensky et Clayberger 2009). Lysis by perforin-granzyme is an extremely rapid process since it allows the cell lysis in just a few minutes (J. Li et al. 2014; Prager et al. 2019).

Activation of NK cells also expose death receptors ligands on their surface. NK cells express FasL and TRAIL (Suda et al. 1995; Kayagaki et al. 1999), which can engage their respective receptors FasR and TRAIL-R1/R2 on the surface of the target cells. Engagement of death receptor triggers the apoptosis pathways in the targeted cell. In addition, NK cells produce TNF α which also induces cell death when binding to TNFR-1 and 2 receptors (Ross et Caligiuri 1997). The apoptosis triggered by the engagement of death receptor is slower than the one triggered by lytic granules, of the order of a few hours. Moreover the threshold to induce target killing by lytic granule may be lower than the one by death receptor, as a study suggests that the release of 2 to 4 lytic granules is sufficient to induces target cell death (Gwalani et Orange 2018). NK cells are also capable of lysing multiple targets sequentially, a process referred to as serial killing (Bhat et Watzl 2007; Choi et Mitchison 2013; Vanherberghen et al. 2013). When engaging with a target cell, NK cells release the majority of their cytotoxic granules during this first contact. Therefore, the following targets will preferentially be lysed by the engagement of death receptors (Prager et al. 2019).

Numerous mechanisms allow NK cells to be protected from their own cytotoxicity. CD107a (LAMP-1), present in the membrane of cytotoxic granules, is one of them. Upon cytotoxic granules release, CD107 is expose to the surface of the NK cells and prevents the attachment of perforin to NK cell surface (Cohnen et al. 2013). Of note, CD107a is often used as a marker of degranulation of cytotoxic granules.

3.2. Secreting function

In addition to their cytotoxic ability, NK cells also produce various cytokines and chemokines important to coordinate the immune response. NK cells produce large quantities of IFN- γ , which promotes antitumor and antiviral immunity. IFN- γ is a pleiotropic cytokine, which induces among other things, macrophage activation, T helper 1 (Th1) lymphocytes differentiation, immune cell migration to the infection site and increased MHC-I expression on various cell types (Ivashkiv 2018). Unlike in T cells, IFN- γ locus is epigenetically primed in NK cells and directly accessible, promoting rapid IFN- γ transcription and thus production upon activation (Tato et al. 2004; Stetson et al. 2003). IFN- γ production by NK cells is further induced by cytokines, notably IL-12 and IL-18 in synergy.

NK cells also produce growth factors, such as FLT3L, GM-CSF or M-CSF, which regulate the recruitment, maturation and survival of myeloid cells, including dendritic cells (Barry et al. 2018; Cuturi et al. 1989). They also secrete numerous chemokines such as CCL5 and XCL1/2 which participate in the recruitment of immune cells to the inflammation site (Fauriat et al. 2010; Böttcher et al. 2018). Finally, in some settings, it has been described that NK cells are capable of producing certain anti-inflammatory cytokines such as IL-10 (Perona-Wright et al. 2009; Maroof et al. 2008) or TGF β (Esplugues et al. 2003; Gray et al. 1998).

4. Regulation of NK cell activity

NK cell effector functions are regulated by different receptors and cytokines which enable a proper cellular response to its surrounding environment.

4.1. Inhibitory and activating receptors

Unlike T and B lymphocytes, NK cells do not possess a unique antigen receptor resulting from somatic recombination. Instead, they express a set of germline-encoded receptors allowing them to recognize multiple signals on target cells. These receptors can be activating or inhibitory, the balance between the two determining the activity of the cell. Two scenarios can thus tilt the balance toward activation and lead to NK cell activation: the decrease in inhibitory signals (missing self) or the increase in positive signals (stress induced self) (**Figure 4**).

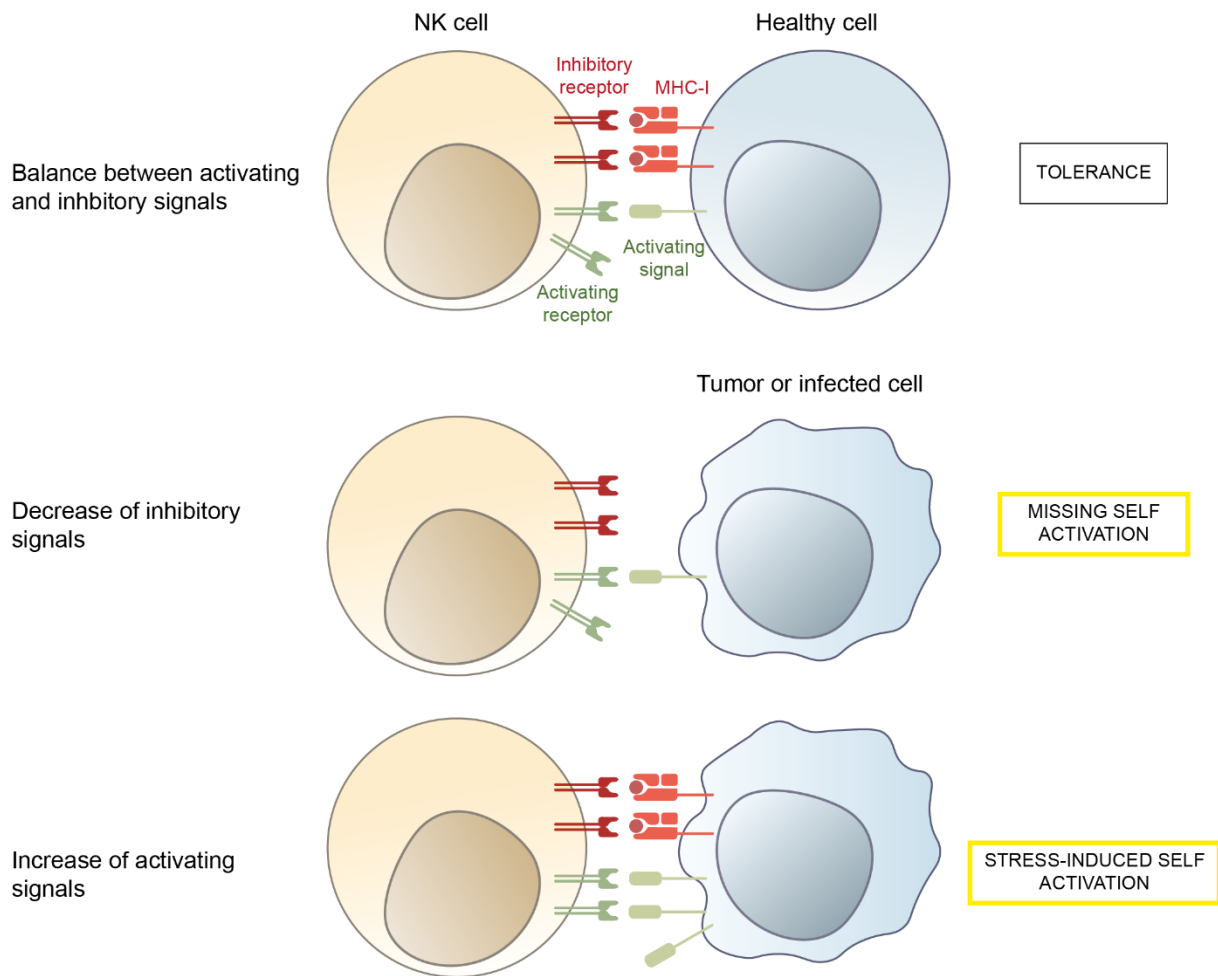


Figure 4: Model of NK cell activation. NK cells express a range of inhibitory and activating receptors. At steady state, the balance between the receptors results in tolerance. A decrease in inhibitory signal, through loss of MHC-I expression by infected or transformed cells, leads to NK cells activation (missing self). In contrast, tumor or infected cells can upregulate some stress signals, which also induces NK cell activation (stress-induced self). Adapted from (Vivier et al. 2012)

a. Decrease in inhibitory signal: missing self

In 1986, Karre and collaborators observed that MHC-I deletion on the RMA tumor cell line rendered them more susceptible to NK cell elimination (Kärre et al. 1986). Indeed, it would be shown later that NK cells express a set of inhibitory receptors specifically recognizing MHC class I molecules. At steady state, cells express MHC-I which protects them from NK cell targeting. By contrast, decrease in MHC-I expression is often observed on infected or transformed cells as a mechanism to avoid T cell recognition. This however triggers a situation referred to as missing self, unmasking them to NK cells (Vivier et al. 2012). Another situation of missing-self is induced when the MHC-I expressed by a given cell is mismatched, i.e. not recognized, with the inhibitory receptors expressed by the NK cell facing it (Ruggeri et al. 1999).

In humans, certain receptors of the KIR (Killer Ig-like receptor) family are NK cell inhibitory receptor and bind MHC-I HLA-A, B and C (**Table 1**). They are named according to the

number of Ig domain they contain, which could be 2 (KIR2D) or 3 (KIR3D). Moreover, most of inhibitory KIRs have a long cytoplasmic domain, and are referred accordingly (e.g. KIR2DL, L for long). KIR receptors constitute a multigenic family of polymorphic genes, with individual KIR recognizing distinct subsets of MHC-I allotype (Campbell et Purdy 2011). Their functional equivalent in mice are certain receptors of the Ly49 family, including Ly49A,B,C,E,G and Q (Schenkel, Kingry, et Slayden 2013).

Another important NK cell inhibitory receptor is NKG2A-CD94 heterodimer. NKG2A-CD94 is expressed in both humans and mice (Lazetic et al. 1996) and its ligand is HLA-E in humans and Qa1b in mice (Braud et al. 1998; Vance et al. 1998).

At the molecular level, inhibitory receptors share an inhibitory motif called ITIM (immunoreceptor tyrosine-based inhibitory motifs) on their intracellular domain, allowing the recruitment and activation of the inhibitory phosphatases SHP-1 and SHP-2 through their SH2 domain (Long 2008). SHP-1/2 tyrosine phosphatase activity prevent activating phosphorylation arising from activator signal such as activating receptors. The clustering of inhibitory receptors and their signaling component in an inhibitory synapse is sometime referred to as supramolecular inhibitory cluster (SMIC) (Vyas et al. 2001; Davis et al. 1999; Burshtyn et al. 2000; Orange 2008).

b. Increase in activating signal: Stress induced self-recognition

NK cells also express a set of activating receptors that recognize stress signals on target cells surface (**Table 1**). Unlike inhibitory receptors, these receptors are generally associated with adaptor molecules containing ITAM motif (Immunoreceptor tyrosine-based activation motifs), similarly to the signaling taking place downstream the TCR or the BCR. ITAM motifs allow the recruitment of kinases belonging to the Src family and the transmission of an activating signal.

The prototypical example of activating receptors is the NCR family which comprises NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3) (Pende et al. 1999; Cantoni et al. 1999; Pessino et al. 1998). NKp46 is expressed both in mice and human whereas NKp44 and NKp30 are not present in inbred mice (Hollyoake, Campbell, et Aguado 2005). A variety of NCR ligands have been reported, including molecules derived from virus, bacteria or parasite. However most of them remain poorly characterized in part because of their absence in mice. Among the best described, B7-H6, a marker of tumoral transformation, is a ligand of NKp30 (Brandt et al. 2009; Yili Li, Wang, et Mariuzza 2011).

As mentioned above, some receptors from the KIR family in human and Ly49 in mice are activating receptor. Unlike inhibitory receptors, activating KIRs have mostly a short cytoplasmic domain (e.g. KIR2DS, S for Short). They also recognize HLA molecules and may be activated by non-self HLAs or HLAs whose expression is increased by infection or tumor transformation. In C57BL/6 mice, Ly49D and Ly49H are also activating receptors. In particular, Ly49H recognizes the m157 glycoprotein from the murine cytomegalovirus (MVMV) (J. C. Sun, Beilke, et Lanier 2009) while Ly49D recognizes H2D^d (George et al. 1999).

In the same family as NKG2A, the NKG2C, NKG2E and NKG2D receptors are activators. NKG2C and NKG2E exist as a heterodimer with CD94 and recognize the HLA-E complex in human/ Qa1 in mice. NKG2D forms an homodimer and recognizes a set of molecules expressed by tumor and infected cells such as MIC1, MICB, ULBP in human and Rae1, H60 and MULT1 in mice (Lewis L. Lanier 2015).

DNAM-1 is another activating receptor recognizing CD112 (PVRL2, nectin2) and CD155 (poliovirus receptor, PVR) (Bottino et al. 2003). DNAM-1 is therefore important for the recognition of infected cells but also transformed cells as mouse deficient for DNAM-1 are more susceptible to tumors (Iguchi-Manaka et al. 2008).

NK cell also express receptors for the antibody constant region Fc, including the IgG receptors CD16 (FcγRIII) and CD32 (FcγRII) (L. L. Lanier, Ruitenberg, et Phillips 1988; Metes et al. 1998). These receptors possess ITAM region and therefore trigger NK cell activation, making NK cells main effectors of Antibody-Dependent Cell-mediated Cytotoxicity (ADCC). NK cell ADCC mediates the elimination of cells coated by antibody, either natural or therapeutic ones in therapy involving antibodies such as rituximab (anti-CD20) or trastuzumab (anti-HER2) (W. Wang et al. 2015).

Other molecules are also important for NK cell activity, such as adhesion molecules. The integrin LFA-1, for example, binds to ICAM-1 and allows the formation of the immunological synapse and the activation of the cell (Urlaub et al. 2017; Barber, Faure, et Long 2004). Finally, certain co-receptors also expressed by T lymphocytes participate in the activation of NK cells. SLAM family proteins such as 2B4 and its ligand CD48 belong to the latter category, and favor NK cell activation and SMAC formation in concert with other activating receptors (Chuang, Kumaresan, et Mathew 2001; Altvater et al. 2009; Bryceson, Ljunggren, et Long 2009) (**Table1**).

Receptor	Inhibitory	Activating	Ligand
KIR (human)	X	X	MHC-I
Ly49	X	X	MHC-I
NKG2A	X		HLA-E(human), Qa1(mouse)
NKG2C		X	HLA-E(human), Qa1(mouse)
NKG2E		X	HLA-E(human), Qa1(mouse)
NKG2D		X	MIC1, MICB, ULBP, Rae, H60 and MULT1.
NKp46(NCR1)		X	unknown
NKp44 (NCR2)		X	unknown
NKp30 (NCR3)		X	B7-H6
DNAM-1		X	PVRL2 (CD112) and PVR (CD155)
FcγRIII		X	IgG Fc

Table 1 : NK cell receptors and their ligands. NK cells express a set of germline-encoded receptors. Inhibitory receptor bind to self MHC-I molecules. Activating receptors recognized a large set of molecules upregulated by transformed cells or infected one. Coreceptor and adhesion molecules are not presented but also participate in NK cell activation (adapted from (Morvan et Lanier 2016)).

c. NK cell education

Through MHC-I ligation, NK cells have the ability to distinguish self from non-self-cells. This capacity is constantly maintained through a process that has been called education and which is tightly linked to the expression of functional inhibitory receptors (Anfossi et al. 2006; Johansson et al. 2005; Fernandez et al. 2005; S. Kim et al. 2005). Indeed, in a MHC-I-deficient environment, NK cells are unresponsive to activating signals (Zimmer 1998, Kim 2005). Likewise, NK cells missing self-MHC-specific inhibitory receptors or expressing inhibitory receptors unable to signal (ITIM deleted or SHP1 deficient cells) have a decreased response to MHC-I deficient target cells (Fernandez et al. 2005; Viant et al. 2014). Several models have been successively proposed to explain NK cell education. The first one, the arming model, proposed that NK cells are first hyporesponsive and acquired their reactivity after ligation of their inhibitory receptors. The disarming model on the contrary postulates that NK cells have a basic reactivity which is lost following chronic engagement of activating receptors, unless it is balanced by the simultaneous engagement of inhibitory receptors. This last model is easier to reconcile with classic inhibitory receptor function and therefore more accepted. Finally, the great functional heterogeneity within the NK cell population suggests that rather than a binary model, education is a continuous process, the number and strength of inhibitory receptors engagement resulting in a continuum of NK cell reactivity. This last view of NK cell education is often referred to as the rheostat model (Höglund et Brodin 2010).

4.2. Regulation by cytokines

Alongside with activating and inhibitory receptors, NK cells activity is also modulated by various cytokines. As this PhD work is centered on the activation of NK cells by IL-15 and IL-18, an entire chapter will be devoted to them. Thus I will only detail here the role of other major cytokines that regulate NK cell biology: IL-12, TGF β and type 1 IFNs (**Figure 5**).

IL-12. IL-12 is a heterodimeric cytokine, composed of an alpha p40 and beta p35 chain covalently linked by disulfide bridges. It belongs to a larger family, the IL-6 super family which also regroups IL-23, IL-27 or IL-35. These cytokines are all heterodimers, having either pro-inflammatory (IL-12 and IL-23) or inhibitory (IL-27 and IL-35) roles. IL-12 is produced by antigen-presenting cells such as dendritic cells and macrophages. Its receptor consists of two IL-12R β 1 and β 2 chains and is constitutively expressed by NK cells. The binding of IL-12 to its receptor leads to its phosphorylation and the recruitment of the kinases TYK2 and JAK2 which leads to the activation of the transcription factor STAT4 (Vignali et Kuchroo 2012; Marçais et al. 2013). IL-12 has a major role in the induction of IFN- γ production by NK cells, in association with IL-18. NK cells derived from IL-12 deficient mice show a reduction in IFN- γ production but unchanged cytotoxicity (Nguyen et al. 2002). IL-12 also induces IL-2 receptor expression on NK cells and can anecdotally promote NK cell proliferation (S.-H. Lee, Fragoso, et Biron 2012; Shemesh et al. 2022; Lauwerys, Renaud, et Houssiau 1999).

TGF β . TGF β is a cytokine belonging to the bone morphogenetic protein (BMP)–activin family. The predominant isoform, TGF β 1, can be produced by virtually all cells of the immune system. In producing cells, TGF β is produced in dimer and associated with

different proteins which control its activity. TGF β dimer are associated with Latency-Associated Protein (LAP) and forms a first complex, the Small Latent Complex (SLC). This SLC can be secreted or associated with a second protein, the Latent TGF β Binding Protein (LTBP) and thus form another larger complex, the Large Latent Complex (LLC). The association of TGF β in the SLC or LLC complex prevents its ligation to its receptor and must therefore be released from its complex to be active. TGF β 1 signals via a tetrameric receptor composed of two TGFBR type I and two TGFBR type II. The binding of TGF β to its receptor leads to the phosphorylation of type I receptors by type II receptors, which then phosphorylates the transcription factors R-SMAD, SMAD2 and 3. SMAD2/3 then reach the nucleus, where they bind to SMAD4 and other factors and regulate the expression of different genes. Beyond this canonical SMAD pathway, TGF β also activates different other pathways, including MAPKs or the PI3K-AKT pathway. The importance of these pathways varies depending on the cell type and cellular context. In NK cells, TGF β has an inhibitory role, limiting both cell proliferation and functionality (Akhurst et Hata 2012). TGF β notably inhibits the action of IL-2 on cell division (Ortaldo et al. 1991; Malygin, Meri, et Timonen 1993), and that of IL-15 on cell metabolism (Viel et al. 2016). TGF β also antagonizes the effect of IL-12 on the effector functions of NK cells, via SMAD proteins which inhibit the expression of T-bet and IFN- γ directly (J. Yu et al. 2006). Finally, TGF β promotes the transition of NK cells towards an ILC1-like phenotype, which promotes tumor development in certain preclinical murine models (Cortez et al. 2017; Gao et al. 2017).

Type I IFN. Type I IFNs IFN- α and IFN- β are proteins first identified for their role in promoting cell resistance state to viral infections. They are produced by numerous cells, of the immune system or not. IFN- α and IFN- β signal via a common receptor, IFNAR1/2, also expressed by most of the cells in the organism, including NK cells. The binding of IFNs I to their receptor leads to TYR2 and JAK1 activation, followed by STAT1/2 transcription factors activation, which associates with the IRF9 protein. The STAT1/2-IRF9 complex then reaches the nucleus where it induces the expression of ISGs (Interferon-stimulated genes) (Platanias 2005). In NK cells, IFNs I activate proliferation and cytotoxicity, through both direct and indirect mechanisms. Notably, IFN receptor deficiency can be compensated by IL-15 injection, in the context of a CMV infection (Nguyen et al. 2002), showing that certain effects of IFNs are in part due to the activation of IL-15 production by third party cells. Nevertheless, certain studies confirm a direct effect of IFNs I on NK cells, in particular on cytotoxicity (Martinez, Huang, et Yang 2008).

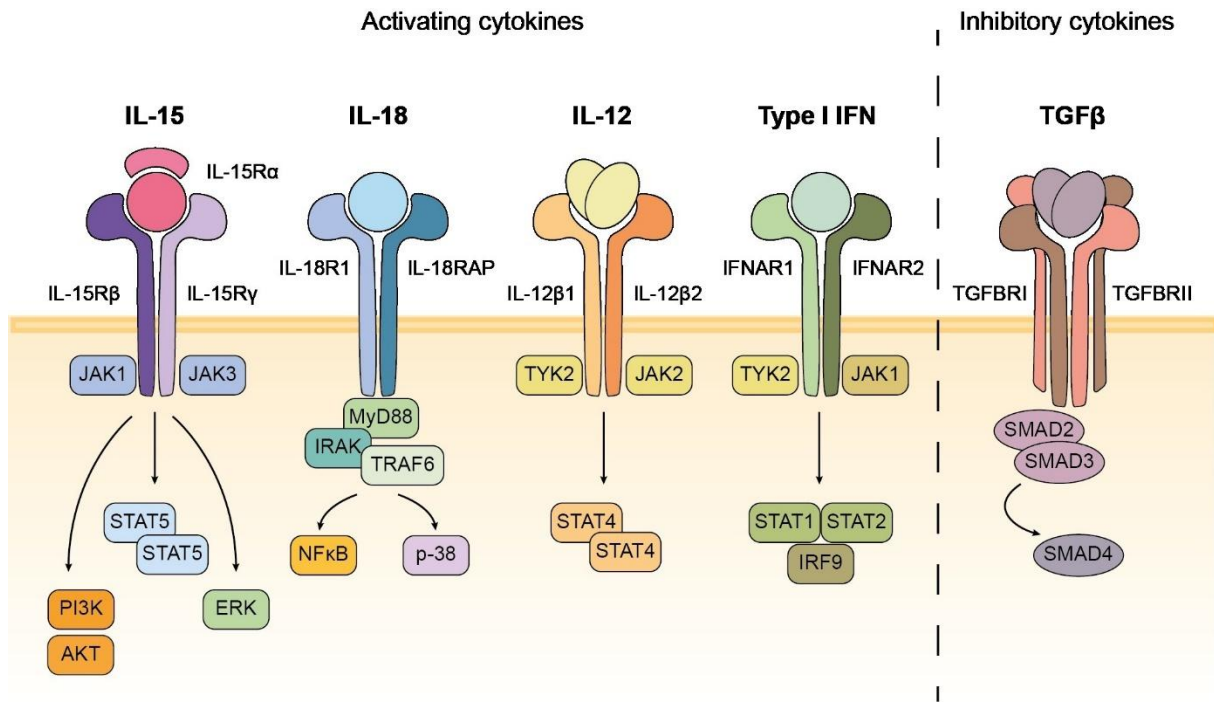


Figure 5 : NK cell regulation by cytokines. NK cell activity is regulated by different cytokines. IL-15, IL-18, IL-12 and IFN I are positive regulators whereas TGFβ is a negative regulator of NK cell functionality. Cytokine receptors and the main molecules involved in their signal transduction are presented.

4.3. Chronic stimulation and NK cell dysfunction

Under chronic stimulation, several studies have reported a decrease in IFN-γ production and cytotoxic capacity of NK cells after *in vitro* re-challenge. This loss in functionality have been called dysfunction, and is particularly described in tumor context or chronic viral infections such as hepatitis virus B (HBV) infection. Although different, NK cell dysfunction is for some aspects reminiscent of T cell exhaustion, with which it is frequently compared. NK cell dysfunction is acquired rapidly upon chronic antigen stimulation. A recent study in mice show that reduction in cytotoxicity, production of IFN-γ and chemokine (CCL5) appears in less than 24 hours contact with the tumor (Dean et al. 2024). NK cell dysfunction has been associated with different phenotypic changes, including a decrease in the expression of certain activating receptors such as NKG2D (Gill et al. 2012; C. Sun et al. 2012; Marotel et al. 2021) and an increase in the expression of Immune Checkpoints (ICPs) such as TIGIT (Q. Zhang et al. 2018; Chauvin et al. 2020), Tim3 (W. Jiang et al. 2022; Seo et al. 2017), Lag3 (Ohs et al. 2017) or PD-1 (Seo et al. 2017) in tumor context or Tim3 in chronic HBV (Ju et al. 2010).

Dysfunction can result in part from the altered environment. In tumor microenvironment, decrease in pH, nutrients and oxygen as well as the presence of regulatory immune cells impact the functionality of the cells. Similarly, chronic infection can also be associated with a decrease in nutrients and an increase in inhibitory molecules. Upon contact with tumor, several studies also show that NK cells acquire a more tissue-like ILC1 phenotype as mentioned earlier, expressing notably the CD49a integrin (Dean et al. 2024; Gao et al.

2017). This transition to ILC1, mediated in particular by TGF β , is also associated with poorer tumor control (Gao et al. 2017). TGF β has also been associated with NK cell dysfunction in chronic HBV infection. TGF β and IL-10 blockade in this context partially restore NK cell function (Peppas et al. 2010; C. Sun et al. 2012). Therefore, environmental inputs and chronic stimulation need both to be taken into account.

Distinct from immunosuppressive environment, chronic stimulation of NK cell receptor alone is sufficient to drive dysfunction (Tripathy et al. 2008; Bolanos et al. 2011; Oppenheim et al. 2005). To study the importance of chronic stimulation in tumor context, our team developed a new tumor model, the RMA-KR line, KO for MHC-I and expressing Rae1- β , a ligand for the NKG2D activating receptor. These two signals allow the RMA-KR line to be recognized by NK cells specifically whereas the WT RMA line is not recognized by NK cells, and therefore allows to distinguish the impact of the tumor environment from chronic stimulation of NK receptors. At equal tumor load, NK cell dysfunction is observed in the presence of the RMA-KR line only, characterized by a reduction in cytotoxicity, a reduction in IFN- γ production and a reduction in the response to cytokines. This loss of functionality is associated with an increase in the expression of ICPs and the transcription factor Tox, important for the establishment of the exhausted lineage in T cells (Pouxvielh et al. 2024). Thus chronic stimulation of NK cells by NK cell receptor in tumor context is sufficient to produce a dysfunctional state.

Cytokine stimulation has a dual role in NK cell dysfunction. Indeed, several reports show that cytokine treatment reverses NK cell dysfunction. *In vivo*, treatment with the cytokines IL-12+IL-18 (Ardolino et al. 2014), IL-21 (Seo et al. 2017), or IL-15 (Dean et al. 2024), restores NK cell functionality. On the contrary, chronic treatment with cytokines, akin to chronic receptor stimulation, renders NK cells dysfunctional. Felices and collaborators compared human NK cells treated continuously or discontinuously with IL-15. They showed that continuously treated cells divide more but produce less IFN- γ and are less cytotoxic. Moreover, expressing an equivalent level of IL-15 receptor, the level of activation of the transduction pathways downstream of IL-15 is lower for continuously treated cells. They also show that IL-15 chronic treatment is associated in the end with lower metabolic capacity (Felices 2018). *In vivo*, NK cells from mice chronically treated with IL-15 for 14 days also exhibit poorer cytotoxicity and produce less IFN- γ than those from mice treated only once 2 days before (Alvarez et al. 2019). Therefore, doses and frequencies impact NK cell response to cytokines.

5. NK cell integration in antitumoral and antiviral immunity

NK cells are effectors of type 1 immunity as indicated by their cytotoxic capacities and their secretion of IFN- γ and TNF α . They also belong to innate immunity and therefore have a pivotal role in orchestrating immune response in its early phases.

5.1. Evidences of NK cell role in antitumoral and antiviral immunity

NK cell antitumoral role is indicated by their ability to recognize cancer cells *in vitro* (Kiessling, Klein, et Wigzell 1975). *In vivo*, NK cell depletion, through anti-NK1.1 or anti-GM1 depleting antibodies, results in a decreased control of certain tumors (Cerwenka,

Baron, et Lanier 2001; Diefenbach et al. 2001). In humans, an epidemiological study also shows a correlation between suboptimal NK cell activity and the risk of developing cancer (Imai et al. 2000).

NK cells also have a role in antiviral immunity and have been extensively studied in the context of infections with hepatitis virus, influenza virus, flavivirus, HIV, or SARS-Cov-2 (Björkström, Strunz, et Ljunggren 2022). NK cells seem particularly important for the control of herpes viruses, notably cytomegalovirus (CMV) (Biron, Byron, et Sullivan 1989). Transfer or depletion of NK cells in mice has been associated with resistance or increased susceptibility to mouse cytomegalovirus (MCMV) infections respectively (Bukowski, Woda, et Welsh 1984; Bukowski et al. 1985). Likewise in humans, a deficiency in NK cells is associated with a greater susceptibility to viral infections, particularly to herpes viruses (Orange 2006; 2013).

5.2. Orchestration of adaptive immunity by NK cells.

NK cells belong to the innate response and therefore can be mobilized quickly. NK cell can directly lyse or induce apoptosis of their target cells, although given their small number compared to effector T cells for instance, this role may be rather marginal. On the other hand, NK cells have an important role in orchestrating the immune response during the early phases of inflammation (Bald et al. 2020). NK cells are a major source of IFN- γ and can be activated rapidly before adaptive immunity takes over (Mah et Cooper 2016). They also promote the activation of antigen-presenting cells, particularly dendritic cells (DC).

Numerous studies have documented the existence of cross-talk between NK and DC cells, favoring the establishment of an adaptive type 1 response (**Figure 6**). NK cells promote the development of conventional dendritic type 1 cells (cDC1), specialized in the activation of cell-mediated immunity, through the secretion of FLT3L (Barry et al. 2018). TNF α in synergy with IFN- γ also induces DC maturation (Piccioli et al. 2002). The cytotoxic activity of NK cells also takes part in the NK-DC cross-talk. DCs can be lysed by NK cells, immature DCs being more targeted, which participate in the maturation and homeostasis of DC populations (Wilson et al. 1999; Ferlazzo et al. 2002). In addition, NK cells, through their cytotoxic activity, promote cross-presentation by DCs. The chemokines CCL5 and XCL1/2 secreted by NK cells participate in the attraction of DCs to the tumor bed and favor the tumor control (Böttcher et al. 2018). Conversely, dendritic cells promote NK cell activation, in particular through the production of the cytokines IL-15, IL-12, IL-18 and type I IFN and chemokines such as CCL3-5 and CXCL9-11 (Walzer et al. 2005). This cross-talk between NK and DC promotes the activation of naïve T cells which are important adaptive effectors of antitumoral and antiviral immunity (Bald et al. 2020).

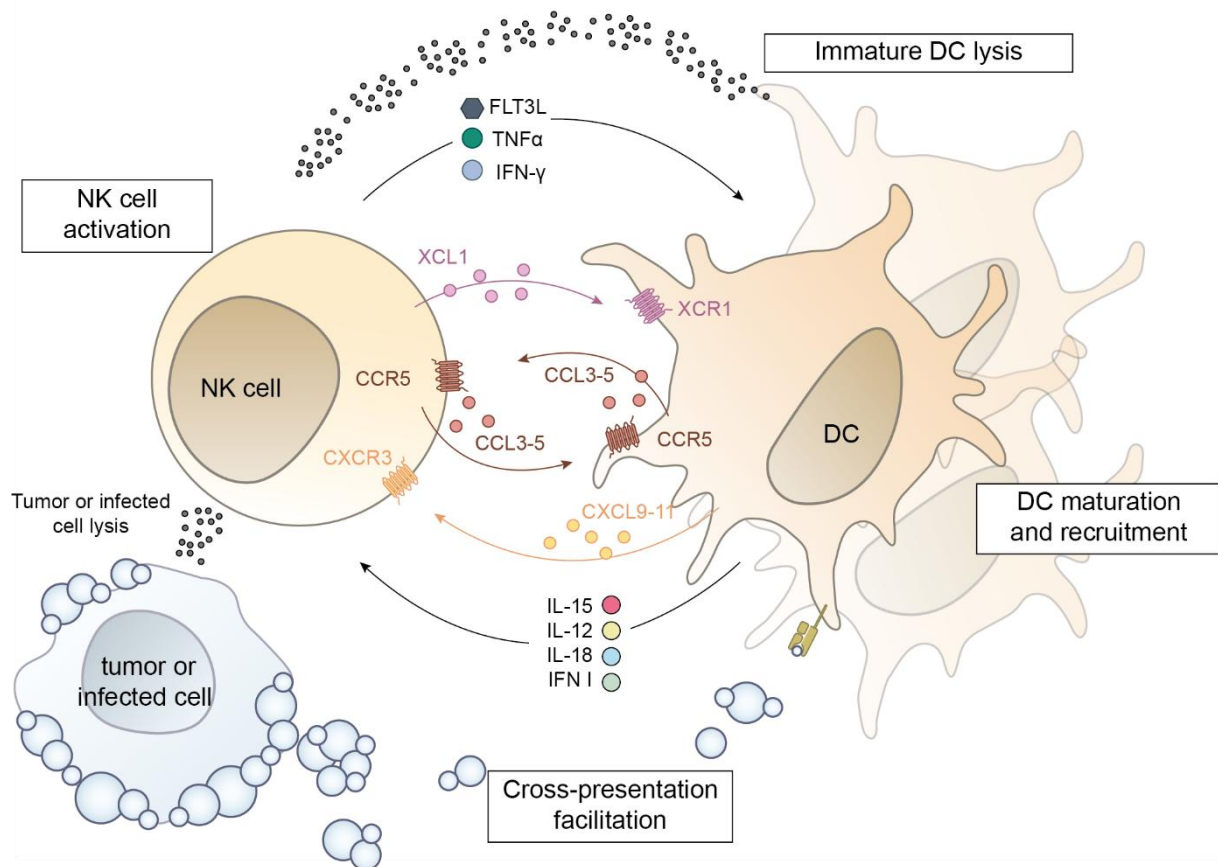


Figure 6: NK cell-DC cross-talk. NK cell favor DC maturation through FLT3L, TNF α and IFN- γ secretion and by killing immature DCs. NK cell also favor cross-presentation through their cytotoxicity against target cells. NK cell secrete also chemokine recruiting DC to inflammation site. Conversely, DC favor NK cell activation through cytokine and chemokine secretion. NK-DC cross-talk orchestrate type 1 immunity during early phase of the immune response to tumor and virus.

Regulatory roles for NK cells have also been described. Indeed, NK cell are capable of producing anti-inflammatory cytokines, particularly in response to high viral load. Studying a panel of various viral infections, Perona-Wright and collaborators showed that systemic infection leads to high production of IL-12 by DCs, inducing IL-10 secretion by NK cell. This IL-10 response was not observed for local infection which involved lower levels of IL-12 (Perona-Wright et al. 2009; Vivier et Ugolini 2009). NK cell can also directly kill lymphocytes, which may participate in the regulation of the lymphocyte pool (Fort, Leach, et Rennick 1998; Noval Rivas et al. 2010; Waggoner et al. 2012). These “regulatory” NK cells have therefore been proposed to play a role in tolerance and limitation of systemic inflammation immunopathology.

This first part presented fundamental aspects of NK cell biology and highlight their role in cellular immunity. It also introduced cytokines as central regulators of NK cell maturation and function. In particular, IL-15 and IL-18 are two major positive regulators of NK cell response, which are attracting increased interest with the development of NK cell based immunotherapy.

II. NK cell regulation by the interleukin 15 and 18

1. Biology of IL-15

Interleukin 15 (IL-15) belongs to the γ -chain cytokine family, which also regroups IL-2, IL-7, IL-4, IL-9 and IL-21. IL-15 is essential for NK cell homeostasis, as demonstrated by the absence of NK cells in IL-15 deficient mice (Kennedy et al. 2000). IL-15 is structurally close to IL-2, but has a unique role in the development and maintenance of NK cells. Indeed, no NK cell defects were evidenced in mice deficient for other members of the γ chain cytokine family (Vosshenrich et al. 2005).

1.1. IL-15 production and trans-presentation

In 1994, two groups independently identified a 14-15kDa protein with properties similar to IL-2, which they named IL-15 (Burton et al. 1994; Grabstein et al. 1994). Close in structure to other four α -helix bundle cytokines such as IL-2, IL-15 and IL-2 share nevertheless only very little sequence similarities (19%). The IL-15 gene is located on chromosome 4 in humans and chromosome 3 in mice and codes in both species for two isoforms obtained by alternative splicing. These two isoforms differ in their signal peptide length, the long one being predominant (Budagian et al. 2006; Waldmann 2006). IL-15 mRNA is constitutively expressed by a large number of cells including hematopoietic and non-hematopoietic ones. However, IL-15 is poorly translated due to the presence of multiple AUGs sites upstream the translation initiation codon, which reduces the efficiency of translation, a signal peptide sequence inefficiently translated and the presence of negative regulator in 5'UTR. Removal of these various checkpoints greatly enhance IL-15 translation (Fehniger et al. 2001). Therefore, despite an abundance of transcripts in various tissues and cell types only some efficiently produce the protein, including monocytes, macrophages, DCs and epithelial cells (Jabri et Abadie 2015; G. Cui et al. 2014). IL-15 expression is further increased by LPS or type I IFN stimulation in DCs (Mattei et al. 2001). Other studies report the importance of NF- κ B and IRF-1 to enhance IL-15 transcription (Washizu et al. 1998; Azimi et al. 2000; Ogasawara et al. 1998).

IL-15 receptor is composed of three chains: the γ chain (IL-2/15R γ /CD132) common to all cytokines of the family, the β chain (IL-2/IL-15R β /CD122) common to IL-15 and IL-2 only, and an α chain (IL-15R α /CD215) specific to IL-15. IL-15 and IL-2 thus share two out of the three chains of their receptors, the third chain of the IL-2 receptor being IL-2R α /CD25. IL-15R α is a type 1 transmembrane protein, similar in structure to IL-2R α . IL-15R α and IL-2R α are both characterized by the presence of sushi domains (one for IL-15R α and two for IL2R α) which are consensus motifs for the binding of cytokines. Compared to IL-2 and IL-2R α , IL-15 has a much higher affinity for its receptor IL-15R α (Budagian et al. 2006). This property is fundamental to understand IL-15 signaling. Indeed, IL-15 and IL-15R α are co-expressed by the producing cells where IL-15 is loaded directly onto IL-15R α in the endoplasmic reticulum. The IL-15/IL-15R α complex is then exposed to the cell surface, and presented to responding cells expressing IL-15R γ and IL-15R β . This particular mechanism is called trans-presentation and is necessary for IL-15 signaling *in vivo* (Dubois et al. 2002; Burkett et al. 2004; Huntington et al. 2009; Castillo et al. 2009) (**Figure 7**). Trans-presentation explain why NK cells deficient for IL-15R α can survive when

transferred into WT but not into IL15Ra deficient mice (Koka et al. 2003). Several soluble forms of IL-15Ra, have been reported, natural or induced, with agonist or antagonist roles (Mortier et al. 2004; 2006). In particular, the development of a truncated soluble form of IL15Ra containing the sushi domain potentiates the effects of IL-15 and has been used for therapy development (Mortier et al. 2006; Rubinstein et al. 2006; Stoklasek, Schluns, et Lefrançois 2006). The presentation of IL-15 in cis by NK cells themselves has been suggested, as NK cells expressing IL-15 and IL-15Ra have been described (Zanoni et al. 2013) but it is still debated.

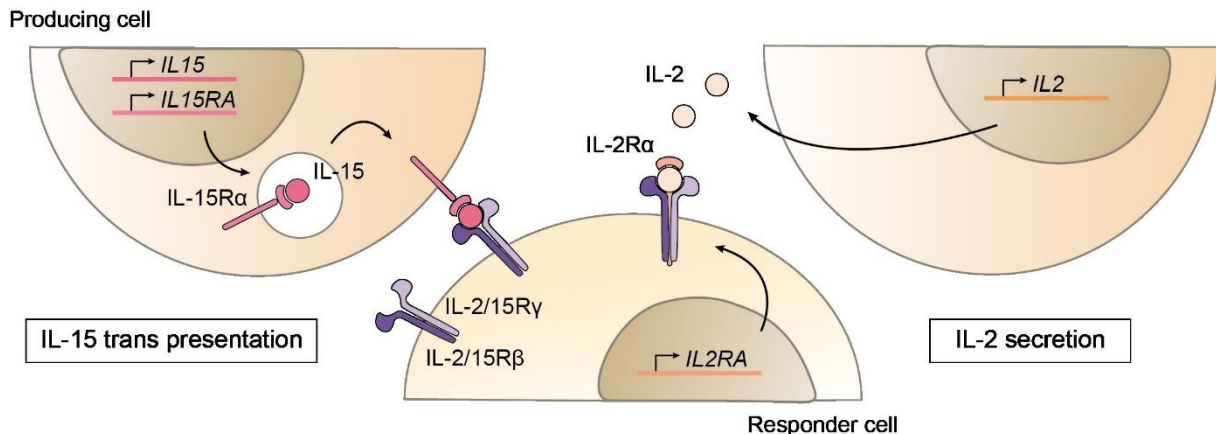


Figure 7: IL-15 trans presentation and IL-2 secretion. IL-15 and IL-2 shared the IL-2/15R γ chain and the IL-2/15 β chain. The two cytokines differentiate by their receptor α chain. IL-2R α is expressed by the responder cells whereas IL-15Ra is produced by the producing cell, allowing trans presentation of IL-15/IL-15Ra complex to the responder cell.

1.2. IL-15 roles in NK cell biology

IL-15 has a fundamental role in NK cell development as indicated by the absence of NK cells in IL-15 or IL-15Ra KO mice. Beside NK cells, IL-15 deficient mice have also reduced NKT cells, CD8⁺ T cell especially memory CD8⁺ T cells and TCR $\gamma\delta$ intraepithelial lymphocytes (Kennedy et al. 2000; Lodolce et al. 1998). To sustain NK cell development and maturation, IL-15 is trans presented by neighboring cells. IL-15Ra deficiency in DC or macrophages reduces by two the total number of NK cell. However, deleted IL-15Ra in both DC and macrophage does not recapitulate the phenotype of total IL-15Ra KO, suggesting the importance of other cell subset such as epithelial cells (Mortier et al. 2009). IL-15 is also required for the survival of mature NK cells. Mature NK cells transferred into mice that do not produced IL-15 are not maintained (Megan A. Cooper et al. 2002). The transfer of NK cells constitutively expressing the anti-apoptotic factor Bcl2 partially restores NK cell survival, even in the absence of IL-15 (Megan A. Cooper et al. 2002; Ranson et al. 2003). A study by Huntington and colleagues indicates that treatment with low doses of IL-15 limits the accumulation of the pro-apoptotic factor Bim and induces the expression of the anti-apoptotic Mcl-1 (Huntington et al. 2007). Interestingly, inhibition of Bcl-2 in this study did not influence NK cell survival in response to IL-15, suggesting that Bcl-2 can protect NK cells from apoptosis but is not the primary target of IL-15. Conversely, deprivation of IL-15 leads to an increase in Bim and Noxa and a

decrease in Mcl-1, resulting in apoptosis of NK cells (Huntington et al. 2007). Higher doses of IL-15 also support NK cell division, both *in vitro* and *in vivo* (Wiedemann et al. 2020).

IL-15 also potentiates NK cell functions (**Figure 8**). *In vitro*, NK cells pretreated with IL-15, display better effector functions upon activation by a second signal, a mechanism called priming by IL-15. Effector function of resting NK cells are actually quite low particularly so in the mouse. Indeed, resting murine NK cells express only little granzyme B and perforin proteins, two key factors in cytotoxicity (Fehniger et al. 2007). A 48h treatment with IL-15 *in vitro* increases granzyme B transcription and translation and perforin translation, resulting in better cytotoxicity against target cells. Therefore, IL-15 priming directly impacts cytotoxicity. IL-15 priming also increases cellular metabolism. 18h treatment with IL-15 *in vitro* upregulates mouse NK cell metabolism, with a higher impact on glycolysis (Marçais et al. 2014). This increase probably participates to the impact of IL-15 on effector functions, by sustaining energy production for effector component synthesis. IL-15 priming has also been observed in human cells. Human NK cell treated 16h with IL-15 *in vitro* and then challenged with tumor show an increase in cytokine IFN- γ and TNF α production, and an increase in cell cytotoxicity, measured by granzyme B and perforin expression and killing assay (Wagner et al. 2017). The most immature cells (CD56^{bright}) seem to respond more strongly to IL-15 priming than the mature ones (CD56^{dim}). In this study, the authors also show that IL-15 priming increases the expression of activating receptors and adhesion molecules such as LFA-1, promoting a better recognition of target cells and their killing (Wagner et al. 2017). A key study from Andreas Diefenbach's group addressed the physiological relevance of IL-15 priming *in vivo* and highlight the importance of DC in this process (Lucas et al. 2007). The authors observed that NK cell priming is induced by injection of different TLR agonists in mice. Similarly to IL-15 priming *in vitro*, NK cells primed by TLR injection are characterized by an increase in IFN- γ and granzyme B production and increased cytotoxic capacities. Ablation of the TLR response in NK cells specifically did not reduce priming, however depletion of dendritic cells specifically blocks the increase in NK cell functions, therefore showing that DCs are responsible for the priming of NK cells in response TLR stimulation. The authors then studied the spatial dynamic of DC and NK cells. Local TLR stimulation led to the rapid recruitment of NK cells in the corresponding draining lymph node (4h) followed by their increase in the periphery (8h). Blocking the entry of NK cells into the draining lymph nodes reduced NK cell number in the lymph node and in the periphery and reduced NK cell priming. Thus, as for T cells, the model proposed is that DC activation leads to their recruitment to the secondary lymphoid organs where they can prime NK cells, which then return to the periphery. Finally, the author identified IL-15 as the key molecule mediating NK cell priming by DCs, and showed that priming was dependent on cell-cell contact, suggesting the importance of trans-presentation for priming *in vivo* (Lucas et al. 2007).

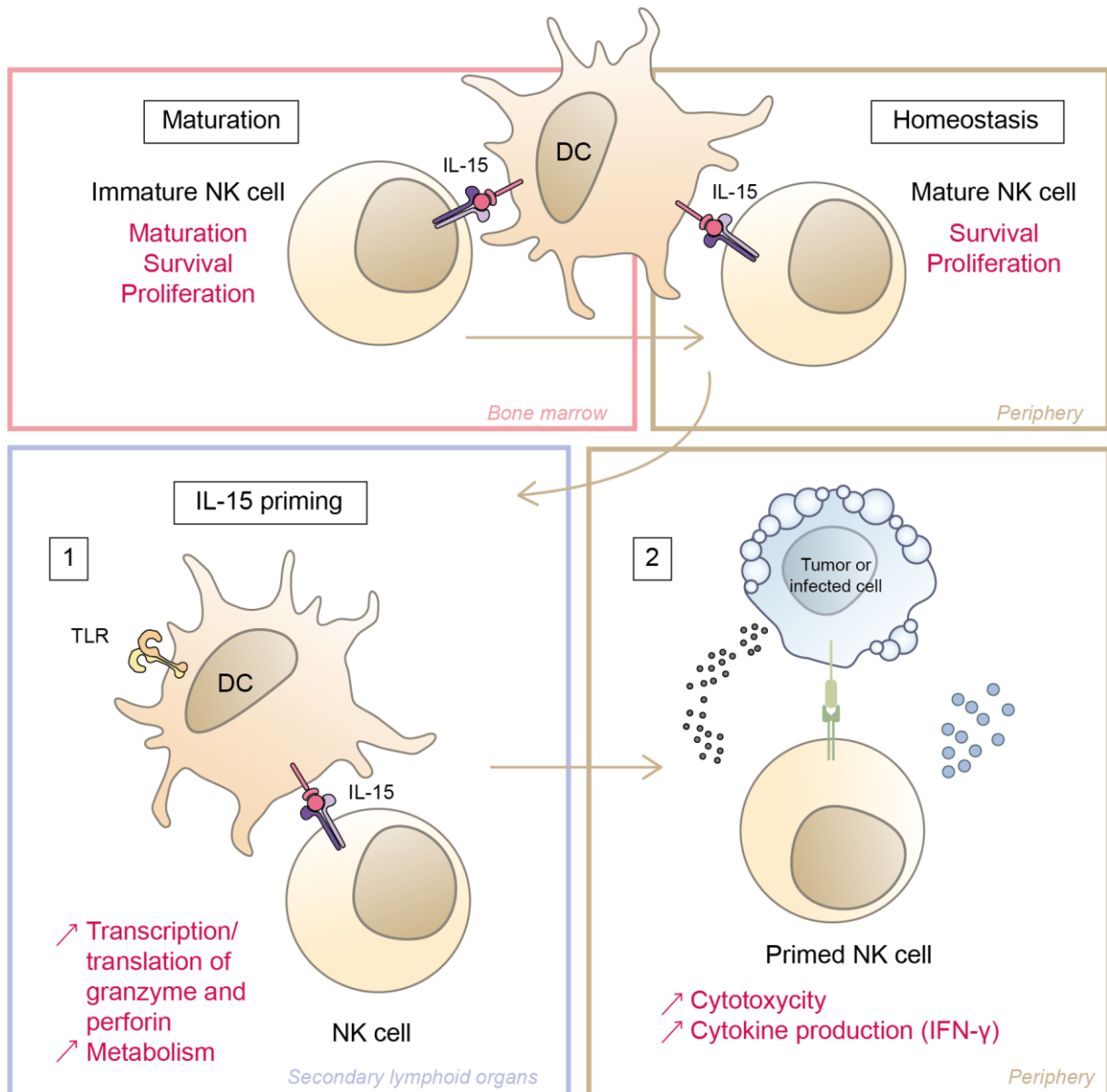


Figure 8: Roles of IL-15 in NK cell biology. IL-15 promotes NK cell development and survival. Priming with IL-15 also increase expression of cytotoxicity factor and increase cellular metabolism inducing a better effector response of primed NK cell. Effects of IL-15 on NK cells are indicated in red.

1.3. Signaling downstream IL-15 receptor

Due to their great similarities, a large part of the knowledge concerning IL-15 signaling comes from the study of IL-2, the archetype of γ chain cytokine.

IL2/15R β and IL2/15R γ do not have their own kinase activity. The binding of the cytokine to their receptors leads to the recruitment of the JAK1 and JAK3 proteins to the β and γ receptor chains respectively. JAK1/3 proteins then phosphorylate several tyrosine residues on the receptor β chain, creating attachment sites for SH2 domain protein. Hence, the IL-2/15 receptor engagement activates 3 main pathways namely the STAT5 pathway, the PI3K-AKT pathway and the MAPK ERK pathway (**Figure 9**). The very proximal

position of the JAK proteins is highlighted by a total absence of NK cells in mice deficient for JAK3 (Park et al. 1995).

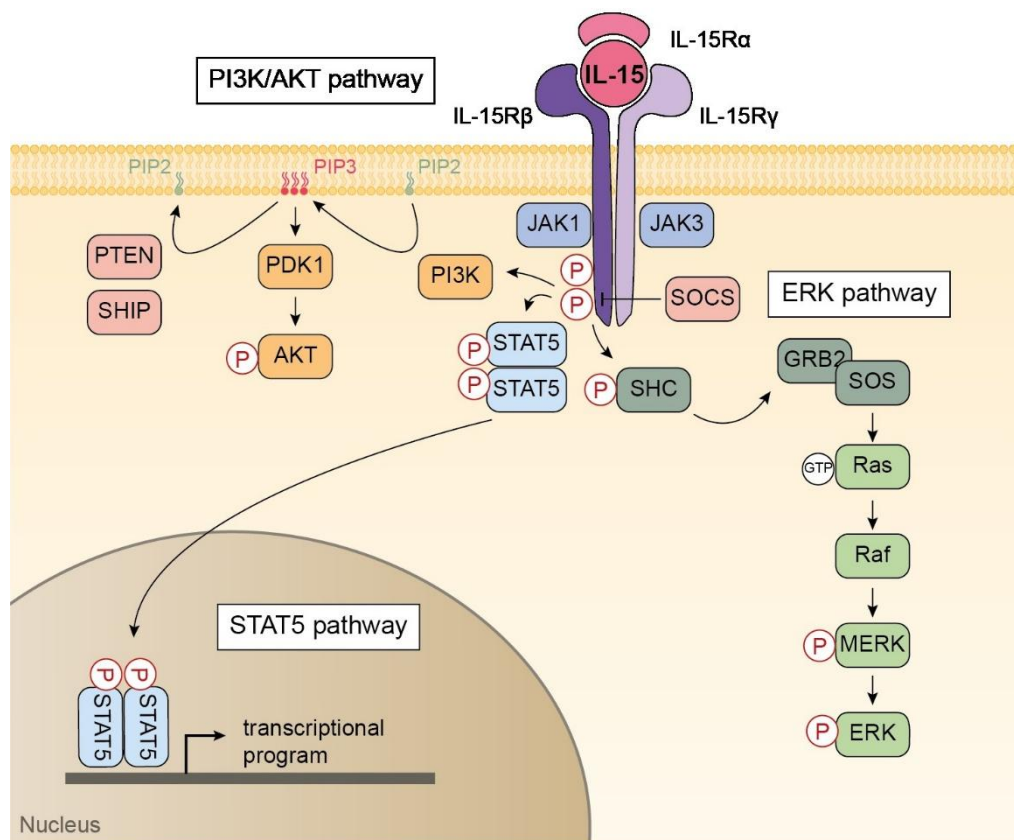


Figure 9: IL-15 signaling. Upon receptor engagement, IL-15 induces the activation of JAK1 and JAK3, which phosphorylates different residues and the β chain of the receptor, creating a platform for SH2 domain protein recruitment. This triggers the activation of three main pathways: the PI3K-AKT pathway, The STAT5 pathway and the MAPK-ERK pathways. PTEN, SHIP and SOCS are negative regulators.

STAT5 pathway. Activation of JAK1/3 leads to the recruitment and phosphorylation of the transcription factors STAT5a and STAT5b. Once phosphorylated, STAT5s dimerize and are then translocated to the nucleus where they bind to different sequences including gamma interferon-activated sequences (GAS), and induced a specific transcription program. STAT5s also exist as tetramers which are specifically required for T and NK cell homeostasis and response to cytokines (Lin et al. 2012). STAT5a and STAT5b double KO leads to an absence of NK in the periphery and the remaining cells are blocked at the precursor stage (Eckelhart et al. 2011). Specific deletion of STAT5b alone results in a more severe phenotype than deletion of STAT5a alone, characterized by a more severe decrease in NK cell number and a stronger impairment response to IL-2 and IL-15 for STAT5b deficient cells than STAT5a ones (Imada et al. 1998). STAT5 has an important role in NK cell survival. Absence of STAT5 is correlated with a decrease in the Bcl2 and Mcl-1 expression (Gotthardt et al. 2016). More recent studies with partial or inducible KO, indicate that STAT5 also participates in the transcription of a large set of genes which are

important for the differentiation and functions of NK cells. These genes include T-bet, KLRG1, the Ly49 receptors, granzyme A, IFN- γ or even the IL-15 receptor itself (Villarino et al. 2017). Finally activation of STAT5 downstream of IL-15 is needed for the survival and the generation of adaptative NK cells, which will be described later, following MCMV infection. STAT5 conditional deletion leads to greater susceptibility to MCMV and a reduced proportion of Ly49H⁺ cells (Wiedemann et al. 2020).

JAK-STAT signaling is negatively regulated by suppressor of cytokine signaling (SOCS) proteins such as CIS and SOCS1-7. These proteins also have a SH2 domain, and can bind to the IL2/15 receptor β chain, which directly inhibits JAK protein attachment or can also mediate proteasomal degradation of bound proteins (Linossi et al. 2013). CIS deficient NK cells show an increase in proliferation, survival and cytotoxicity in response to IL-15. Mechanistically CIS targets JAK1 and promotes its proteasomal degradation, inhibiting signaling downstream IL-15. CIS deficiency in NK cells favor antitumor NK cell function and metastasis control *in vivo* in a model of mouse breast cancer (Delconte et al. 2016). Similarly, SOCS2 deficiency has been shown to increase NK cell number and inhibits JAK signaling through JAK2 interaction (W. S. Kim et al. 2017).

PI3K pathway. Class I PI3Ks catalyze the conversion of phosphatidylinositol (4,5) biphosphate (PI(4,5)P₂) (PIP₂) into PI(3,4,5)P₃ (PIP₃). PI3K class I are subdivided into subclasses A and B. Class IA PI3Ks consist of a p110 catalytic subunit and a p85, p55 or p50 regulatory subunit. Three variants of p110 have been described, p110 α (*Pik3ca* gene), p110 β (*Pik3cb* gene), and p110 δ (*Pik3cd* gene). Class IB PI3Ks are formed by the association of p110 γ (encoded by the *Pik3cg* gene) and a regulatory subunit p101 or p84. The conversion of PIP₂ to PIP₃ creates a recruitment site for proteins possessing a pleckstrin homology (PH) domain such as AKT or PDK1. p110 α and β are widely expressed whereas the p110 δ and p110 γ expression are restricted to lymphocytes. Phosphorylation of the IL-15R β chain also forms a recruitment site for the PI3K regulatory subunit. Several studies have characterized the impact of inhibition of p110 δ and γ subunits on NK cell biology. NK cells deficient for PI3K δ show a defect in maturation and in cytokine (IFN- γ) and chemokine production (H. Guo et al. 2008). The double KO p110 δ and p110 γ is characterized by an even more severe phenotype, with an additional lack of cytotoxicity and proliferation (Tassi et al. 2007; N. Kim et al. 2007). Of note, many signals can activate PI3K including activating receptors, and the phenotypes described in these studies may be due to other factors than IL-15 signaling deficiency. Inhibition of PI3K by pharmacological inhibitors have also been shown to interfere with IL-15 priming (Wagner et al. 2017; Nandagopal et al. 2014).

The reverse reaction, the conversion of PIP₃ back to PIP₂, is catalyzed by the phosphatase PTEN which metabolizes PI(3,4,5)P₃ into PI(4,5)P₂. In addition, phosphatases of the SHIP family (SHIP-1 and 2) convert PI(3,4,5)P₃ into PI(3,4)P₂, thus also counteracting PI3K activity. PTEN deficiency in NK cells increases PI3K signaling following IL-15 stimulation, measured by AKT phosphorylation, indicating that PTEN regulates PI3K activity downstream IL-15 in NK cell (Leong et al. 2015). In terms of phenotype, PTEN deficiency has been associated with increased NK cell cytotoxicity upon target stimulation and

alteration of NK cell trafficking but the link with the IL-15 signaling has not been assessed in these studies (Leong et al. 2015; Briercheck et al. 2015).

ERK pathway. Finally, engagement of the IL-15 receptor drives the recruitment of Shc. Phosphorylation of Shc recruits the GRB2-sos complex. Sos is a Guanine nucleotide Exchange Factor (GEF) for GTP-ase Ras, which is the entry point of the MAPK ERK pathway. Like the PI3K pathway, the ERK pathway can be induced by many stimuli. The importance of ERK downstream IL-15 in NK cell has been assessed indirectly through the generation of specific Shp-2 deletion in NK cell (Niogret et al. 2019). Shp-2 (encoded by Ptpn11) is a phosphatase with an atypical positive effects on various growth factor signaling, the best described being its positive regulation of ERK. Shp-2 deficiency in NK cell leads to an increase in the number of NK cells, especially of the mature ones. These cells also exhibit a defect in proliferation and superior metabolism in response to IL-15. Shp-2 deficiency is associated with a decrease in ERK activation, both at basal state and after IL-15 stimulation. Inhibition of ERK by pharmacological inhibitors decreases WT NK cell proliferation in a similar manner as the Shp-2 deficiency (Niogret et al. 2019). Other studies using inhibitors also show the importance of ERK for survival downstream of IL-15 (Huntington et al. 2007) and cell priming (Wagner et al. 2017; Nandagopal et al. 2014).

Dose effect. How IL-15 promotes such a large amount of effect on NK cell? Similarly, how IL-2 and IL-15 produce distinct signal with such close receptors? The importance of cytokine doses and receptor engagement strength has emerged in several report as a key aspect of IL-15 signaling. Low dose and high dose of IL-15 does not produce the same effect on NK cells. IL-15 low dose is sufficient to engage the STAT5 pathway and cell survival, while proliferation or metabolism increase are triggered by higher doses of IL-15 (Marçais et al. 2014). In the same sense, another study from Ring and colleagues indicates that IL-2 and IL-15 receptor have an extremely close structure (Ring et al. 2012). At saturating doses, IL-2 and IL-15 are even able to produce the same proximal signaling with the same kinetic. However, that is no longer true at lower concentration. Therefore, IL-2 and IL-15 differences would lie mostly in the quantity of cytokine available and strength of their receptor engagement (Ring et al. 2012).

1.4. NK cell regulation by other cytokines of the γ -chain family

IL-2. IL-2 recapitulates many of the effects of IL-15 *in vitro* (**Figure 10**). For instance, IL-2 is used for the *in vitro* expansion of NK cells. The cells obtained are called LAK (Lymphokine Activated Killer cells) and are commonly used for NK cell analysis. The relevance of IL-2 activity on NK cells *in vivo* however, is more difficult to assess. NK cells do not express the IL2R α without stimulation and NK cells develop normally in IL-2 deficient mice, suggesting that IL-2 is not required for NK cell homeostasis (Vosshenrich et al. 2005). IL-2 may however participates to NK cell activation in pro-inflammatory settings. IL-2 have for instance been shown to promote IFN- γ production by NK cells in response to E.coli (Granucci et al. 2004), or *Leishmania major* (Bihl et al. 2010). The pro-inflammatory cytokine IL-12 also increases the expression of CD25 on NK cells and conversely, IL-2 potentiates the effects of IL-12 (Bihl et al. 2010; K. S. Wang, Frank, et Ritz 2000). Regulatory T cells (Treg) may modulate NK cell activity by a mechanism dependent

on IL-2. Several reports show that Treg depletion increases IL-2 availability which in turn activates NK cell function (Gasteiger, Hemmers, Firth, et al. 2013; Sitrin et al. 2013; Gasteiger, Hemmers, Bos, et al. 2013).

IL-21. Discovered in 2000, IL-21 is another homolog of IL-2 and IL-15 whose receptor is expressed by B cells, T cells, DCs and NK cells (Parrish-Novak et al. 2000). Deletion of the IL-21 receptor does not alter NK cell development (Kasaian et al. 2002). IL-21 alone is not sufficient to sustain NK cell survival *in vitro* but potentiates NK cell maturation and effector functions such as IFN- γ production and cytotoxicity (Kasaian et al. 2002; Brady et al. 2004). IL-21 also boosts NK cell antitumoral functions *in vivo* and has been shown to reverse NK cell dysfunction in a murine tumor model (Seo et al. 2017).

IL-7. IL-7 is an important cytokine for the early phases of B and T cell development. Although NK cell progenitors also express the IL-7 receptor, NK cells are normal in IL-7 KO mice (Y. W. He et Malek 1996; Vosshenrich et al. 2005).

IL-4. IL-4 impact on NK cell is rather controversial. IL-4 deletion in mice does not impact NK cell development (Vosshenrich et al. 2005). Several studies have reported an inhibitory role of IL-4, such as inhibition of cytotoxic capacities and a reduction in the expression of NKG2D in IL-4 treated NK cell (Brady et al. 2010; Marcenaro et al. 2005). However, other studies have described a rather activating role for IL-4, particularly by promoting IFN- γ production by NK cells (Morris et al. 2006; Kiniwa et al. 2016).

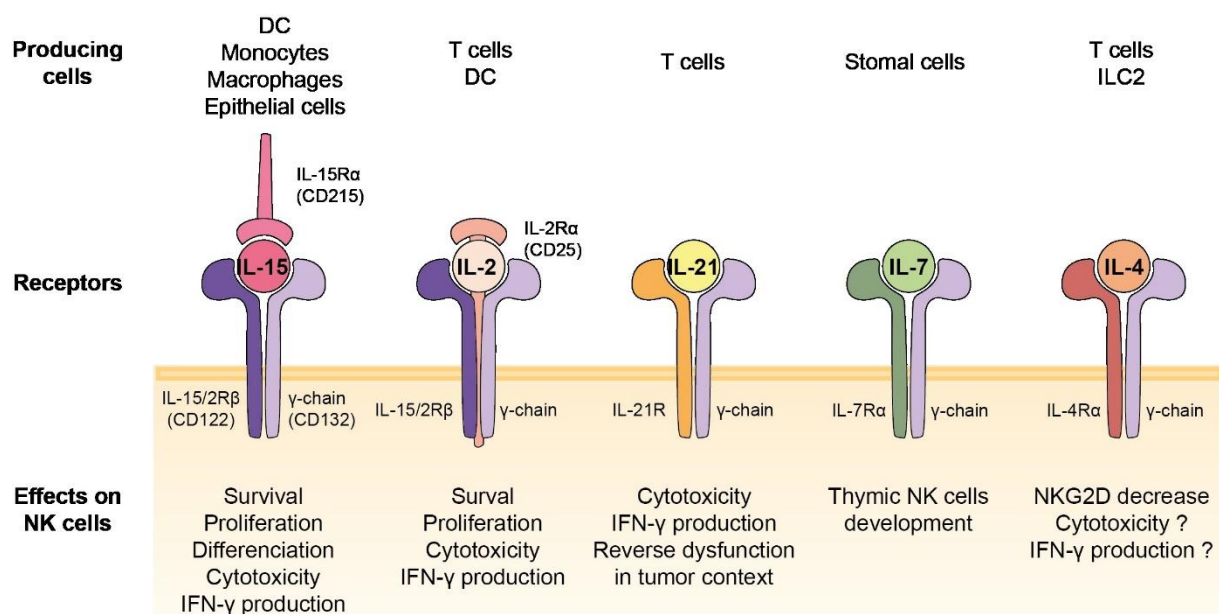


Figure 10: Cytokines of the γ -chain family in NK cell biology. Cytokines belonging to the γ -chain family share the same receptor γ chain but have different functions on NK cells. Producing cells, receptor structures and impact on NK cell biology are reported. Adapted from (Marçais et al. 2013).

2. Biology of IL-18

IL-18 was first described in 1989 and named “IFN- γ inducing factor” due to its capacity to enhance IFN- γ production by lymphocytes. After isolation and cloning, the name was changed for IL-18 in 1995 (Okamura et al. 1995). IL-18 belongs to the IL-1 family, as IL-1 β with which it shares a lot of similarities. It is a proinflammatory cytokine, which promotes type 1 immunity in various models. In particular IL-18 in combination with cytokines signaling via JAK-STAT increases IFN- γ production in type 1 immunity effectors, such as T cells, NK cells and ILCs (Landy et al. 2024).

2.1. IL-18 production

IL-1 cytokine family regroups 11 members, including the pro-inflammatory cytokines IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β and IL-36 γ . The family also contains 4 members with anti-inflammatory properties: the IL-1 antagonist receptor (IL-1Ra), the IL-36 receptor antagonist (IL-36Ra), IL-37 and IL-38 (Dinarello 2019; Gaballa et al. 2024). The cytokines of the IL-1 family are further divided into 3 subfamilies based on their affinity with their receptor. The IL-1 subfamily is composed of IL-1 α , IL-1 β and IL-33 and binds to the IL-1R3 receptor (IL1RAP), the IL-18 subfamily is formed with IL-18 and IL-37 and engages IL-1R5 (IL-18R), and the IL-36 subfamily is composed of IL-36 α , IL-36 β , IL-36 γ and IL-38 and binds to IL-1R6 (IL-36R). Unlike most IL-1 family genes which are located on chromosome 2 in humans, the IL-18 gene is located on chromosome 11 in humans and chromosome 9 in mouse (Taylor et al. 2002; Sims et Smith 2010). Like other members of the IL-1 family including IL-1 β , IL-18 is first produced as a pro-peptide and needs to be cleaved into its mature form. However, unlike pro IL-1 β , pro IL-18 is constitutively expressed by many cells, including myeloid cells such as macrophages, DCs, neutrophils and monocytes, and non-myeloid cells such as epithelial cells (Landy et al. 2024).

As for the pro IL-1 β , the best characterized maturation mechanism for IL-18 is the one involving caspase 1 and its activation by the inflammasome (Ghayur et al. 1997; Fantuzzi et al. 1998; Y. Gu et al. 1997) (**Figure 11**). Inflammasomes are oligomeric protein platforms, composed of a receptor, an adaptor and the caspase 1, the effector. The inflammasome receptors perceive PAMP (pathogen associated molecular pattern) or DAMP (damage associated molecular pattern) signals which trigger the recruitment of the adapters, followed by the recruitment of pro-caspases 1 through their interaction between homotypic domains. Oligomerization of pro-caspases 1 leads to their autoproteolytic cleavage and their activation. Activated caspases 1 then catalyze the cleavage of different protein, including pro IL-18 into its mature form. Inflammasome are named according to the receptor they involved. IL-18 can be activated virtually downstream any inflammasomes, the one reported so far being NLRP3, pyrin, NLRC4, NLRP6 or NLRP9B inflammasomes (Canna et al. 2014; Levy et al. 2015; Zhu et al. 2017; Landy et al. 2024). Independently from the inflammasome, other proteases can also cleave pro IL-18, such as the caspase 8 downstream of Fas/FasL (Bossaller et al. 2012), the proteinase 3 (Sugawara et al. 2001) or granzyme B (Omoto et al. 2010; Akeda et al. 2014).

IL-18, like IL-1 β , lacks a signal peptide. Thus several secretion mechanisms have been proposed for these cytokines. Activation of the inflammasome and caspase 1 also leads to the cleavage of gasdermin D which oligomerizes into pores into the plasma membrane. These pores are associated with a particular cell death, the pyroptosis, but could also serve as a passive exit pathway for cytokines such as IL-1 β and IL-18 (Tapia et al. 2019; Xia et al. 2021). Other caspases such as caspases 4 and 5 can also activate gasdermin D but appear to have limited activity in the maturation of IL-1 β and IL-18 (Landy et al. 2024). IL-1 β and IL-18 secretion can also be induced by active mechanisms. In particular, one study showed that the interaction between NK cells and DC promotes IL-18 transport to the immunological synapse and then its polarized secretion into lysosomes towards NK cells (Semino et al. 2005).

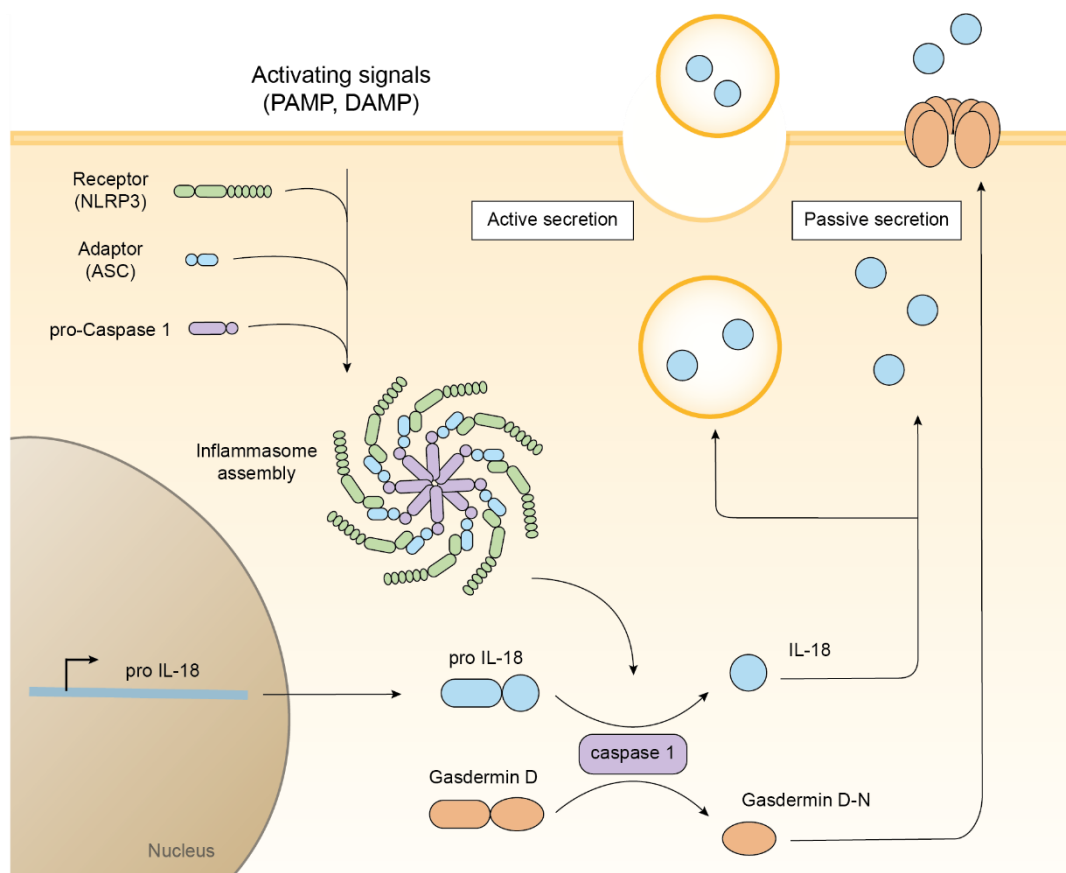


Figure 11: Production and secretion of IL-18. IL-18 is first produced as a pro peptide (pro IL-18) and required to be cleaved into its mature form. Several proteases can catalyze pro IL-18 conversion into mature IL-18, the best described are caspase 1 which are themselves activated following inflammasome assembly. Inflammasome activation also leads to gasdermin D activation. IL-18 can then be secreted either passively through pores form by activated gasdermin or actively through lysosome. Adapted from (Dinarello 2019)

2.2. Signaling downstream IL-18 receptor

Most of the data concerning signaling downstream of IL-18 come from studies on IL-1 β (**Figure 12**). The IL-18 receptor consists of two chains, IL-1R5 and IL-1R7. IL-18 binding to IL-1R5 leads to the recruitment of IL-1R7, and the autophosphorylation of their TIR domains. Phosphorylation of the receptor drives the recruitment of the proteins MyD88, IRAK4, 1 and 2 sequentially, into a structure called the Myddosome, also involved in signal transduction downstream of the TLRs. Myddosome assembly allows activation and oligomerization of the E3 ubiquitin ligase TRAF6. In association with the E2 ligase UBC13 and UEV1A, TRAF6 catalyzes the K63 poly-ubiquitination of various proteins including TAB2/3, IRAK and TAK1. TAK1 kinase then assembles into different signaling complexes, such as the one composed of TAB or MEKK3 proteins, and leads to the activation of different pathways including the MAPK c-Jun-N-terminal kinase (JNK), the MAPK p38 and the NF- κ B pathway (Weber, Wasiliew, et Kracht 2010).

IL-18 activity is also regulated extracellularly by the IL-18 binding protein (IL18BP). IL18BP is a negative regulator of IL-18 signaling as its binding to the cytokine prevents the ligation of IL-18 to its receptor (Novick et al. 1999). IL-18BP binds IL-18 with high affinity, therefore in healthy donors, more than 95% of IL-18 is associated with IL-18BP (Landy et al. 2024; Ha et al. 2016). IL18BP is constitutively expressed by a large number of cells and IFN- γ , the main molecule downstream IL-18, is a strong activator of the IL-18BP, resulting in a negative feedback loop (Harel et al. 2020).

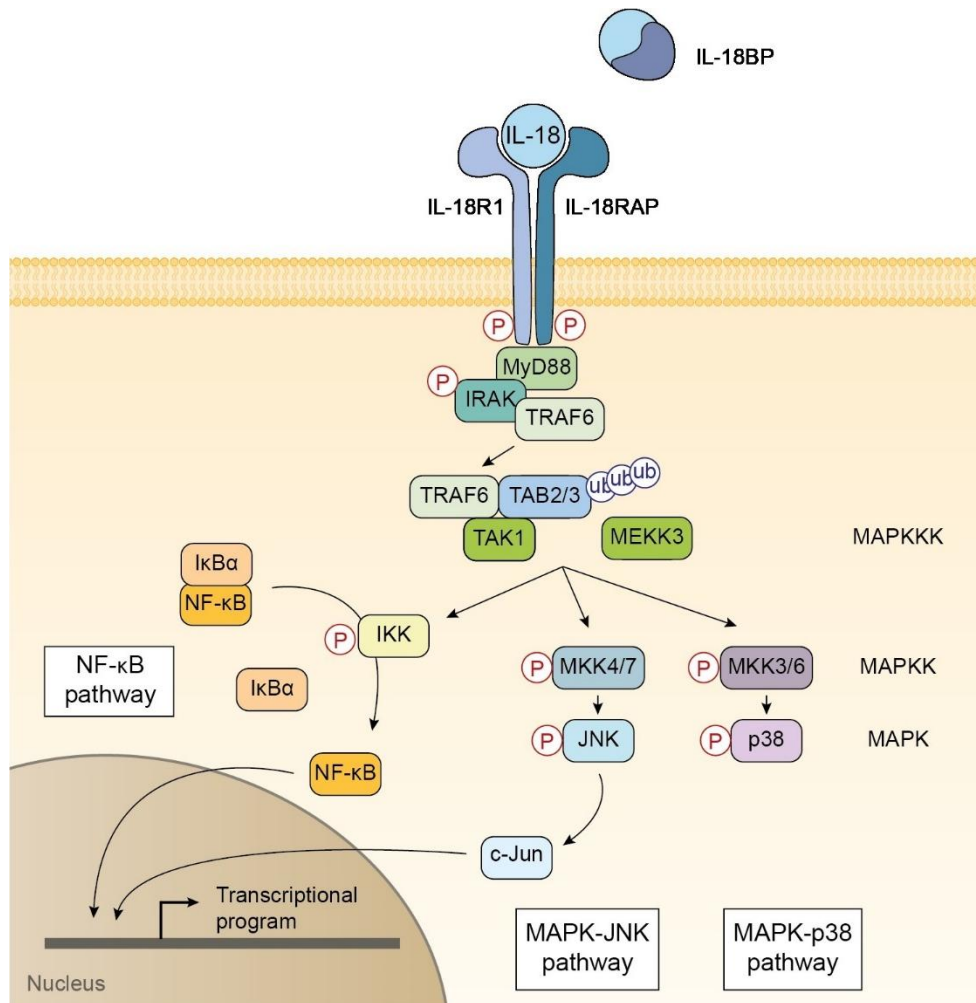


Figure 12: IL-18 signaling. IL-18 binding to its receptor leads to their phosphorylation on their TIR domain and the recruitment of MyD88 and IRAKs in a structure call the Myddosome. This allows the activation of the E3 ubiquitin ligase TRAF6 and the activation of three main pathways: the NF-κB pathway, the MAPK JNK pathway and the MAPK p38 pathway. IL-18 activity is also negatively control by the IL-18BP.

2.3. IL-18 roles in NK cell biology

IL-18 receptor is constitutively expressed by NK cells. NK cells lacking IL-18 signaling component show no defect in number or maturation (Chaix et al. 2008). However, their functionality (cytotoxicity and IFN-γ secretion) is affected by deletion of the IL-18 receptor (Hoshino et al. 1999) or proteins composing the Myddosome (Suzuki et al. 2003; Kanakaraj et al. 1999; Adachi et al. 1998; Chaix et al. 2008), highlighting the importance of IL-18 for NK cell effector functions.

IL-18 is a major activator of IFN-γ secretion in synergy with JAK-STAT cytokines and in particular with IL-12. These two cytokines are therefore often studied together, making sometimes difficult to assess their individual role. IL-12 (and IL-18 alone to a lesser extent) activates IFN-γ transcription. Indeed, IL-18 engages the NF-κB and JNK/c-jun pathway (Kojima et al. 1999; Nakahira et al. 2002) and IL-12 activates the STAT4 transcription factor, which are both positive regulators of IFN-γ transcription. Besides, several studies

describe synergic cross-talk between IL-18 and IL-12 at different level of IFN- γ production. IL-18 induces the expression of I κ B ζ , the deletion of which reduces IFN- γ production downstream of IL-12/18 stimulation (Miyake et al. 2010; Kannan et al. 2011). I κ B ζ expression promotes IFN- γ mRNA accessibility to the transcription factor STAT4, downstream IL-12 and histone 3 (H3K9) acetylation which has been shown to promote IFN- γ transcription (Miyake et al. 2010). I κ B ζ also binds directly to the IFN- γ promoter and to NF- κ B, which could participate in the transcriptional regulation of IFN- γ downstream of IL-18 (Kannan et al. 2011). Mavropoulos and colleagues also report post-transcriptional control of IFN- γ by IL-18. IFN- γ mRNA and protein level are strongly reduced by treatment with a p38 inhibitor downstream of IL-12/18 stimulation. The authors subsequently show that IL-18 allows the stabilization of the IFN- γ mRNA 3' UTR through the induction of p38 and its downstream target MK2 (Mavropoulos et al. 2005). Altogether these studies show that IL-12 and IL-18 synergize at multiple level of IFN- γ production in NK cells (**Figure 13**).

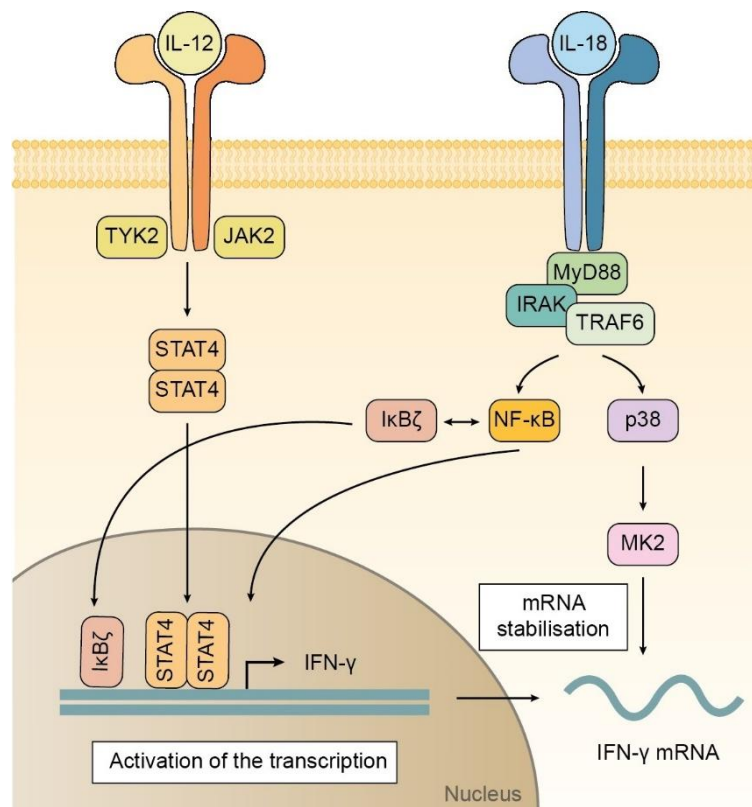


Figure 13: Synergy between IL-18 and IL-12 for IFN- γ production. IL-18 and IL-12 both activates IFN- γ transcription in a synergic manner. IL-18 also stabilized IFN- γ mRNA.

Besides its role on IFN- γ , IL-18 also regulates various other genes, alone or in combination with IL-12. IL-18 notably induces the transcription of factors important for cellular metabolism (Myc), cross-talk with myeloid cells (GM-CSF, XCL-1) or even cytotoxicity (Granzyme B, perforin) (A. Cui et al. 2024; Fehniger et al. 2007). This transcriptional role could account for IL-18 broad positive effect on NK cell effector functions. *In vitro*, the combination of IL-12 and IL-18 at high doses could also induces NK cells proliferation (Lauwerys, Renaud, et Houssiau 1999).

IL-18 positive impact on effector functions has also been further confirmed *in vivo*. IL-18 induced IFN- γ production by NK cells in various infectious contexts (Rowland et al. 2006; Kawakami et al. 2000; Pien et al. 2000). IL-18 also favor NK cell function in certain tumor settings, activating both direct cytotoxicity (Dupaul-Chicoine et al. 2015) and the production of IFN- γ by NK cells (Ardolino et al. 2014; Ni et al. 2020).

2.4. NK cell regulation by other cytokines of the IL-1 family

IL-1 β . IL-1 β has very similar structure to IL-18 and activates similar signaling pathways. NK cells constitutively express the IL-1 receptor (IL-1R1). In addition, treatment with IL-1 β and IL-12, as for IL-18/IL-12, strongly induces the transcription and production of IFN- γ in NK cells (M. A. Cooper, Fehniger, Ponnappan, et al. 2001) (**Figure 14**).

IL-33. IL-33 is a dual-function cytokine, like IL-1 α , which signals both intranuclearly by binding directly to DNA and extracellularly by binding to its ST2/IL-1R4 receptor. IL-33 has historically been associated with the type 2 response and the activation of Th2 and ILC2 cells which express the IL33 receptor, ST2. However, more recent studies have shown that NK cells can also express ST2 (Smithgall et al. 2008; Bourgeois et al. 2009; Qi et al. 2020), in particular ST2 expression is promoted by IL-12 treatment (Eberhardt et al. 2023). IL-33 in association with IL-12 activates the production of IFN- γ by NK cells (Kearley et al. 2015; Smithgall et al. 2008; Bourgeois et al. 2009). Combined IL-12/IL-33 treatment also promotes the NK cell antitumor activity *in vivo*, notably through the production of IFN- γ (Eberhardt et al. 2023).

IL-37. IL-37 is a cytokine of the IL-1 family with anti-inflammatory properties. A study shows that NK cells cultured in the presence of Treg exhibit a reduction in IFN- γ production and expression of the proliferation marker Ki67. Culture in the presence of IL-37 receptor blocking antibody restored IFN- γ secretion and Ki67 expression, suggesting a role for IL-37 in the suppression of NK functions by Tregs (Sarhan et al. 2018).

IL-36. IL-36 signals through the IL-36 receptor (IL-1R6) and IL-1R3, like IL-1 and IL-33. A study showed that IL-36 γ in combination with IL-12 also induces IFN- γ production by NK cells (X. Wang et al. 2015).

Sun and Lanier then demonstrated the existence of memory NK cells in a viral context (J. C. Sun, Beilke, et Lanier 2009). In C57BL/6 mice, about 50% of the NK cells express the activating receptor Ly49H at basal state, which recognize the m157 glycoprotein of the MCMV. Mice infection with MCMV leads to the specific expansion of Ly49H⁺ NK cells. This NK cell population contracts after infection but a sizable Ly49H⁺ NK cell population with improved capacities remains for several months. Indeed, the isolation and re-stimulation of these Ly49H⁺ NK cells by m157 results in an amplified response, characterized by a higher percentage of cells producing IFN- γ and displaying CD107a. These memory Ly49H⁺ NK cell can be also distinguished from naive Ly49H⁺ NK cell based on their differential expression of several surface marker such as their higher level of KLRG1. This response has a physiological role as transfer of memory Ly49H⁺ cells protects newborn mice from lethal MCMV infection. In human too, HCMV infection leads to the expansion and persistence of a specific NKG2C⁺ population, showing greater functionality toward a second HCMV infection (Gumá et al. 2004; Schlums et al. 2015; Foley et al. 2012). These memory NKs, sometimes called adaptive NK cells by analogy with T lymphocytes, have since been described for other viruses than CMV, including vaccinia virus (Gillard et al. 2011), influenza virus (Paust et al. 2010), HIV (Paust et al. 2010; Reeves et al. 2015) or the herpes virus HSV-2 (Abdul-Careem et al. 2012).

3.2. Cytokine Induced Memory Like NK cells

In parallel to these studies, Cooper and Yokoyama reported that murine NK cells treated with a cocktail of IL-15, IL-12 and IL-18 exhibit memory like properties (Megan A. Cooper et al. 2009). In this pioneering study, NK cells were treated 16h with a combination of cytokine IL-15, IL-12 and IL-18, or IL-15 alone, before transfer into Rag KO mice. After initial proliferation, cells return to a resting state about a week after transfer. However, in response to a second challenge *in vitro*, the NK cells that have been treated with IL-15/12/18 show a higher IFN- γ production than the cells treated with IL-15 alone or endogenous NK cells. This response is cell-intrinsic, passed to daughter cells and lasts for several weeks after transfer. These results indicate that IL-15/12/18 treated cells are endowed with memory like properties and were thus named Cytokine Induced Memory Like (CIML) NK cells (Megan A. Cooper et al. 2009). Besides increase IFN- γ production capacities, later studies demonstrated that CIMLs also display a higher cytotoxic potential measured by the expression of granzyme B and perforin. This probably explains their better antitumor activity *in vivo*, as CIML injection combined with radiation therapy reduced tumor growth and increased mice survival in an MHC-I deficient RMA model (Ni et al. 2012). Memory like responses following IL-15/12/18 treatment has also been studied in human in particular by the Fehniger's group (Romee et al. 2012; 2016). NK cells activated overnight with IL-15, IL-12 and IL-18 or with IL-15 alone were then maintained for 1 to 3 weeks with low dose IL-15 *in vitro*. After a week no difference in IFN- γ production at basal state was observe between control IL-15 or IL-15/12/18 treated cells. However, upon a secondary challenge with cytokines or tumor cells one to three weeks later, the IL-15/12/18 treated NK cells have a superior IFN- γ secretion capacity than control cells only treated with IL-15. This response is observed in both CD56^{dim} and CD56^{bright} cells and is also preserved during cell divisions. Unlike hapten or virus memory which are quite

similar to adaptive immunity, the memory like response to cytokines is more reminiscent of the concept of training immunity mentioned above as CIMLs have an amplified response to multiple stimuli and not specifically to a given one. Furthermore, the physiological importance of this mechanism in the context of infection has not been shown, although CIMLs have important therapeutic applications which will be discussed in the following section.

Several molecular mechanisms can explain the improved functionality of CIMLs (**Figure 15**). Phenotypically, CIMLs displays few change compared to non-treated NK cells. A mass cytometry analysis of human CIMLs establishes that CIMLs show an increase in the expression of certain markers such as the adhesion molecules CD56, CD62L, the proliferation marker Ki67, the IL-2 receptor CD25, the activation marker CD69, and the NKG2A, NKG2D, NKp30 or NKp44 receptors (Romee et al. 2016). CIML also display increased expression of effector molecules such as TRAIL, perforin and granzyme B. The same group also confirmed that these phenotypical changes were conserved after CIMLs transfer *in vivo* in patient treated with CIML therapy (Berrien-Elliott et al. 2019). In particular, they highlight a correlation between the expression of the inhibitory receptor NKG2A and treatment failure in certain patients.

Compared to IL-15 treated cells, IL-15/12/18 treatment induces an increase in the IL-2 receptor CD25 expression, making CIMLs responsive to IL-2 *in vitro* and *in vivo*. This might explain that two studies show that CIML survival and expansion *in vivo* is dependent on IL-2 (Leong et al. 2014; Ni et al. 2012).

Finally, epigenetic modification can account for CIML higher activity. In particular, a study reports demethylation of the conserved non-coding sequence 1 (CNS1) of the IFN- γ locus promoting transcription of the gene in CIMLs (Ni et al. 2016).

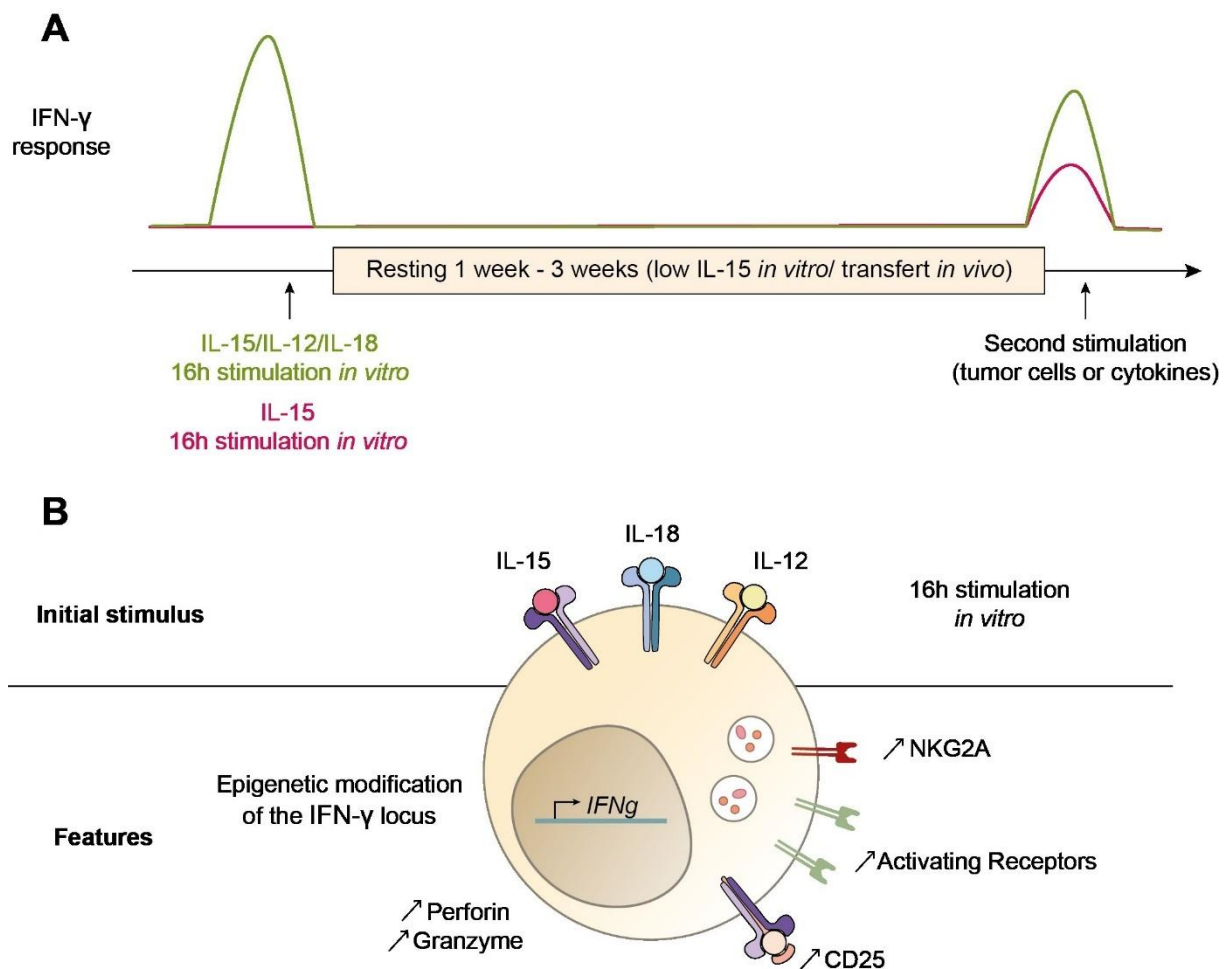


Figure 15: Generation and features of Cytokine Induced Memory-Like (CIML) NK cell. A. Schematic representation of CIML generation and response. NK cells are treated with IL-15 alone (control) or IL-15 in combination with IL-12 and IL-18 (CIML) for 16h and then maintained for several weeks either under low dose of IL-15 for survival or by transfer *in vivo*. CIMLs show an increase in IFN- γ response following stimulation. After a return to basal state, CIMLs show a superior IFN- γ production than control ones upon second stimulation. Adapted from the results of (Romee et al. 2012). **B.** Principal features of CIML NK cells explaining their improved capacities.

4. Use of IL-15 and IL-18 for therapy

Immunotherapy has revolutionized clinical oncology. As both IL-15 and IL-18 activate CD8⁺ T cells and NK cells, their use for antitumoral therapy is under investigation. This section discusses the potential of cytokines for antitumor therapy.

4.1. Recombinant cytokine and agonist

Because of its effect on lymphocyte proliferation, IL-2 was the first cytokine approved by the Food and Drug Administration (FDA) in 1992 for the treatment of certain cancers (S. A. Rosenberg et al. 1994; S. A. Rosenberg et al. 1998; Steven A. Rosenberg 2014). However, its high toxicity (Skrombolas et Frelinger 2014; Panelli et al. 2004; Chavez et al. 2009) and its capacity to induce Tregs and lymphocyte activation-induced cell death (AICD) limits the effects of IL-2 treatments (Ahmadzadeh et Rosenberg 2006; Jensen et al. 2009; Sim et al. 2014). In contrast, IL-15 also induces NK cells and CD8⁺ T cells proliferation, but

without activation of Tregs or AICD (X. Zhang et al. 1998). IL-15 has also shown antitumor effect in various pre-clinical model (Y. Zhou et al. 2022). In 2015, recombinant human IL15 (rhIL-15) was tested for the first time in human for the treatment of patients with metastatic malignant melanoma or metastatic renal cancer (K. C. Conlon et al. 2015). IL-15 treatment induced expansion of NK cell and memory CD8⁺ T cells, associated with moderate toxicity. However no real response was observed in the treated patients.

Several challenges remain with the use of IL-15 in cancer therapy (**Figure 16**). IL-15 can promote the growth of certain tumors (Doucet et al. 1997; J. Chen et al. 2012; Tinhof et al. 2000). Likewise, NK/T leukemia have been reported in mice overexpressing IL-15 (Fehniger et al. 2001). On the contrary, a study shows that chronic stimulation of NK cells by IL-15 leads to their dysfunction (Felices et al. 2018). These studies highlight the importance of IL-15 doses and cellular context for its use in therapy. Soluble IL-15 also has a short half-life which may limit its therapeutic applications. To overcome this challenge, numerous agonists have been developed, based on the mechanism of trans presentation of IL-15 by IL-15R α (S. Ma, Caligiuri, et Yu 2022). IL-15 has been used in combination with the alpha chain of its receptor in a heterodimer IL-15/IL-15R α (hetIL-15, NIZ985) (Rubinstein et al. 2006; Dubois et al. 2008; K. Conlon et al. 2021). IL-15 fused to the IL-15R α sushi domain by a linker has been also developed under the name receptor-linker-IL-15 (RLI, SO-C1) (Mortier et al. 2006; Desbois et al. 2020). Finally a super agonist IL-15 N72D linked to IL-15R α sushi domain and an immunoglobulin IgG1 Fc to increase its stability has been also tested in therapy (formerly ALT-803 now N-803) (Han et al. 2011; W. Xu et al. 2013). Beside stability, targeted delivery instead of systemic infusion would also improve cytokine therapies. In this sense a new IL-15 agonist, the pro-IL-15, has been developed (J. Guo et al. 2021). Pro-IL-15 is composed of the IL-15-IL-15R α -Fc fused to IL-15R β which restrain its activity by blocking N-terminus of IL-15-IL-15R α -Fc. The peptide used as a linker is specifically cleaved by a Matrix Metalloproteinase (MMP) enriched in the tumor microenvironment. Therefore pro-IL-15 is fully activated only when is cleaved by the MMP at the tumor site. As a proof of concept, pro-IL-15 have been shown to induce NK cells and CD8⁺ T cells proliferation only intratumorally and not peripherally. It also limits the toxicity of the molecule while maintaining the same antitumor activity as IL-15-IL-15R α -Fc in mouse.

In addition to IL-15, several phase I/II clinical trials have also been carried out using recombinant IL-18(rhIL-18, SB-485232). Like IL-15, IL-18 monotherapy shows limited effects (Robertson et al. 2006; Tarhini et al. 2009). One study shows a 10- to 100-fold increase in the concentration of IL-18BP in the serum of patients treated with recombinant IL-18, which may partly explain the lack of response (T. Zhou et al. 2020). Therefore the same group developed a decoy-resistant IL-18 (DR-18) not targeted by IL-18BP. DR-18 increase both T cell and NK cell antitumor activity *in vivo* in tumor model in mice. In particular, concerning NK cell activation, DR-18 increase mouse survival and decrease tumor burden in mice injected with different MHC-I deficient tumor line compared to recombinant IL-18. DR-18 treatment results in an increase in NK cell number, KLRG1 expression, and NK cell production of IFN- γ and granzyme B (T. Zhou et al. 2020).

IL-15 and IL-18 in monotherapy shows overall limited effects, therefore their use in combination with other molecules is also evaluated (Waldmann et al. 2020). In particular, IL-15 combined with ICP blocking antibody such as anti-PD-1 and anti CTLA-4, have been shown to improve tumor control in mouse pre-clinical model and are now tested in human (W. Dong et al. 2019; P. Yu et al. 2010).

4.2. Cytokine and killer engager

Cytokines can be combined with other recombinant molecules on engaging systems, which are antibodies modified to bind both tumor antigens and immune activating receptors. Engager brings together targets and effectors cells and favor the formation of immunological synapses between the two. As an example, the bispecific killer engager (BiKE) CD16-CD33 was modified with the addition of human IL-15 in a new trispecific engager (TriKE). TriKE increases the functions and survival of NK cells *in vitro* and the control of tumor growth in a mouse tumor model compared to the BiKE (Vallera et al. 2016; Felices et al. 2020)

4.3. Cytokine and adoptive cell transfer

The use of cytokines also benefits the development of CAR (Chimeric Antigen Receptor) therapies. The first CAR therapies were developed using T cells (CAR-T cells) and referred to T cell modified to express an engineered CAR, redirecting the recognition and the cytotoxicity of the modified T cell against cancer cells expressing the CAR antigen. CAR-T cell therapies show effective activity in the treatment of hematological malignancies and are authorized by the FDA for the treatment of certain B lymphomas and myelomas. The use of other cell types such as NK cells is also gaining attention. Compared to T cells, NK cells show a natural cytotoxicity against numerous targets, particularly those not expressing MHC-I, which may be complementary to targeting mediated by CAR. CAR-NK cells induce less toxicity than CAR-T, probably due to a different cytokine repertoire specific to these two cell types. Finally, the allogeneic transfer of NK cells is easier than the one of T cells due to the absence of graft-versus-host-disease (Dagher et Posey 2023; Vivier et al. 2024). Due to IL-15 ability to induce the proliferation of CD8⁺ T cells and NK cells, several groups have developed both CAR-T and CAR-NK cells expressing this cytokine. For NK cells, the addition of IL-15 expression module has been evaluated in the context of CD19-CAR (E. Liu et al. 2018; Enli Liu et al. 2020), CD123-CAR (Christodoulou et al. 2021) or NKG2D-L-CAR (Du et al. 2021). The major drawback of NK cell therapies being their low persistence, addition of IL-15 favors CAR-NK cells survival and supposedly their efficiency.

Cytokine pretreatment is also considered for therapy development. In particular, CIMLs have been tested for the treatment of acute myeloid leukemia (AML). In a first clinical trial, 4 out of 9 patients showed complete remission after treatment with allogeneic CIMLs (Romee et al. 2016). An IL-15, IL-12, IL-18 agonist molecule has also been developed for the generation of CIML *in vitro*, inducing a phenotype comparable to the use of recombinant cytokine (Becker-Hapak et al. 2021). Finally, the combination of CIML treatment with CAR therapies has also been studied. CD19-CAR NK cells pretreated with IL-15/12/18 have better anti-tumor functionality *in vitro* and *in vivo* in mice than untreated

CD19-CAR NK cells (Gang et al. 2020). Likewise, CIMLs expressing a CAR directed against NPM1, frequently mutated in AML, have better efficacy than WT CIMLs in a mouse model of NPM1 mutated AML. The persistence of NPM1-CAR CIML is further increased by the expression of a membrane-bound IL-15 (H. Dong et al. 2022).

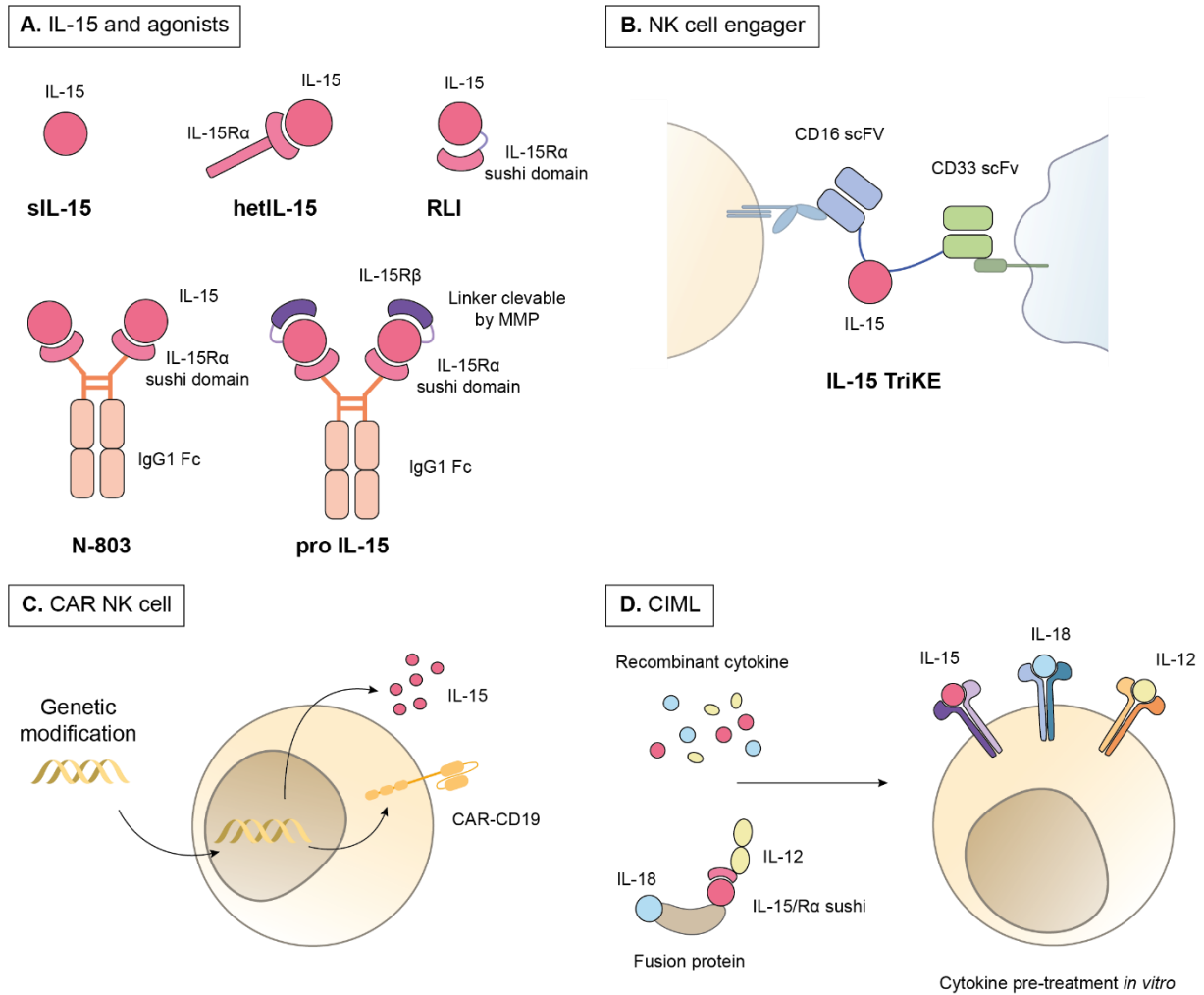


Figure 16: Overview of cytokine based innovative immunotherapies. A. Several IL-15 agonists have been developed base on IL-15 capacity to be trans presented by IL-15Rα. **B.** Trispecific killer engager (TriKE) is a killer engager fused to IL-15. **C.** IL-15 expression module have been used for improved CAR-T and CAR-NK therapy. **D.** Cytokine pre-treatment improve NK cell function used for adoptive transfer. Adapted from (S. Ma, Caligiuri, et Yu 2022)

To sum up, IL-15 and IL-18 are both important cytokines in NK cell biology, sustaining homeostasis and effector function. Beyond their direct action on NK cells, these cytokines can prime NK cells acutely or for longer period of time to induce memory like properties (Divangahi et al. 2021). A key aspect determining NK cell reactivity is these cells ability to mobilize the energy required to sustain their functionality. Metabolic programs are controlled by a few hubs, which integrate a large set of inputs, among which cytokines, and in turn control multiple aspects of the cell biology.

III. Role of the mechanistic target of rapamycin (mTOR) pathway in lymphocyte biology

Metabolism describes the intracellular chemical reactions which allow the conversion of nutrients and endogenous molecules into energy and biomass. Metabolic needs are variable, as a resting cell or a growing activated cell do not have the same needs. Therefore, metabolic programs are tightly controlled and influence cellular function and differentiation. Likewise, nutrient availability and activating signals influence the functionality of the cell.

Lymphocytes, such as NK cells, perceive a large number of signals, activating or inhibitory, which must be integrated so that the cell engage a metabolic program adapted both to its function but also to the availability of nutrients. The mechanistic target of rapamycin (mTOR) is one of the principal metabolic checkpoint responsible for this integration.

1. mTORC1 and mTORC2 complexes

In 1964, a macrolide produced by the bacteria *Streptomyces hygroscopicus* was isolated from soil samples from Easter Island. This compound, later called rapamycin, Rapa Nui being the name of the island in the local language, showed anti-fungal properties (Vézina, Kudelski, et Sehgal 1975). In 1990, Schreiber and collaborators showed that rapamycin forms a complex with the FKBP12 protein, and inhibits cell growth and proliferation (Bierer et al. 1990). Then in 1994, three groups independently identified the target of the FKBP12-rapamycin complex, which they called the mTOR protein (formerly “mammalian” or today “mechanistic Target of Rapamycin”) (Sabatini et al. 1994; Brown et al. 1994; Sabers et al. 1995). These studies also revealed homology between mTOR and the yeast TOR proteins, previously identified in genetic screens for rapamycin resistance (Heitman, Movva, et Hall 1991).

mTOR is a 289kDa serine/threonine kinase which belongs to the PI3K-related kinase (PIKK) family. In mammals, mTOR is the catalytic subunit of two complexes mTOR Complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which differ in their protein composition, their sensitivity to rapamycin as well as their substrates and functions

1.1. mTORC1 structure

mTORC1 is composed of three core complex proteins: the mTOR kinase, mLST8 (mammalian lethal with SEC13 protein 8, also named GβL) (D.-H. Kim et al. 2003) and the scaffold protein Raptor (Regulatory-associated protein of mTOR) (D.-H. Kim et al. 2002; Kenta Hara et al. 2002). mLST8 is common to mTORC1 and mTORC2 and is thought to have a stabilizing role for the mTOR kinase domain (H. Yang et al. 2013). However, a study shows that mLST8 depletion has no effect on mTORC1 substrates phosphorylation *in vivo*, but that it is essential for the structure and function of mTORC2 (Guertin et al. 2006). Raptor is a scaffolding protein specific to the complex 1. It allows the recruitment of mTOR substrates that possess a TOS domain (Schalm et al. 2003; Nojima et al. 2003) and participates in the complex intracellular localization. mTORC1 core complex is associated with two inhibitory proteins, PRAS40 (proline-rich Akt/PKB substrate 40 kDa)

(Sancak et al. 2007; Vander Haar et al. 2007) and DEPTOR (DEP-domain-containing mTOR interacting protein) (Peterson et al. 2009). In the cell, mTORC1 core complex is found as a dimer, with dimerization taking place along the mTOR HEAT domain and the mTOR-Raptor interface. Structural approaches have also shown that the rapamycin-FKBP12 complex binds directly to the mTOR FRB domain which partially occludes the active site, preventing the access of some of the substrates to the kinase domain (H. Yang et al. 2013).

1.2. mTORC2 structure

Like mTORC1, mTORC2 core complex is composed of mTOR and mLST8, and the protein Rictor, instead of Raptor (Jacinto et al. 2004; Sarbassov et al. 2004). Rictor is also a scaffolding protein and allows the binding of mSIN1 (MAPK-interacting protein 1) (Frias et al. 2006; Jacinto et al. 2006; Q. Yang et al. 2006), DEPTOR (as for mTORC1) (Peterson et al. 2009) and PROTOR1/2 (protein associated with rictor 1/2 also named PRR5/PRR5L) (L. R. Pearce et al. 2007; Woo et al. 2007). mSIN1 contains a PH domain (pleckstrin homology domain) allowing mTORC2 recruitment to the membrane which participates to its activation (Yuan et Guan 2015). Like mTORC1, mTORC2 associates in dimer. The structure of the complex shows that Rictor blocks the rapamycin-FKBP12 binding site on mTOR, explaining why mTORC2 is insensitive to direct rapamycin inhibition (X. Chen et al. 2018, 2; Stuttfeld et al. 2018) (**Figure 17**).

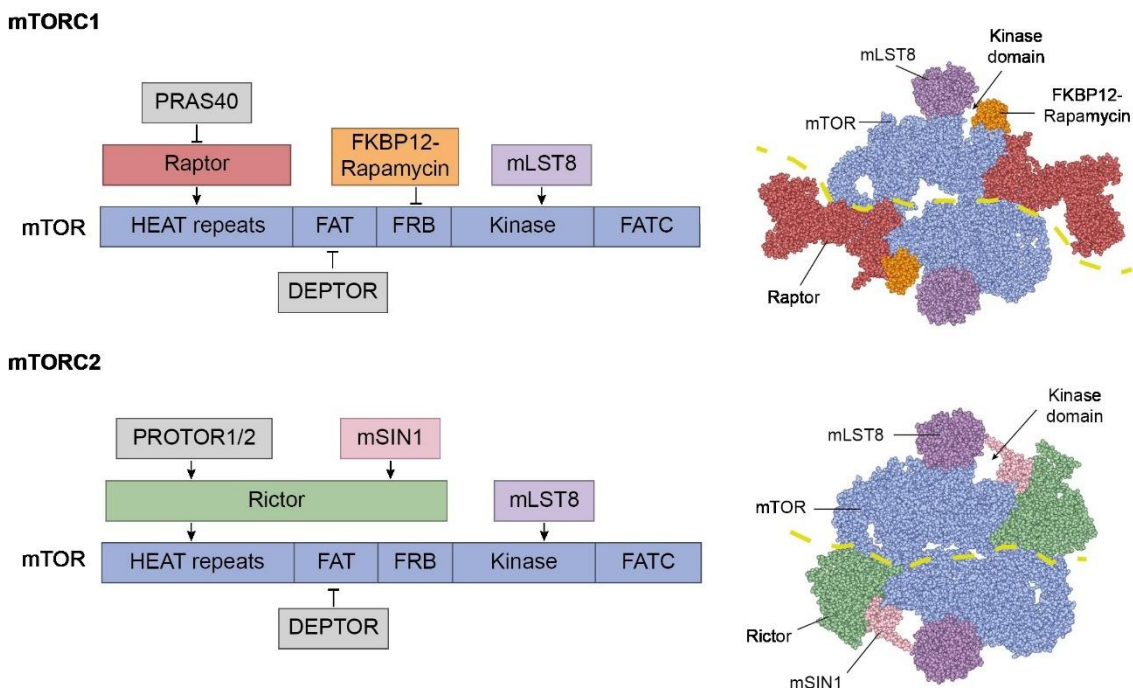


Figure 17: mTORC1 and mTORC2 structures. Left: mTORC1 is composed of mTOR, Raptor, mLST8, PRAS40 and DEPTOR. mTORC2 is composed of mTOR, Rictor, mLST8, mSIN1, PROTOR1/2 and DEPTOR. Right: 3D-Reconstructions of mTORC1 (without PRAS40 and DEPTOR) and mTORC2 (without PROTOR and DEPTOR) are shown. FKBP12–rapamycin partially blocks mTORC1 kinase domain. Each complex dimerizes as indicated by the yellow line. From (G. Y. Liu

2. mTOR downstream targets

mTOR activation engages the cell towards cell growth and proliferation. Thus mTORC1 and, to a lesser extent, mTORC2 control a set of pathways acting on cellular metabolism and new compounds synthesis to support cell division (**Figure 18**).

2.1. mTORC1 targets

mTORC1 increases cell anabolism through two processes; (i) activation of bioenergetic pathways and new compound synthesis (namely proteins, lipids and nucleotides synthesis), and (ii) inhibition of catabolism and autophagy.

mTORC1 probably best-characterized role is the activation of protein synthesis, mainly through phosphorylation of two proteins: 4E-BP and S6K1. In the absence of phosphorylation, 4E-BP inhibits protein translation by binding to the initiation factor eIF4E which is an essential factor for the eIF4F cap-binding complex. Phosphorylation of 4E-BP releases eIF4E and facilitates mRNAs cap-dependent translation (Brunn et al. 1997; K. Hara et al. 1997). In particular, transcriptome-scale ribosome profiling reveals that mTORC1 inhibition greatly impact the translation of 5' terminal oligopyrimidine (TOP) mRNAs in a 4E-BP dependent manner. As TOP motif is preferentially present on mRNA which encodes protein that are essential for protein synthesis, this would also account for mTORC1 broad effect on protein translation (Thoreen et al. 2012; Hsieh et al. 2012). mTORC1 also phosphorylates the S6K1 kinase. S6K1 participates in activation of the translation through different mechanisms, including the increase in ribosomal RNA transcription (Hannan et al. 2003), the activation of the initiation factor eIF4B (Holz et al. 2005) or the repression and degradation of PDCD4, a negative regulator of protein translation (Dorrello et al. 2006). mTORC1 phosphorylation of S6K1 also enhance its kinase activity towards the S6 protein, a component of the 40S subunit of the ribosome (Burnett et al. 1998; G. Y. Liu et Sabatini 2020). However, the exact role of this phosphorylation is still unknown.

mTORC1 also activates lipid production by regulating the activity of the transcription factors SREBP1/2 and PPAR γ . SREBP1/2 controls lipids and cholesterol synthesis and is under the control of lipin-1 which is phosphorylated and inhibited by mTORC1 (Peterson et al. 2011). PPAR γ regulates fatty acid storage and is also activated by mTORC1 (J. E. Kim et Chen 2004). mTORC1 also enhances nucleotide synthesis. Indeed, mTORC1 activates the transcription factor ATF4, promoting purine production (Ben-Sahra et al. 2016) and inhibits CAD, a negative regulator of pyrimidine biogenesis, through phosphorylation by S6K1 (Ben-Sahra et al. 2013). Finally, mTORC1 activates the transcription factors HIF1 α and c-Myc, which activate the synthesis of glycolytic enzymes resulting in increased glycolytic activity (Düvel et al. 2010, 1).

In parallel, mTORC1 inhibits autophagy through inhibition of ULK1 and ATG13, two key factors in autophagy initiation (Hosokawa et al. 2009; J. Kim et al. 2011). mTOR also phosphorylates and inhibits the activity of the transcription factor TFEB as well as other

factors of the MiT-TFE family to which TFEB belongs, which are master transcriptional regulators of lysosome synthesis and autophagy (Martina et al. 2012; Settembre et al. 2012; Roczniak-Ferguson et al. 2012).

2.2. mTORC2 targets

mTORC2 mainly targets kinases belonging to the AGC family. In collaboration with PDK1, it regulates the activity of different PKCs, of SGK1 or AKT. PKC α was the first identified substrate of mTORC2 and mediates effects of mTORC2 on the cytoskeleton (Sarbasov et al. 2004). mTORC2 also activates SGK, a protein kinase that regulates numerous pathways, including the activity of different ion transporters, proliferation or apoptosis (García-Martínez et Alessi 2008). The best studied role of mTORC2 is probably its participation in the activation of the kinase AKT (also known as PKB) a key effector downstream PI3K signaling (Sarbasov et al. 2005). Indeed, PIP3 production by PI3K recruits the PH domain proteins AKT and PDK1, which leads to AKT phosphorylation by PDK1 on the Thr-308. A second phosphorylation of AKT at Ser-473, catalyzed by mTORC2, is necessary for AKT full activation. AKT then phosphorylates and inhibits the transcription factors FOXO1/3 which participate in the control cell metabolism, quiescence and survival (Brunet et al. 1999) and GSK3 β , a kinase important in glucose homeostasis downstream of insulin (Cross et al. 1995). AKT also activates the NAD Kinase which catalyzes the conversion of NAD⁺ to NADP⁺, an essential cofactor of reductive metabolism and lipid synthesis (Hoxhaj et al. 2019). Finally, AKT is an important regulator of mTORC1 by inhibiting TSC, a negative regulator of the pathway (see below). Therefore, with its unique position as an upstream regulator of mTORC1 and downstream target of mTORC2, AKT participates to the cross-talk between the two complexes (Inoki et al. 2002).

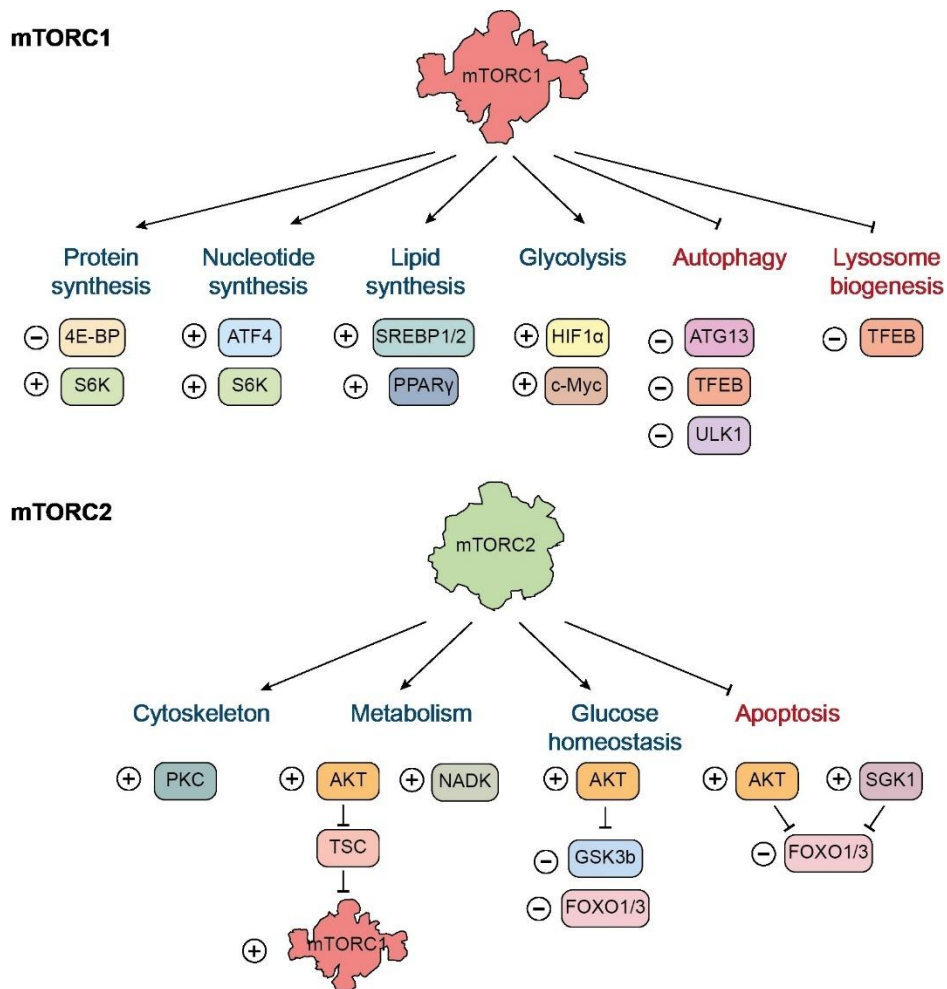


Figure 18: mTORC1 and mTORC2 targets. The cellular processes downstream mTORC1 and mTORC2 are indicated. The molecules downstream each complexes are also presented. Plus and minus sign indicates activation and inhibition respectively.

3. mTOR upstream signaling

3.1. mTORC1 regulation

mTORC1 activation promotes a cellular switch toward anabolism which requires energy and macromolecule building blocks. Therefore, its activation must be tuned with the cell resources. mTORC1 acts as an integrator, and is sensitive both to growth factors signaling such as insulin or cytokines, and to the availability of nutrients such as amino acids or glucose. The classic model of mTORC1 activation is based on the concomitance of these two signals, with growth factors regulating mTOR kinase activity and the nutrients regulating its localization. It involves two sets of GTPases; Rheb which is an allosteric mTORC1 activator anchored to the lysosome surface, and Rags which mediate mTORC1 recruitment to the lysosome (**Figure 19**).

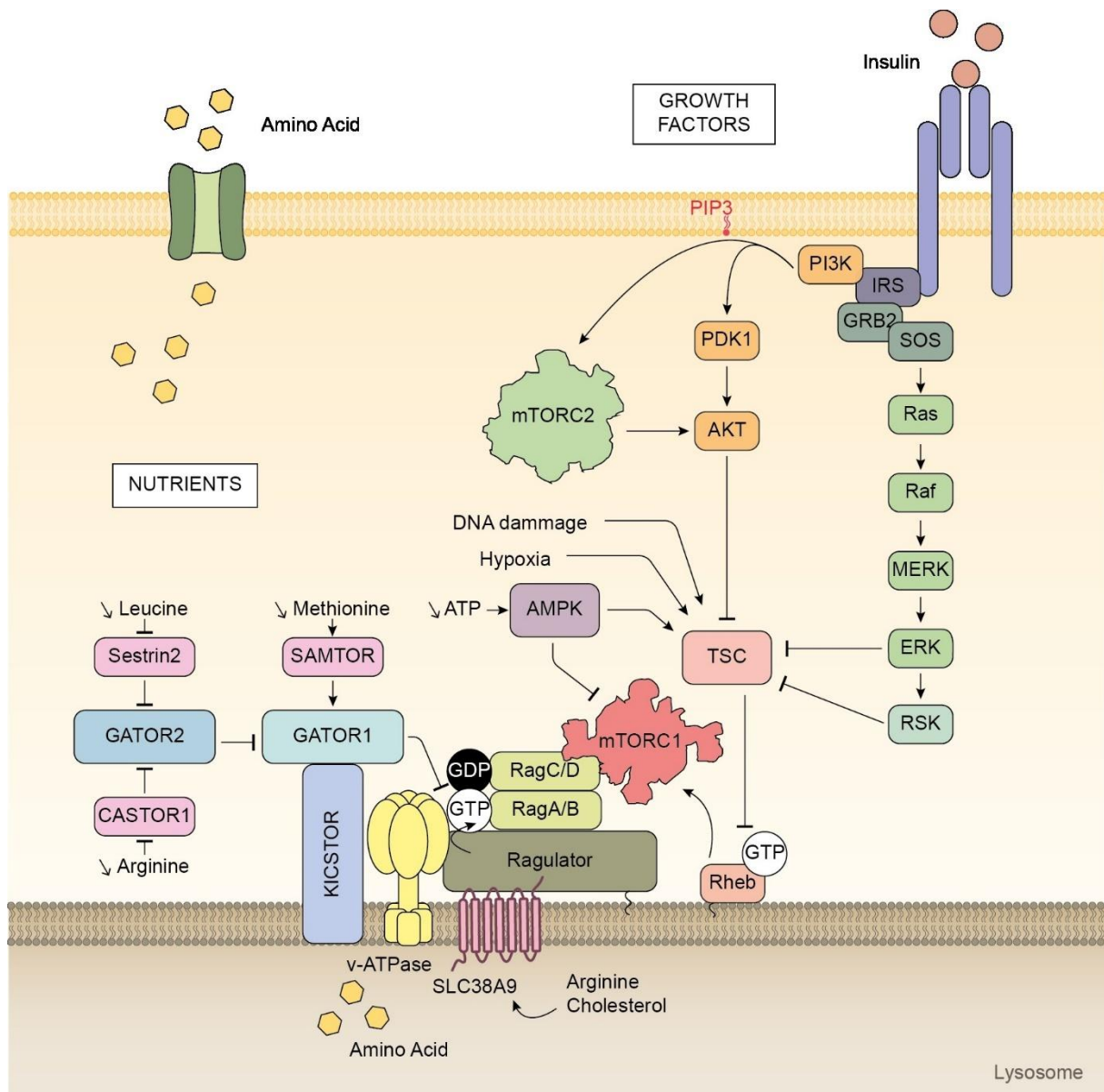


Figure 19: The mTOR pathway. Growth factors such as insulin and cellular stresses such as DNA damage, hypoxia or energy stress converge on TSC in an opposite fashion. TSC inhibition allows Rheb activation which increases mTORC1 kinase activity. Nutrients such as amino acid are detected by different sensors which converge on the control of Rags activation. Active Rags (RagA/B-GTP-RagC/D-GDP) can bind Raptor and therefore recruit mTORC1 to the lysosome surface, close to its activator Rheb. mTORC2 is activated downstream PI3K and participates among other things to AKT activation. See text for further details. Adapted from (G. Y. Liu et Sabatini 2020).

a. mTORC1 control by growth factors

mTORC1 is a downstream target of several mitogenic factors. These pathways converge on the inhibition of the Tuberous Sclerosis Complex (TSC) which is composed of three proteins: TSC1, TSC2 and TBC1D7 (Dibble et al. 2012). TSC acts as a GTPase-activating protein (GAP) for the GTPase Rheb and catalyzes the conversion of the active form of Rheb bound to GTP (Rheb-GTP) to its inactive form bound to GDP (Rheb-GDP) (Inoki et al. 2003; Tee et al. 2003). Rheb is an allosteric activator of mTORC1. Structural analysis showed that

Rheb-GTP binds distally from mTOR kinase site to promote a conformational rearrangement of the complex and increase its kinase activity (H. Yang et al. 2017). TSC inhibition downstream of growth factors releases its negative pressure on Rheb thus allowing mTORC1 full kinase activity. As TSC is a major brake in mTORC1 signaling, it is tightly regulated. Insulin was the first mitogen described in mTORC1 activation, and the signal transduction it elicits is therefore often presented as a general working model for mTORC1 activation (Scott et al. 1998). Insulin receptor is a tyrosine kinase receptor, which activation leads to its autophosphorylation and the recruitment and phosphorylation of the Insulin Receptor Substrate (IRS). IRS then serves as activation platform for SH2 domain proteins, and triggers the activation of PI3K-AKT and Ras-ERK pathways, mentioned earlier when we described the IL-15 pathways. AKT phosphorylates TSC at several sites (Inoki et al. 2002), allowing its inhibition and its release from the lysosome (Manning et al. 2002; Menon et al. 2014; Demetriades, Doumpas, et Teleman 2014). Inversely, to terminate mTORC1 signaling, the mTORC1 substrate S6K1 phosphorylates the IRS which blocks insulin-dependent PI3K-AKT signaling, as a part of a negative feedback loop (Harrington et al. 2004; Shah, Wang, et Hunter 2004). ERK and RSK, a protein downstream of ERK can also phosphorylate TSC to inactivate it (Roux et al. 2004; L. Ma et al. 2005). Likewise, the Wnt and TNF α pathways inhibit TSC although the mechanisms involved are not very well characterized (Inoki et al. 2006; D.-F. Lee et al. 2007).

Besides TSC, growth factors can modulate mTORC1 activity through PRAS40. PRAS40 is a negative endogenous regulator of mTORC1, which binds Raptor and prevents mTORC1 activation by Rheb. AKT also phosphorylates PRAS40 allowing its sequestration by the 14-3-3 protein and the activation of mTORC1 (Sancak et al. 2007; Vander Haar et al. 2007). It should be mentioned that the importance of PRAS40 is still elusive and much less characterized than TSC.

In addition to positive signals arising from mitogen signaling, cellular stress also interferes with the TSC-Rheb axis. Different cellular stresses regulate negatively mTORC1 pathway. For instance, energy stress induced by glucose depletion, leads to a decrease in the ATP/ADP ratio which is sensed by the AMP-activated protein kinase (AMPK). AMPK then inhibits mTORC1 either directly via Raptor phosphorylation or indirectly via TSC2 activation by phosphorylation on residues distinct from the inhibitory ones (Inoki, Zhu, et Guan 2003; Gwinn et al. 2008). Hypoxic stress also increases the expression of REDD1 which activates TSC independently of AMPK (Brugarolas et al. 2004; DeYoung et al. 2008). Finally, stresses linked to DNA damage induce several targets of p53, including AMPK or TSC2 (Feng et al. 2007).

b. mTORC1 control by nutrients

As early as 1998, it was observed that amino acids were required for mTORC1 activation. Hara and collaborator noted that depletion in particular of leucine and arginine prevented activation of the pathway (K. Hara et al. 1998). It was only 10 years later that the mechanism of mTORC1 activation by amino acids were characterized (Sancak et al. 2008). Amino acid control of mTORC1 involves the Rags, which are GTPases functioning

as heterodimers with RagA or B binding RagC or D. When amino acids are present, Rags are in an active configuration where RagA/B is linked to GTP and RagC/D to GDP (RagA/B-GTP, RagC/D-GDP). On the contrary, when amino acid are depleted, Rags are in an inactive inverse form with RagA/B bound to GDP and RagC/D to GTP (RagA/B-GDP, RagC/D-GTP). Rags are anchored to the lysosome via the Ragulator complex, a pentamer composed of the LAMTOR1 to 5 proteins (Sancak et al. 2010). The Ragulator complex has also a guanine nucleotide exchange factor (GEF) activity towards RagA/B (Bar-Peled et al. 2012). Structural data show that Raptor can binds the active form of Rag through a structure sometimes referred as “Raptor claw”, and therefore attach mTORC1 to the lysosome when amino acids are available (Rogala et al. 2019). In parallel, farnesylation of Rheb in C-ter promotes also its anchoring to the endomembrane, particularly to the lysosome (Clark et al. 1997; Takahashi et al. 2005). Therefore, when nutrients are available, mTORC1 is tethered to the lysosome via the Rag-Ragulator complex in close vicinity of Rheb, which allows its activation. On the contrary, when amino acids are scarce, mTORC1 is released from the lysosome and thus cannot be activated by Rheb anymore. This dual control by Rags and Rheb constitute the classic working model for mTORC1 activation by nutrients and growth factors.

Rags regulation involves different GAP/GEF and nutrients sensors. The most proximal component of this machinery is the GAP activity towards the Rags 1 (GATOR1) complex. GATOR1 is composed of three proteins (DEPDC5, NPRL2, NPRL3) and has GAP activity towards RagA/B (Bar-Peled et al. 2013). By hydrolyzing the GTP linked to RagA/B GATOR1 allows the inhibition of the pathway when amino acids are lacking. GATOR1 is itself anchored to the lysosome by the KICSTOR complex, a tetramer composed of KPTN-, ITFG2-, C12orf66-, and SZT2 (Wolfson et al. 2017; Peng, Yin, et Li 2017). The activity of GATOR1 is modulated by the GATOR2 pentamer (composed of MIOS, WDR59, WDR24, Seh1L, and SEC13) which acts as a negative regulator of GATOR1 (Bar-Peled et al. 2013) via a molecular mechanism unknown to date.

Regulation of mTORC1 by nutrients currently constitutes an active field of research and several nutrients sensors have been discovered. The best characterized amino acid sensors so far are leucine sensor sestrin2 and the arginine sensor CASTOR1. During leucine deprivation, Sestrin2 inhibits GATOR2 which prevents the recruitment of mTORC1 to the lysosome and therefore its activation (Saxton, Knockenhauer, et al. 2016; Wolfson et al. 2016). Likewise, the arginine sensor CASTOR1 (cellular arginine sensor for mTORC1) also inhibits GATOR2 in the absence of arginine (Chantranupong et al. 2016; Saxton, Chantranupong, et al. 2016). mTORC1 is also sensitive to lysosomal amino acids concentrations. Early studies on mTORC1 control by nutrients identified v-ATPase, a proton pump involved in the acidification of lysosomes, as an integral part of the Rag-Ragulator complex. The v-ATPase has been shown to act as a lysosomal amino acids sensor and positively regulates the GEF activity of the Ragulator complex (Zoncu et al. 2011; Bar-Peled et al. 2012). More recently, SLC38A9, a lysosomal amino acids transporter, has also been identified as a lysosomal amino acids sensor by binding the Ragulator-Rag complex and participating in its activation in the presence of arginine (S. Wang et al. 2015; Rebsamen et al. 2015; Wyant et al. 2017).

Beyond amino acids, mTORC1 is also sensitive to other nutrients. S-adenosylmethionine (SAM), a methionine derivative, is for example recognized by the SAMTOR sensor. In the absence of methionine, SAMTOR binds GATOR1 and KICSTOR directly and inhibits mTORC1 activation (X. Gu et al. 2017). Another study shows that the transporter SLC38A9 and the protein NPC1 are sensitive to the lysosomal concentration of cholesterol and regulate the activity of mTORC1 (Castellano et al. 2017). Finally, Rags may also participate in the sensitivity of mTORC1 to glucose deprivation, independently of AMPK (Efeyan et al. 2013). In particular, dihydroxyacetone phosphate, an intermediate of glycolysis, could be involved in glucose sensing independently of AMPK (Orozco et al. 2020).

c. Non-canonical mTORC1 signaling

Our knowledge on mTORC1 regulation comes in great part from studies measuring the phosphorylation of S6K and 4E-BP which are classic readouts of mTORC1 kinase activity. However, a growing body of evidences point out that mTORC1 kinase activity is not just on/off, but rather that mTORC1 phosphorylates certain targets specifically downstream certain stimuli. In particular, the regulation of proteins from the MiT-TFE family, to which TFEB belongs, seems to constitute a separate case. Several studies show that MiT-TFE family member phosphorylation by mTORC1 can be inhibited specifically without inhibiting the classic mTORC1 targets S6K and 4E-BP (Napolitano, Malta, et Ballabio 2022). Conversely, the phosphorylation of TFEB is insensitive to the presence of growth factor and Rheb, which is however necessary for the phosphorylation of S6K and 4E-BP (Napolitano et al. 2020).

MiT-TFE family proteins do not contain a TOS motif necessary for canonical recruitment of mTORC1 targets by Raptor. Conversely, they bear a motif later shown to interact with Rag, the Rag binding motif (RBM) (Martina et Puertollano 2013). A recent study by Napolitano and collaborator sheds a new light on TFEB regulation by mTORC1. They show that the phosphorylation of TFEB occurs only when it is recruited by the Rags in their active form: RagA/B-GTP-RagC/D-GDP. The activation status of RagC in particular is crucial for TFEB phosphorylation whereas is appear less important for S6K and 4E-BP phosphorylation. In parallel, several groups have highlighted the role of the folliculin complex FLCN:FNIP in the control of RagC/D with FLCN:FNIP having a GAP activity towards RagC/D (Tsun et al. 2013). When amino acid are scarce, FLCN:FNIP binds RagA/B-GDP to the surface of the lysosome while in amino acid presence, FLCN:FNIP dissociates from the lysosome and catalyzes the conversion RagC/D-GTP to RagC/D-GDP. Interestingly, FLCN:FNIP deficiency leads to TFEB nuclear localization whereas FLCN:FNOP is dispensable for phosphorylation of S6K and 4E-BP. (Lawrence et al. 2019; Napolitano et al. 2020). This study thus proposes a new mode of regulation of mTORC1 targets and an explanation for TFEB very high sensitivity to the presence of nutrients and not to growth factors. In this model, activation of RagA/B would be necessary for the binding of mTORC1 to the lysosome and the phosphorylation of all mTORC1 targets while the activation of RagC/D is required specifically for the recruitment and activation of TFEB (**Figure 20**). This study raises several questions however, in particular on the mechanism of activation of mTORC1 in the absence of Rheb.

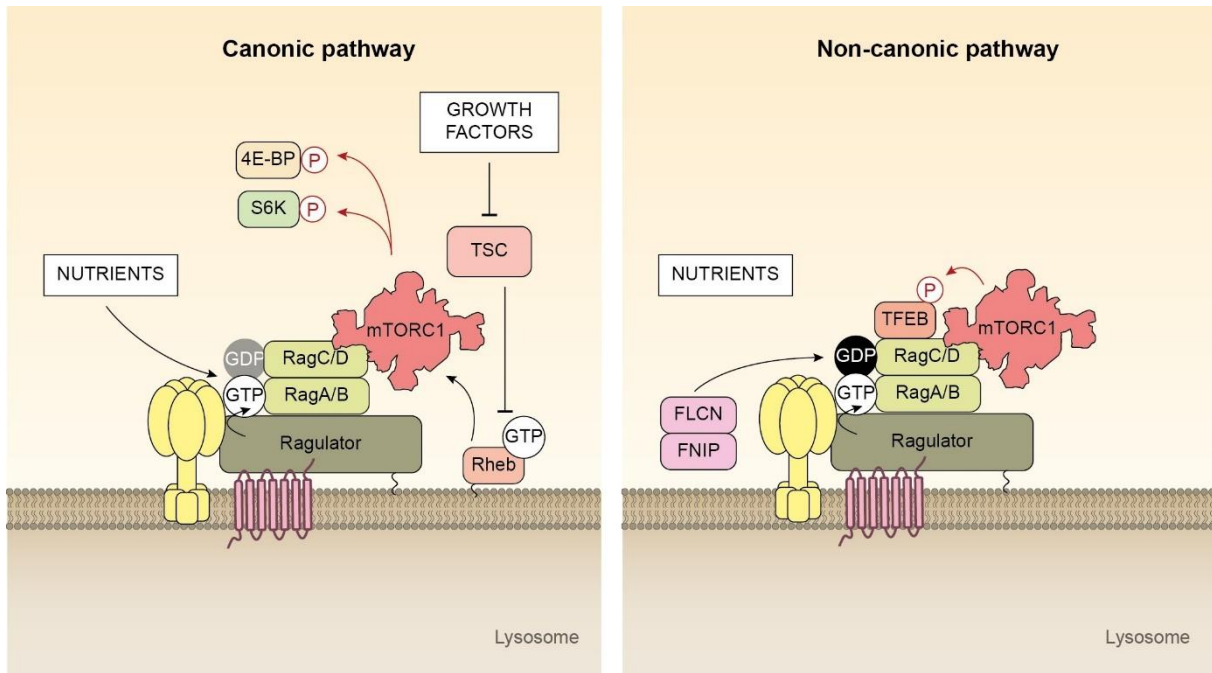


Figure 20: Non-canonical activation of TFEB by mTORC1. 4E-BP and S6 phosphorylation by mTORC1 depends on TSC-Rheb. TFEB phosphorylation by mTORC1 is independent on Rheb but depends strongly on RagC/D activation status. TFEB is phosphorylated and inactivated only when RagC/D is bound to GDP which is regulated by the FLCN:FNIP complex. Adapted from (Napolitano, Malta, et Ballabio 2022).

3.2. mTORC2 regulation

mTORC2 regulation is less characterized than the one of mTORC1 and depends essentially on the PI3K pathway. The endogenous mTORC2 partner mSIN1 contains a PH domain, which allows its binding to the PIP3 of the membrane (Yuan et Guan 2015). The concomitant recruitment of AKT allows its activation by mTORC2 (Ebner et al. 2017). Besides PI3K, several studies also indicate that mTORC2 can be activated downstream of the Ras oncogene (Saci, Cantley, et Carpenter 2011; Khanna et al. 2016; Senoo et al. 2019; Kovalski et al. 2019). Finally, mTORC2 activity has also been shown to be regulated by ribosome association (Zinzalla et al. 2011). mTORC2 has been shown to interact directly with component of the ribosome and ribosome deletion or defect in ribosome maturation pathway impact mTORC2 activity toward AKT. In the same study, the authors show that insulin and PI3K signaling promotes mTORC2-ribosome binding.

4. mTOR in T cells

The immunosuppressive role of rapamycin has been known since the 1970s, even before the identification of mTOR. Therefore, mTOR roles are particularly well documented in T cells and which have served as a basis to understand mTOR regulation in other lymphocytes. In relation to its activating role in cell proliferation and metabolism, mTOR has a fundamental role in cell fate decision and therefore shapes the adaptive response (Chi 2012). Indeed, next to growth factor and nutrients, inflammatory molecules and antigenic cues also modulates mTOR activity in T cell. Engagement of the TCR and its coreceptors such as CD28 leads to the phosphorylation of mTORC1 and mTORC2 targets

and cell proliferation (Colombetti et al. 2006; Y. Zheng et al. 2009). Conversely, the activity of mTOR is repressed by certain inhibitory receptors such as PD-1 (Francisco et al. 2009). mTOR has also been shown to be activated downstream different cytokines in T cell, including IL-2 (Finlay et al. 2012; Macintyre et al. 2011; Villarino et al. 2022), IL-7 (Rathmell et al. 2001; Wofford et al. 2008; A. Xu et al. 2022), IL-4 (Stephenson et al. 2005), IL-12 (Rao et al. 2010), IL-1 (Chang et al. 2013; Gulen et al. 2010), IL-33 (Liang et al. 2022) or IL-36 (Zhao et al. 2019). This next section presents the main roles of mTOR and mTOR key regulators in T cell biology (**Table 2**).

Protein	mTOR activity	Cre expression system	Phenotype	References
mTOR	mTORC1- mTORC2-	CD4	CD4 ⁺ T cell differentiation defect in Th1, Th17, Th2. Spontaneous differentiation in Treg.	(Delgoffe et al. 2009)
RHEB	mTORC1-	CD4	CD4 ⁺ T cell differentiation defect in Th1 and Th17. Th2 differentiation remains.	(Delgoffe et al. 2011)
		CD4	Loss of CD8 ⁺ T cell effector function but conservation of memory characteristic	(Pollizzi et al. 2015)
RAPTOR	mTORC1-	CD4	Contrary to Rheb deficiency, Raptor deficiency also results in defect in Th2 differentiation. Impaired quiescence exit and metabolic reprogramming upon activation	(K. Yang et al. 2013)
		Lck	CD4 ⁺ T cell differentiation defect in Th17 but not Th1	(Kurebayashi et al. 2012)
		Foxp3	Loss of Treg effector function, defect in metabolic reprogramming.	(Zeng et al. 2013)
RICTOR	mTORC2-	CD4	CD4 ⁺ T cell differentiation defect in Th2. Th1 and Th17 differentiation remains.	(Delgoffe et al. 2011)
		dLck	CD4 ⁺ T cell differentiation defect in Th2 and Th1. Th17 differentiation remains.	(K. Lee et al. 2010)
		CD4	Increase in memory CD8 ⁺ T cell	(Pollizzi et al. 2015)
TSC1	mTORC1+	CD4	Loss of quiescence, increase in apoptosis	(O'Brien et al. 2011)
		Lck/CD4		(Q. Wu et al. 2011)
		CD4		(K. Yang et al. 2011)
TSC2	mTORC1+	CD4	CD8 ⁺ terminally differentiated effector cell T cell that failed to transition to memory cells	(Pollizzi et al. 2015)

Table 2: Phenotype associated with deletion of key components of mTOR signaling in T cell.

4.1. mTOR in T cell quiescence and activation

Naive T lymphocytes are actively maintained in a state of quiescence, characterized by cell cycle arrest in G₀ as well as low metabolic, transcriptional and translational activity. Their survival depends on the continuous engagement of their TCR with MHC presenting self-molecules (tonic signal), and certain growth factors such as IL-7 (Chapman et Chi 2018). Depletion of mTOR (CD4-Cre, deletion at double positive stage) has only a minor effect on T cell development and homeostasis in the periphery (Delgoffe et al. 2009). On the other hand, the deletion of negative regulators of the pathway strongly impacts T cell quiescence. In particular, several studies have shown the importance of the negative regulator TSC in maintaining the homeostasis of naive T cells. TSC deficiency in T cells results in their exit from quiescence, characterized by an increase in proliferation and cell size, paradoxically resulting in apoptosis and decrease in lymphocytes number. These cells also show an increase in mTORC1 activity and a decrease in that of mTORC2, which could be explained by the negative feedback loop of mTORC1 on the PI3K-AKT pathway (O'Brien et al. 2011; K. Yang et al. 2011; Q. Wu et al. 2011; Pollizzi et al. 2015). Therefore, low mTORC1 activity is essential for maintaining quiescence of naive T cells.

Antigen and costimulatory receptors engagement results in T cells to exit quiescence and promote subsequent clonal expansion and effector differentiation. If mTOR low activity is essential for T cell quiescence, mTOR activation on the contrary has an essential role in quiescence exit upon antigen activation, in particular through metabolism regulation. Quiescent cells have a catabolic metabolism, centered on fatty acid oxidation and TCA. Quiescence exit is associated with a metabolic switch towards anabolism, characterized by an increase in nutrient import, anaerobic glycolysis, glutaminolysis and the pentose phosphate pathway as well as an increase in synthesis of mitochondria allowing oxidative phosphorylation (OXPHOS) and one-carbon metabolism (Chapman, Boothby, et Chi 2020). mTORC1 is a central player in this metabolic switch as it induces the expression of MYC, SREBP and HIF1 α (R. Wang et al. 2011; Zeng et al. 2013; K. Yang et al. 2013) as well as genes involved in mitochondria activation (Tan et al. 2017). Raptor deficiency in T cell (CD4-Cre) impaired T cell IL-2 production and proliferation in response to TCR stimulation (K. Yang et al. 2013). mTORC2 also participates in the exit from quiescence through the repression of FOXO via AKT, which allows the expression of the glucose transporter GLUT1 (Frauwirth et al. 2002) (**Figure 21**). Interestingly, the deletion of Rheb in T cells does not recapitulate that of Raptor upon activation by the TCR. Rheb-deficient T cells show an intermediate phenotype with a decrease in p-S6 and p-4E-BP, and a decrease in cell proliferation and cytokine production compared to WT but to a lesser extent than what is observed for Raptor KO (K. Yang et al. 2013).

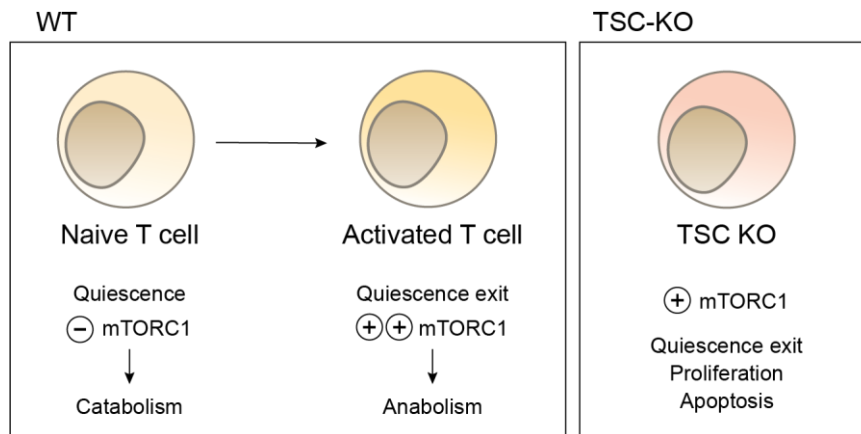


Figure 21: mTORC1 activation is required for T cell quiescence exit. Quiescence exit is associated with a metabolic switch toward anabolism which is orchestrated by mTORC1 in T cell. TSC deficiency in T cell results in an increase of mTORC1 activity at steady states which leads to quiescence exit and apoptosis.

4.2. mTOR in CD4⁺ T cell differentiation

Naive CD4⁺ T cells differentiate into distinct effector cell lineages upon antigen and cytokine stimulation, such as Th1, Th2, Th17 or Treg, which all have different properties and effector functions. The importance of mTOR in CD4⁺ T cell differentiation was highlighted by pioneering experiments from Jonathan Powell's group. mTOR deficient T cells (CD4-Cre, deletion at the double positive stage), fail to differentiate into Th1, Th2 or Th17 effector T in response to the appropriate cytokines, but conversely present a higher propensity to express the transcription factor Foxp3 characteristic of Treg. mTOR deficient cells present a reduction in lineage-specific STAT signaling downstream cytokine receptors, associated with a decrease in lineage-specific transcription factors expression (Delgoffe et al. 2009). This phenotype is not observed in Rheb or Rictor deficient T cell, indicating that inhibition of both complexes is required for Treg differentiation. The same group shows that Rheb deficient T cell (mTORC1 KO) do not differentiate into Th1 and Th17 but manage to differentiate into Th2 (Delgoffe et al. 2011). However another group using Raptor deficient T cells (CD4-Cre) show that they also failed in differentiate into Th2 (K. Yang et al. 2013). Cells deficient for Raptor at an earlier stage of development (Lck-Cre, stage DN2) have also been reported to fail to differentiate into Th17 but not in Th1 (Kurebayashi et al. 2012). Conversely, Delgoffe and colleagues show that Rictor deficient T cell (mTORC2 KO) retain the capacity for differentiation into Th1 and Th17 but not that of Th2 (Delgoffe et al. 2011). Another group demonstrate that Rictor ablation (distal Lck-Cre, deletion at double positive stage) results in a loss in the capacity to differentiate into Th1 too. In this last study, complementation of Rictor KO cells with constitutively active AKT restores T-bet expression and Th1 lineage while constitutively active PKC restores GATA3 transcription factor and Th2 lineage (K. Lee et al. 2010). In summary, mTOR activity is an important parameter for CD4⁺ T cell differentiation, although the exact role of mTORC1 and mTORC2 as proposed by Delgoffe and colleague has since been challenged by later studies (**Figure 22**).

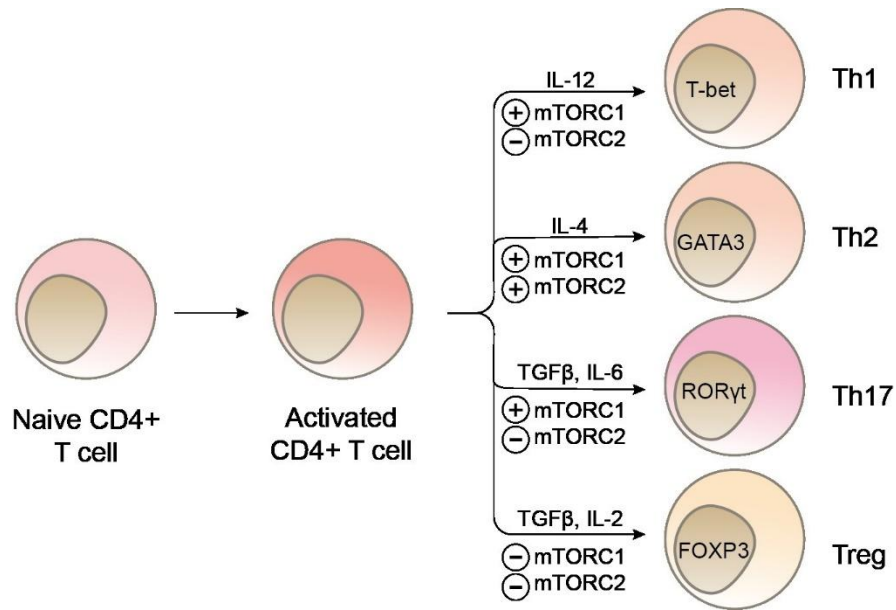


Figure 22: mTOR activity in CD4⁺ T cell differentiation. Naive CD4⁺ T cells differentiate into distinct effector cell lineages upon antigen and cytokine stimulation. The activity of mTOR associated with each lineage commitment is indicated.

4.3. mTOR in memory CD8⁺ T cell differentiation

CD8⁺ T cell activation results in clonal expansion and differentiation into cytotoxic T cells, followed by the contraction phase, where most of the cells die by apoptosis, while those remaining differentiate into memory CD8⁺ T cells. In 2009, Araki and colleague made the surprising observation that rapamycin treatment favor memory CD8⁺ T cell generation in a lymphocytic choriomeningitis virus (LCMV) infection model (Araki et al. 2009). More precisely, rapamycin treatment during the expansion phase increase memory CD8⁺ T cells numbers while rapamycin treatment during the contraction phase improves the functionality of the memory cells. Same results were obtained with RNA silencing Raptor, thereby indicating a specific role of mTORC1 in memory CD8⁺ T cell formation. The positive role of mTORC1 inhibition on memory cell was also discovered independently by Pearce and collaborators (E. L. Pearce et al. 2009). Studying TRAF6 deficient CD8⁺ T cells, they observed that these cells showed a decrease in fatty acid oxidation and a defect in memory cells transition. Treatment with metformin (an AMPK activator) or rapamycin (an mTORC1 inhibitor) restored fatty acid oxidation and memory cell differentiation. Another group also showed that mTOR inhibition by rapamycin decreased the expression of T-bet and increased the one of Eomes, inhibiting the effector functions of CD8⁺ T cells while enhancing their differentiation into memory cells (Rao et al. 2010). More recently Pollizzi and collaborators showed that CD8⁺ T cells deficient for TSC2 presented the characteristics of terminal effector T cells, and that the deletion of Rheb conversely favored the generation of memory cells (Pollizzi et al. 2015) (**Figure 23**).

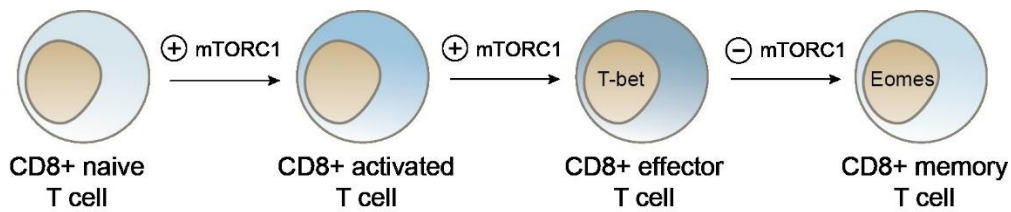


Figure 23: mTORC1 activity in CD8⁺ memory T cell generation. Activated CD8⁺ T cells differentiate into effector T cells which then can transition into memory cells. mTORC1 inhibition favor CD8⁺ memory T cell generation.

5. mTOR in NK cells

mTOR importance in other lymphocytes emerged soon after its identification in T cells. In NK cells, mTOR also regulates key aspect of their biology via metabolic reprogramming and therefore impacting their development and induction of effector functions (**Table 3**).

Protein	mTOR activity	Cre expression system	Phenotype	References
mTOR	mTORC1- mTORC2-	NCR1	<ul style="list-style-type: none"> - Block at early stages of maturation (CD11b^{lo}) - Decrease in T-bet and Eomes expression - Decrease in IL-15 receptor expression - Decrease in proliferation, no viability defect - Decrease in cytotoxic capacities 	(Marçais et al. 2014)
RAPTOR	mTORC1-	NCR1	<ul style="list-style-type: none"> - Block at early stages of maturation (CD11b^{lo}) - Decrease in T-bet and Eomes expression - Decrease in IL-15 receptor expression - Decrease in proliferation, no viability defect 	(C. Yang et al. 2018)
		NCR1	Decrease in cytotoxic capacities	(F. Wang et al. 2018)
RICTOR	mTORC2-	NCR1	<ul style="list-style-type: none"> - Defect in terminal maturation - Decrease in T-bet expression - Proliferation and migration defect, no viability defect. 	(C. Yang et al. 2018)
		NCR1	Increase in cytotoxic capacities	(F. Wang et al. 2018)
TSC	mTORC1+	Vav1	<ul style="list-style-type: none"> - Block at early stage of maturation - Increase proliferation and apoptosis - Increase in T-bet and decrease in Eomes expression 	(M. Yang et al. 2016)
		NCR1	no impaired phenotype	

Table 3: Phenotypes associated with deletion of key components of mTOR signaling in NK cell.

5.1. Phenotypes associated with mTOR depletion in NK cell

In 2014, NK cell specific deletion of mTOR was generated in our team. Deletion of mTOR (Ncr1-iCre) affects NK cell maturation and homeostasis (Marçais et al. 2014). mTOR deficient cells are blocked at the CD11b^{lo} stage, and are characterized by a decrease in the expression of activating receptors normally acquired during maturation, and a decrease in the expression of the transcription factors T-bet and Eomes. Their viability is comparable to WT cells, however their proliferation is reduced, explaining the decrease in NK cell number in periphery. Expression of IL-15 receptor is halved in mTOR deficient cells which is sufficient to allow them to respond normally to low dose of IL-15 required for cell survival, but which affects their maximal response to higher doses of IL-15 necessary for proliferation.

It was later shown that, Raptor specific deletion (NCR1-Cre) results in a phenotype similar to mTOR deficiency. Raptor KO NK cells have normal viability but a proliferation defect and maturation arrest at the CD11b^{lo} stage. As for mTOR KO, Raptor deficient cells are characterized by a decrease in the expression of T-bet and Eomes and a decrease in the expression of the IL-15 receptor resulting in a decreased response to this cytokine (C. Yang et al. 2018; F. Wang et al. 2018). Rictor deletion (NCR1-Cre) also results in proliferation and migration defect, without decrease in cell viability. Unlike Raptor KO, Rictor KO cells show terminal maturation defect with NK cells being blocked at the transition between DP/CD27^{lo} associated with a decrease in T-bet expression (C. Yang et al. 2018; F. Wang et al. 2018). Taken together, these studies show the importance of mTORC1 for NK cell early maturation and respond to IL-15 whereas mTORC2 seems to further promotes late maturation stage.

Impact of the depletion of the mTORC1 inhibitor TSC has also been assessed. Deletion of TSC1 in hematopoietic cells (Vav1-Cre) leads to a decrease in NK cell number, associated with an arrest of maturation at the CD11b^{lo} stage and a defect in cytotoxicity. Cells lacking TSC1 are very proliferative and more apoptotic than WT ones, a phenotype reminiscent of the quiescence exit of TSC1-null T cells. TSC1 deletion results in an increase in mTORC1 activity without notable change in that of mTORC2. However, deletion of TSC1 at later stages (NCR1-Cre) has surprisingly no effect on NK cells number and function (M. Yang et al. 2016). This suggest another mechanism of mTORC1 regulation in mature NK cells which correlates with the decrease in TSC expression with NK cell maturation (M. Yang et al. 2016).

5.2. mTOR activity in NK cells

As for T cell, mTOR activity in NK cell is tightly link to cell metabolism. For instance, mTOR activity decreases during NK cell maturation, which correlates with a decrease in metabolism (Marçais et al. 2014) (**Figure 24**). Indeed, immature CD11b^{lo} cells have a higher metabolism than the CD27^{lo} mature one, as indicated by nutrient receptor expression and metabolic enzyme mRNA level and activity that correlate with mTOR activation status (Marçais et al. 2014; Schaum et al. 2018).

Resting mature NK cell have a low mTOR activity associate with a low basal metabolism (Marçais et al. 2014). Cytokines or activating receptor engagement triggers mTOR

activation in the minutes following stimulation (**Figure 24**). In particular, IL-15 is a potent activator of mTORC1 and mTORC2 in NK cells, as well as the IL-15 related cytokine IL-2 (Marçais et al. 2014; Donnelly et al. 2014; F. Wang et al. 2018). *In vivo*, polyI:C, MCMV or tumoral challenge also increase mTOR activity in NK cell (Marçais et al. 2014; Donnelly et al. 2014; Nandagopal et al. 2014; Pouxvielh et al. 2024). On the contrary, anti-inflammatory signals block mTOR activation, in particular TGF- β inhibits mTORC1 signaling induced by IL-15 stimulation (Viel et al. 2016).

mTOR activity is also tightly link to NK cell basal reactivity (**Figure 24**). A study from our group showed that the basal level of mTOR pathway activation is correlated with cell reactivity, the most responsive cells having the highest level of phosphorylation of mTORC1 and mTORC2 targets protein (Marçais et al. 2017). Reciprocally, gradual inhibition of mTOR with pharmacologic inhibitors tunes down NK cell reactivity. These results indicates that mTOR acts as a molecular rheostat controlling NK cell reactivity.

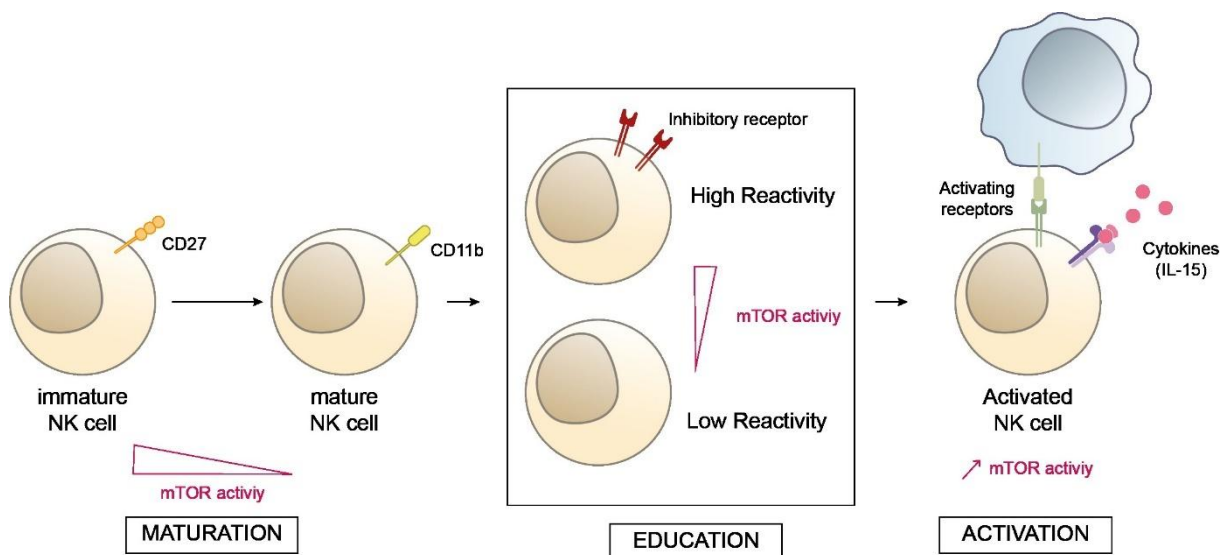


Figure 24: mTOR activity in NK cell. mTOR activity decrease during NK cell maturation. Basal mTOR activity is also corelated with NK cell reactivity. NK cell activation results in an increase in mTOR activity.

5.3. mTOR in NK cell functions

a. Metabolism

Activated NK cells experience an increase in the rates of both glycolysis and OXPHOS, an increase in nutrients transporter and therefore uptake of nutrients, an increase in glycolytic enzymes expression and an increase in mitochondrial mass (O'Brien et Finlay 2019). mTOR deficient mice have impaired metabolic switch upon activation following polyI:C challenge. They show a decrease in some nutrients transporters such as CD98/LAT1 (large amino acid transporter 1) or CD71 (transferrin receptor) and in glucose uptake measured by 2-NDBG incorporation compared to WT (Marçais et al. 2014). Acute mTOR inhibition with rapamycin also abrogates glycolysis and OXPHOS rate increase, 2-NDBG incorporation as well as expression of key glycolytic enzyme induced by 18h

stimulation with IL-2+IL-12 (Donnelly et al. 2014). Therefore, these studies highlight the importance of mTOR for NK cell metabolic reprogramming upon activation.

Two studies from the group of David Finlay shed light on the mechanisms underlying the control of metabolism by mTOR in NK cell. The first one from Assmann and colleagues, described the role of SREBP. They observed that activation with IL-2+IL-12 for 18h led to an increase in NK cells glycolysis and OXPHOS rates which is abolished by the deletion of SREBP. SREBP is a master regulator of lipid metabolism, however lipid synthesis inhibitors did not recapitulate the effect of SREBP deletion, suggesting another role for SREBP than its conventional one in lipid synthesis. The authors then showed that SREBP promotes the conversion of glucose into cytosolic citrate in IL-2/12 activated NK cells, by a metabolic pathway involving the citrate-malate shuttle, and thereby supports glycolysis and mitochondrial respiration. Inhibition of SREBP or the citrate-malate shuttle both led to a reduction in IFN- γ production and cytotoxicity. Rapamycin treatment inhibits SREBP targets, indicating the importance of mTORC1 upstream of the pathway (Assmann et al. 2017). The same group also showed that Myc-deficient NK cells treated 18h with IL-2+IL-12, have a lower glycolysis and mitochondrial respiration rate than the WT ones, associated with a decrease in functions (Loftus et al. 2018). Rapamycin treatment also decreased c-Myc expression in this setting again placing mTORC1 upstream of the metabolic changes. In addition, the authors also showed that the deletion of another mTORC1 target, HIF1 α , known to positively regulate glycolysis does not impact NK cell functions.

b. Effector functions

Several reports indicate that mTOR is important for NK cell cytotoxicity. mTOR deficient NK cells show a decrease in granzyme B and CD107 expression following poly I:C or IL-15 priming or MCMV infection (Marçais et al. 2014). Similarly, Raptor KO NK cells have a defect in CD107 expression and cytotoxicity in response to tumor cells (F. Wang et al. 2018). On the contrary, it appears that Rictor deficient cells have a higher cytotoxic response than the WT ones. The mechanism underlying this increase as well as the associated higher mTORC1 activity in this study is unclear (F. Wang et al. 2018). Impact of mTOR on NK cell cytotoxicity seems to be in great part dependent on mTOR impact on metabolism. Indeed in the studies mentioned above, mTOR and Raptor deficiency alter mTOR function and metabolic parameter in the same manner while Rictor deficiency promotes both (Marçais et al. 2014; F. Wang et al. 2018). Similarly, inhibition of glycolysis with 2DG decreases granzyme production following IL-15 stimulation *in vitro*, which suggests that mTOR activation by IL-15 is essential for effector function priming (Mah et al. 2017).

The role of mTOR in IFN- γ production is less clear. mTOR or Raptor deficiency have no effect on IFN- γ production. The metabolic dependency and therefore mTOR dependency of IFN- γ production seems to vary according to the activating stimuli. In that sense, a study shows that IFN- γ production is not affected by glycolysis or OXPHOS inhibition downstream IL-12/IL-18 stimulation, whereas it is affected downstream activating receptor or IL-15 depending on the dose used (Keppel et al. 2015). This suggests that IL-

IL-12/IL-18 enhanced IFN- γ production mostly through transcriptional control whereas IL-15 priming increase cellular metabolism which then support IFN- γ production. This explains how rapamycin treatment decrease IFN- γ production following polyI:C priming and IL-2+IL-12 treatment *in vitro* (Donnelly et al. 2014).

c. Memory cell generation

Impact of mTOR inhibition has also been evaluated for adaptive NK cell generation, in particular in the case of MCMV infection. mTOR inhibition during the effector phase of the NK cell response results in an increase in mice susceptibility to MCMV (Mah et al. 2017; Nandagopal et al. 2014). On the contrary, mTOR inhibition with rapamycin during the contraction phase results in Ly49H⁺ adaptive NK cell accumulation 28 days post infection. The underlying mechanism evoked is that mTORC1 inhibition increases mitophagy which prevent the accumulation of dysfunctional mitochondria that were acquired by NK cells during the expansion phase (O'Sullivan et al. 2015), a mechanism reminiscent of the one described for T cells.

d. NK cell dysfunction

Several report suggest that NK cell dysfunction is associated with metabolic dysfunction and therefore mTOR modulation could be used to reverse NK cell dysfunction in certain settings. In particular, several studies indicates that limitation of mTORC1 activity could be beneficial to preserve NK cell function in chronic stimulation setting. In this sense, a study indicate that rapamycin treatment decrease accumulation of fragmented dysfunctional mitochondria in infiltrating tumor NK cells, which has been linked with NK cell antitumor activity (X. Zheng et al. 2019). Conversely, unpublished data from our team show that rapamycin treatment decrease Tim3 expression on NK cells although this was not sufficient to reverse NK cell dysfunction and increase mice survival in a model of lymphoma (Marotel PhD manuscript). Dysfunction induced *in vitro* by chronic IL-15 stimulation could also be reversed by rapamycin treatment (Felices et al. 2018). Taken together these study suggest that a precise control of mTOR activity ensures proper NK cell function and that NK cell functions can be tuned by mTOR activation or inhibition.

PART II : RESULTS

I. Working objectives

NK cells are natural effectors of both antitumor and antiviral responses, and their use in therapy is gaining momentum. In particular, NK cells can be primed by different cytokines *in vitro* among which IL-15, IL-18 and IL-12 show the best potential for antitumor treatment. Metabolism activation is a crucial step in sustaining different aspects of NK cell biology and is under the regulation of the master integrator mTOR. Since mTORC1 deficiency recapitulates most of the defects observed in mTOR deficient NK cell, our study had focused mostly on this complex.

IL-15, and IL-18 to a lesser extent, both activate mTORC1 in NK cell (Marçais et al. 2014). However, the pathways leading to mTORC1 activation in primary cells in general and in NK cells in particular are still poorly defined. Indeed, most of the mechanisms described come from studies using immortalized cell lines *in vitro*, which are easy to manipulate but have very different features from primary cells. This is illustrated by several reports, mostly in T cells, pointing out some discrepancies with the canonical pathways described so far (Pollizzi et Powell 2015). For instance, the deletion of Rheb in T cells does not recapitulate that of Raptor upon activation by the TCR (K. Yang et al. 2013). Similarly, Rheb or Raptor ablation in CD4⁺ T cell do not results in the same differentiation defects (Delgoffe et al. 2011; K. Yang et al. 2013). These results suggest the existence of additional control checkpoints independent of Rheb. The reliance on AKT activation for mTORC1 signaling has also been called into question in T cells (Finlay et al. 2012; Macintyre et al. 2011). Indeed, AKT inhibition or expression of an inactive form of PI3K δ by cytotoxic T cell does not result in a decrease in mTORC1 activation following IL-2 stimulation.

Therefore, our objectives were to better characterize the signaling pathways leading to mTORC1 activation by IL-15 and IL-18 and to test the impact of combining both cytokines. More specifically, our study was driven by the following questions:

- What is the impact of IL-15 and IL-18 treatment alone or in combination on mTORC1 activation?
- What are the pathways involved in IL-15 and IL-18 activation of mTORC1?
- What are the consequences of IL-15 and IL-18-dependent mTORC1 activation on NK cell biology *in vitro* and *in vivo*?

II. Research Article

Article, **Interleukins 15 and 18 prime Natural Killer cell antitumor function synergistically by activating mTORC1 in a non-canonical manner.** Fallone et al., submitted.

Title: Interleukins 15 and 18 prime Natural Killer cell antitumor function synergistically by activating mTORC1 in a non-canonical way

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Abstract: mTORC1 is essential for the increase in protein synthesis and bioenergetic metabolism resulting in cellular proliferation. The pathways leading to mTORC1 activation have been largely defined using cell lines, but remain to be studied in primary cells. Here, we investigated the mechanisms of mTORC1 activation by IL-15 and IL-18 in Natural Killer (NK) cells, that are important innate effectors of the antitumoral response. Through genetic and pharmacological approaches, we show that IL-15 activates mTORC1 via PI3K/Akt/ERK whereas IL-18 signals through p38/MK2 in both murine and human primary NK cells. Both pathways synergize to promote mTORC1-dependent NK cell proliferation and effector functions. Moreover, they both operate in a TSC-independent manner, revealing a non-canonical mode of mTORC1 activation by cytokines. In vivo activation of NK cells by IL-15/18 in combination leads to increased NK cell number and improved anti-tumoral activity, which could be exploited to boost NK cell potential in therapeutic settings.

One Sentence Summary: IL-15 and IL-18 synergically activate mTORC1 in NK cell through non-canonical pathways, to prime antitumor functionality.

INTRODUCTION

Natural Killer (NK) cells are innate lymphocytes with antitumoral and antiviral abilities. Their activity is triggered by a set of activating receptors and is regulated by different pro or anti-inflammatory cytokines. Among them, IL-15 and IL-18 are strong positive NK cell regulators. IL-15 belongs to the IL-2 family, with which it shares two receptor subunits, the IL-2/IL-15 receptor β chain (CD122) and the common γ chain (CD132) (1). IL-15 receptor also comprises a third unique chain, IL-15R α , which trans-presents IL-15 from neighboring producing cells to responding cells (2). IL-15 is essential for NK cells homeostasis and maturation but also primes NK cells in various physiological setting. A brief IL-15 treatment enhances NK cell cytokine production and cytotoxicity, resulting in better antiviral and antitumor capacities (3–5).

IL-18 is a cytokine of the IL-1 family, which receptor is highly expressed by NK cells and composed of two chains, IL-18R1 and IL-18RAP (6). Along with IL-12, IL-18 is best described for its ability to induce IFN- γ production by NK, and memory T cells (7, 8). Mice deficient for IL-18 signaling have a normal number of NK cells but these cells show decreased IFN- γ secretion and cytotoxic capacity upon *ex vivo* stimulation (9, 10). It thus appears that both IL-15 and IL-18 participate in the priming of NK cells *in vivo*, preparing them to display robust effector functions upon challenge. Interestingly, their impact on NK cells is not redundant but rather complementary as underlined by their association in the IL-12/15/18 cocktail used to obtain so-called “cytokine induced memory-like” (CIML) NK cells (11–13). Indeed, short-term stimulation of NK cells with this cytokine cocktail leads to the generation of effector cells with superior cytokine secretion and cytotoxic ability associated with ameliorated survival upon transfer *in vivo*. Notably, these properties have been exploited for the treatment of relapse/refractory AML and complete clinical remission has been observed upon patient infusion with CIML NK cells (12). However, the molecular basis of the complementary effect of these cytokines on NK cells is still incompletely understood.

We and others have shown the importance of the metabolic checkpoint kinase mechanistic Target Of Rapamycin (mTOR) in NK cell homeostasis and activation (*14, 15*). mTOR is a serine/threonine kinase, and constitutes the catalytic subunit of two complexes, mTORC1 and mTORC2. mTORC1 acts as a molecular switch, promoting cell growth, proliferation and metabolism when nutrients and growth signal are both present, whereas mTORC2 participates in the activation of the kinase Akt (*16*). Later studies have shown that the functional defect observed in mTOR deficient NK cells could be ascribed to mTORC1 (*17*). Of note, IL-15 and, to a lesser extent, IL-18 activate mTORC1 in NK cells (*14*). However, the pathways leading to mTORC1 activation downstream these two cytokines are still poorly defined. Moreover, the impact of IL-15 and IL-18 combination on mTORC1 activity is unknown.

The canonical pathway leading to mTORC1 activation downstream of growth factor stimulation has been deciphered in cell lines (*16*). Growth factors activate PI3K, the product of which, PIP3, recruits PDK1 and mTORC2 to the plasma membrane. This cooperatively triggers activation of Akt through phosphorylation on T308 by PDK1 and S473 by mTORC2 (*18, 19*). Akt in turn phosphorylates TSC2 to inactivate the TSC complex, thus releasing a small GTPase, Rheb, from TSC inhibitory activity (*20*). As a result, GTP-loaded Rheb activates lysosome-bound mTORC1 (*21*). A secondary pathway involves the MAPK ERK and also converge on TSC inactivation (*22*). Therefore, TSC inhibition elicits unrestrained mTORC1 activation (*23, 24*). However, whether these pathways are in use upon IL-15 or IL-18 stimulation is unclear.

Here, we used various pharmacological and genetic approaches to dissect the molecular mechanisms leading to mTORC1 activation by IL-15 and IL-18 in NK cells. We also studied the consequences of this activation on proliferation and antitumor activity of NK cells.

RESULTS

IL-15 and IL-18 activate mTORC1 synergistically in primary NK cells

We hypothesized that the efficacy of the CIML cocktail in boosting NK cell anti-tumor activity resulted from its action on mTORC1. To test this point, we first stimulated primary resting murine NK cells with IL-12, IL-15 and IL-18, alone or in combination and we measured S6 phosphorylation on Ser235-236 (p-S6), as a readout of mTORC1 activity. As shown in Figure 1A, IL-15 and IL-18 treatment both increased p-S6 levels while IL-12 was inert, confirming previous findings (14). Interestingly, an additive effect was observed when IL-15 and IL-18 were combined. We thus focused on these two cytokines in the rest of the study. The increase in p-S6 was prevented in NK cells deficient for the mTORC1 specific scaffold protein Raptor ($Ncr1^{iCre+/-} \times Rptor^{lox/lox}$ (NK-Raptor KO) mice), upon IL-15 or IL-18 treatment, demonstrating the role of mTORC1 in this process (Fig. 1B). In addition, we also observed phosphorylation of other downstream targets of mTORC1 (p-S6 Ser240-244, p-4EBP1 Thr37-Thr46) and of mTOR itself (p-mTOR Ser2448, p-mTOR Ser2481) following IL-15 or IL-18 treatment (Fig. S1A). Moreover, capillary western-blot analysis of S6 phosphorylation on Ser235-S236 led to similar conclusions (Fig. S1B). mTORC1 activation was blocked by an antibody targeting CD122 when NK cells were stimulated with IL-15, but not with IL-18 (Fig. 1C). Conversely, an antibody blocking IL-18 inhibited mTORC1 activation following IL-18 but not IL-15 stimulation (Fig. 1D). The action of IL-15 and IL-18 on NK cells are thus independent of each other.

In terms of kinetics, both cytokines triggered mTORC1 activation in the minutes following exposure, IL-18 stimulation resulting in a more transient pattern of activation compared to IL-15 or the IL-15/18 combination (Fig. 1E). We next titrated both cytokines and measured their combined effect on mTORC1 activity. While IL-15 and IL-18 displayed an additive effect at saturating concentrations (100ng/ml and 5ng/ml respectively), a synergistic effect was

unmasked at lower concentrations, which was defined as IL-15 and IL-18 combined effect being higher than the sum of their effects taken separately (Fig. 1F and G). Overall, these data show that both cytokines activate mTORC1. The synergistic effect observed at limiting IL-15 concentrations suggests that two independent and complementary pathways are triggered.

IL-15 and IL-18 induction of NK cell metabolism and proliferation depends on mTORC1

mTORC1 activates translation and the bioenergetic metabolism, leading to proliferation and improved cell functionality. This prompted us to test the impact of IL-15 and IL-18 on these parameters in the presence or in the absence of Torin, a mTOR ATP-competitive inhibitor. Importantly, we titrated Torin to only inhibit mTORC1 activity while sparing mTORC2 (Fig. S2A). An overnight treatment of NK cells with these cytokines led to an increased level of p-S6, denoting their prolonged effect on mTORC1 (Fig. 2A). This increase was inhibited in a dose dependent manner by Torin as expected (Fig. 2A). Using a puromycin incorporation assay (25), we observed that protein translation levels were only significantly increased upon treatment with both cytokines in combination, further underlying their synergistic effect (Fig. 2B). Importantly, Torin treatment or Raptor deficiency both reduced translational activity, showing that the increase observed was dependent on mTORC1 (Fig. 2B, Fig. S2B). We next measured NK cell metabolism using Seahorse extracellular flux analysis. IL-15 and IL-18 increased both glycolysis and mitochondrial respiration, measured by ECAR (extracellular acidification rate) and OCR (oxygen consumption rate) respectively, with a comparatively higher effect on ECAR (Fig. 2C and D). IL-15/18 combination further increased basal ECAR while basal OCR was already maximal. Torin treatment halved ECAR induced by the IL-15/18 combination while it had a marginal impact on OCR, underscoring the importance of mTORC1 in glycolysis activation (Fig. 2C and D). We next studied the impact of IL-15/18 stimulation on NK cell proliferation and the role of mTORC1 in this process. To this end, CTV-labeled NK cells were cultured for 3 days with cytokines in the presence or in the absence of Torin. IL-15

and IL-18 similarly promoted NK cell division, a parameter only mildly affected by their combination (Fig. 2E). However, combination of both cytokines led to a clear synergistic effect on NK cell accumulation in a mTOR-dependent way (Fig. 2F). We then tested how NK cell functions were regulated in the different conditions. NK cells were cultured overnight with IL-15 and IL-18 in the presence or not of Torin, and their IFN- γ production as well as their cytotoxic capacity toward target cells were measured. As expected, IL-18 induced IFN- γ secretion by NK cells, an effect complemented by IL-15 (Fig. 2G). mTORC1 inhibition, however, did not negatively affect this parameter. IL-15 and IL-15/18 combination induced a similar increase in cytotoxic activity while IL-18 effect was milder (Fig. 2H). Torin treatment abrogated the increase. Taken together, these results unmask the synergistic impact of IL-15/18 on NK cells translation, metabolism, proliferation and effector function; an impact abrogated by mTORC1 inhibition except for IFN- γ production.

IL-15 and IL-18 activate mTORC1 through both canonical and non-canonical pathways

The synergy between IL-15 and IL-18 in mTORC1 activation suggested the existence of two distinct signaling pathways arising from IL-15 and IL-18 receptors. We first analyzed the constitution of mTOR containing complexes in different activating conditions. We took advantage of a mouse strain expressing an endogenously One-Strep-tagged (OST) version of mTOR (MTOR^{OST} mice) to perform mTOR complexes isolation in primary NK cells. The resulting affinity purification was analyzed by mass spectrometry (AP-MS) as previously described (26). mTOR itself, the scaffolding proteins Raptor, as well as the other mTORC1 components, mLST8 and PRAS40 were significantly enriched (Fig. 3A). Partners of mTOR in mTORC2 (Rictor, SIN1, Prr5 and Prr5l) were also identified, in addition to 14.3.3 η . However, none of the treatments tested had any impact on the complexes composition nor stoichiometry. Thus, it appears that mTOR complexes are formed prior to cytokine stimulation and not affected thereafter.

Next, we explored the signaling pathways leading to mTORC1 activation by both cytokines. IL-15 can engage the JAK1-3/STAT5, PI3K/Akt and MAPK ERK pathways (1, 27), while IL-18 is known to activate the p38 MAPK as well as IKK/NF- κ B (6, 27). Therefore, we monitored the activation of these pathways following cytokine stimulation, as well as the impact of their inhibition on p-S6 activation. IL-15 stimulation, alone or in combination with IL-18, elicited an increase in PIP3, p-Akt T308, p-ERK and p-STAT5. In contrast, IL-18 did not show any effect on these pathways but specifically induced p38 phosphorylation and I κ B α degradation, an important negative regulator upstream NF- κ B (Fig. 3B and S3A). This correlated perfectly with the impact of the various inhibitors we tested. Indeed, IL-15-induced p-S6 was impaired by inhibitors targeting JAK (Tofacitinib), panPI3K or PI3K δ (Wortmannin and IC87114), and, to a lesser extent, Akt (AKTi) and ERK (PD98059) while inhibition of p38 α / β (BIRB796 and SB203590) and its downstream effector MK2 (PF3644022) were inefficient. Conversely, p-S6 was conserved upon inhibition of JAK, PI3K, Akt and ERK upon IL-18 stimulation but decreased by p38 α / β and MK2 inhibition (Fig. 3C). As expected, Rapamycin and Torin inhibited both signaling pathways.

To confirm these pharmacologic data, we generated primary NK cells deleted for p38 α and three of its downstream targets MK2, MK3 and MK5, using CRISPR-Cas9 gene deletion and stimulated them with IL-18 prior to measuring p-S6 levels. We verified loss of protein expression by capillary western blot (Fig. S3B). Loss of p38 α reduced p-S6 by 60% following IL-18 treatment (Fig. 3D). Among p38 α targets, MK2 deletion had the most effect in accordance with its higher expression level in NK cells (Fig. 3D and S3C). However, a stronger reduction in p-S6 level, recapitulating p38 α deficiency, was observed upon concomitant targeting of MK2 and 3 (Fig. 3D and S3B) suggesting partial compensation. No further effect was observed upon KO of MK5. Taken together, these results show that IL-15 activates

mTORC1 in a conventional manner while IL-18 activates mTORC1 through a unique p38 MK2/3 pathway in primary NK cells.

Signaling arising from growth factor such as cytokines, classically converge on TSC inhibition, which acts as a major brake in mTORC1 signaling in cell lines (28, 29). We therefore sought out to test the role of TSC in NK cells following IL-15/18 stimulation. To this end, we established a mouse strain deficient for TSC1 ($Ncr1^{iCre} \times Tsc1^{lox/lox}$) in NK cells specifically (NK-TSC1 KO). TSC1-deficient NK cells displayed a higher basal level of p-S6 and p4EBP1 (Fig. S3D), reflecting a higher mTORC1 activation level at steady state. This was confirmed by the increased cell size of TSC1-deficient cells (Fig. S3E). We next tested the impact of TSC deficiency on mTORC1 activation downstream IL-15 and IL-18 stimulation. Although the basal level of p-S6 was slightly higher in TSC deficient NK cells compared to WT NK cells, cytokines alone or in combination induced a massive increase in this level regardless of TSC1 expression (Fig. 3E). This indicated that TSC deletion is not sufficient to mimic IL-15 nor IL-18 stimulation and that TSC is not the principal node controlling mTORC1 activation in NK cells (Fig. 3F) contrary to cell lines and other primary cell types (24, 30, 31).

p38 is required for human CD56^{Dim} NK cell activation of mTORC1 by IL-18

We then wanted to investigate whether our findings on the regulation of mTORC1 by cytokines in murine NK cells apply to human NK cells. In human, peripheral blood (PB) NK cells can be divided according to their level of CD56 expression in CD56^{Dim} and CD56^{bright} subsets. Upon cytokine stimulation, CD56^{bright} NK cells upregulated p-S6 levels in response to IL-15 but not IL-18 (Fig. 4A). In contrast, CD56^{Dim} NK cells responded to both cytokines by activating mTORC1 in a manner that closely matched the situation seen in murine NK cells (Fig. 4A). We next analyzed the impact of mTORC1 inhibition on NK cell proliferation induced by IL-15 and IL-18 treatment. Peripheral blood human NK cells were cultured with IL-15 and IL-18 for 5 days in the presence or absence of various doses of Torin. IL-15 and IL-18 alone did not induce

NK cell proliferation (Fig. 4B). Proliferation was only induced upon combination of both cytokines and inhibited by Torin treatment in a dose-dependent manner (Fig. 4B). This indicates that IL-15/18 induced proliferation depends both on mTORC1 in human and murine NK cells alike.

Human PB NK cells were then treated with cytokines in the presence of increasing doses of Torin or the p38 α/β inhibitor SB203580. As expected, Torin prevented p-S6 induction (Fig. 4C). In contrast, SB203580 treatment did not affect p-S6 induced by IL-15, but it significantly inhibited p-S6 induction following IL-18 stimulation in CD56^{Dim} (Fig. 4C). Therefore, similarly to murine NK cells, mTORC1 activation by IL-18 involves p38 in human CD56^{Dim} NK cells.

IL-15 and IL-18 co-injection enhances NK cell proliferation and anti-tumor function in vivo.

Our results suggest that the combination of IL-15/IL-18 could be particularly effective in enhancing the in vivo anti-tumor activity of NK cells by acting synergistically on mTORC1. To test this point, we first treated C57BL/6J mice at day 0 and day 2 with IL-18 and/or IL-15 in complex with the IL-15R α chain (hereafter called IL-15cplx) (32), and analyzed splenic NK cells at day 3 (Fig. 5A). Interestingly, only the combination of IL-15cplx and IL-18 significantly increased NK cell number and frequency (Fig. 5B, C). Similarly, only the combination induced a robust NK cell proliferation, as measured by CTV dilution of co-transferred NK cells (Fig. 5D). This was reflected by a higher percentage of Ki67⁺ cells when both cytokines were administered (Fig. 5E). This correlated with activation of mTORC1 as denoted by an increased cell size and p-S6 level (Fig. 5F and G). We also measured IFN- γ production at basal state and after 4h re-stimulation *ex vivo* with NK1.1 or RMA-KR, a tumor cell line devoid of MHC-I expression and expressing Rae1 β , a ligand of NKG2D (33). IL-15cplx + IL-18 treatment increased IFN- γ production in all conditions while IL-18 and IL-15cplx alone only induced a

significant increase in IFN- γ upon RMA-KR stimulation (Fig. 5H). Killing of RMA-KR target cells was also increased by IL-18 alone or in combination with IL-15cplx (Fig. 5I). We then assessed the impact of IL-15cplx + IL-18 treatment in a preclinical murine tumor model. Mice were grafted intravenously with RMA-KR tumor cells and treated the following day and 2 days after with IL-15cplx and IL-18, alone or in combination (Fig. 5J). Mouse survival was monitored during 50 days. Only the IL-15cplx + IL-18 treatment had a positive effect on mouse survival compared to the control group (Fig. 5K). Collectively, we show that IL-15 and IL-18 cooperate in vivo, inducing NK cell proliferation and better functionality, associated with high mTORC1 activity, which results in improved tumor control by NK cells.

DISCUSSION

In this study, we describe that IL-15 and IL-18 cooperate to activate mTORC1 in primary mouse and human NK cells. This cooperation stems from the activation by both cytokines of two distinct signaling pathways converging on mTORC1. Indeed, while IL-15 activates mTORC1 via PI3K δ /Akt and ERK, IL-18 activates mTORC1 through p38 α and its downstream effectors MK2 and 3. Our results on the role of PI3K are corroborated by earlier reports. Indeed, NK cells deficient in PDK1, the kinase just downstream of PI3K, show impaired phosphorylation of S6 upon IL-15 stimulation (34, 35). In addition, NK cells doubly deficient in both the p110 δ and p110 γ catalytic subunits of PI3K (36), deficient in p110 δ alone (37, 38) or deficient in PDK1 (34, 35) have a phenotype partially convergent with that of mTOR deficient NK cells. These findings indicate that the PI3K/PDK1 is involved in the control of mTORC1 downstream of IL-15. However, the fact that p110 δ or PDK1 inactivation only partially phenocopies mTOR deficiency in NK cells, suggests that another parallel pathway is involved in NK cells. According to our results, ERK provides this alternative, increasing the robustness of the network. This redundancy could also contribute to explain that an earlier study failed to detect an effect of Akt inhibition on mTORC1 activation in in vitro activated NK cells (39). Strikingly, IL-18 activates a fully alternative pathway that involves p38 α and MK2/3. This pathway has also been involved in the activation of mTORC1 downstream TCR stimulation in CD4 T cells (40). Intriguingly, mTORC1 activation was completely independent of MK2/3 in CD8 T cell, underlying the plasticity of this network.

Our results also show that both cytokines activate mTORC1 in a non-canonical way that is independently of TSC1. This finding is puzzling since this complex is described as a key integrator of various signals upstream of mTORC1 (41). To our knowledge, it is to date the only well-established regulator of Rheb and as such, its deletion leads to unrestrained mTORC1

activity in transformed cells as well as primary tissues, often leading to cancer (30, 31). For instance, TSC1 deletion in T cells leads to strong basal mTORC1 activity leading to a cell-intrinsic loss of quiescence and hyperactive response to TCR stimulation (42). Why this does not happen in NK cells is currently unclear but betrays the existence of yet another layer of positive or negative regulators of mTORC1 in NK cells. In this respect, a similar finding was reported in hematopoietic stem cells (43). In these cells, TSC1 deficiency only induced unrestrained mTORC1 activity when combined with a deficiency in SZT2, a protein taking part in the regulation of nutrient-dependent mTORC1 activation (44, 45). Whether nutrients such as amino-acids participate in the regulation of mTORC1 activation by cytokines remains to be determined.

Molecularly, we show that the composition of the mTOR interactome was not modified following stimulation by either of the cytokines. The molecular mechanisms that link Akt, ERK and MK2/3 kinases to mTORC1 thus remain elusive. Of note, direct phosphorylation of Raptor by ERK and p38 β on serine residues has been reported (46, 47). These modifications were proposed to participate in mTORC1 activation. Regarding the pathway activated by IL-18, MK2 was described to activate mTORC1 through TSC inhibition (48). Perhaps more relevant for our study, another mode of regulation based on both phosphorylations and ubiquitinations of various proteins of the pathway including mTOR itself and involving TRAF6 and p38 δ has been described in 293 cells (49, 50). However, given the prominent role of TSC in cell lines, these modes of mTORC1 activation have received little attention. Whether these post-translational modifications or others are involved in NK cells thus remains to be determined.

The principal physiological consequence of the concomitant activation of mTORC1 by IL-15 and IL-18 was a synergy in terms of cell accumulation, *in vitro* and *in vivo*, both in the mouse and human systems. This is probably linked to several causes. First, we observed a synergistic effect of both cytokines on the activation of mTORC1 itself, an effect that was mostly detectable

at limiting IL-15 concentrations. This could be particularly relevant in the in vivo settings where local cytokine concentration might be limiting. Secondly, for translational activity, a process sustaining cellular proliferation, we observed a non-linear relationship between mTORC1 activity (as measured by p-S6) and its impact on the measured readout. Indeed, mTORC1 activity below a certain threshold was unable to increase translational activity. Such a behavior could allow the cell to filtrate irrelevant signals as noise and only elicit a coherent biological response when a certain threshold of activation is met, as previously described in other biological systems (51). Finally, it is clear that other pathways specifically triggered by each cytokine independently (such as STAT5 for IL-15 or NFκB for IL-18) have a complementary role to play. For instance, the effect of cytokine combination on IFN-γ secretion capacity was independent on mTOR, in accordance with our previously published work (14).

In a therapeutic perspective, we also injected both cytokines separately or together to mice previously implanted with an RMA cell line sensitive to NK cells. While injection of IL15cplx or IL-18 separately did not improve mouse survival despite the high doses employed, their co-injection led to better tumor control.

In conclusion, our data demonstrate that IL-15 and IL-18 both activate mTORC1 using dedicated pathways in human and mouse NK cells, in vitro and in vivo. Their combination leads to a synergistic effect on the activity of the complex that results in improved cellular metabolism and effector functions and culminates in cellular proliferation. In vivo, administration of these cytokines induces NK cell proliferation and primes their effector functions leading to a better control of MHC-I deficient lymphoma. These findings highlight the potential of IL-15/IL-18 combinations for clinical applications in oncology.

MATERIALS AND METHODS

Study Design

The objective of this study was to investigate the mechanisms underlying NK cell priming by IL-15 and IL-18 and the importance of mTORC1 regulation in this process. Pharmacological and genetic approaches were used for in vitro analysis of both murine and human NK cells. Impact of cytokine priming was also assessed in a tumor mouse model in vivo. Unless specified, experiments were performed with at least three independent biological replicates. The number of replicates as well as the statistical analysis performed are indicated in the figure legends. The in vivo study was validated by local and national ethic committees. Human NK cells were isolated from healthy donor whole blood samples obtained from national blood agency. No randomization was performed, with the exception of experiments in which animal survival was monitored, and for which the person in charge of monitoring did not know the treatment used. For the survival analysis, an endpoint based on a set of criteria was used, beyond which the mice were sacrificed. Data collection was stopped after 50 days. No data were excluded.

Mice and in vivo Procedures

C57BL/6J mice were purchased from Charles River Laboratories (L'Arbresle). $Ncr1^{iCre+/-}$ x $Rptor^{lox/lox}$ (NK-Raptor KO) (52, 53), $Ncr1^{iCre+/-}$ x $Tsc1lox/lox^{-/-}$ (NK-TSC1 KO) (54) were generated and bred in our animal facility. $Ncr1^{iCre+/-}$ littermate controls and CD45.1/CD45.2 heterozygous mice were also bred in our animal facility. For the MTOR^{OST} (B6-Mtor^{tm1Ciphe}) mouse, the generic approach already described in (26) was used to construct the targeting vector permitting to introduce a Twin-Strep-tag-coding sequence (OST) at the 5' side of mTOR. Female and male mice 8 to 24-week old were used. All experimental procedures were approved by the local ethic committee (CECCAPP) according to the French recommendations in the

Guide for ethical evaluation of experiments using lab animals and the European guidelines 86/609/CEE.

For in vivo cytokine stimulation, mice were given intraperitoneal injection of 4 μ g of IL-15cplx (32, 55), and/or 1 μ g of IL-18 (Sino Biological) at day 0 and day 2. To assess NK cell proliferation in vivo, NK cells enriched by negative selection from the spleen of CD45.1/CD45.2 heterozygous mice were stained with Cell Trace Violet (Thermo-Fischer). 1x10⁶ CTV-labeled NK cells were injected intravenously to recipient mice.

The RMA-KR lymphoma cell line was generated in our team by inactivation of the B2m gene and lentiviral transduction of Rae1 β (RMA-KR) (33). 5x10⁶ RMA-KR cells were injected intravenously to C57BL/6J mice.

Human Sample

Whole blood samples from healthy subjects were obtained from the French blood agency (Etablissement Français du Sang, Lyon, France). Human PBMCs were separated by Ficoll gradient centrifugation (Eurobio Scientific). NK cells were then purified using NK cell Isolation kit (Miltenyi), according to manufacturer's instructions. The following cytokines were used, rhIL-15 (100ng/ml, Peprotech) and rhIL-18 (100ng/ml, Invivogen).

Murine NK cell Purification

Purified NK cells were obtained by negative selection using a cocktail of biotinylated antibodies and anti-biotin microbeads (Miltenyi). NK cells were then enriched by magnetic separation using an AutoMACS (Miltenyi). NK cell purity was comprised between 60 and 80%. For some experiments, NK cell purification was followed by cell sorting on an Aria II (BD-Biosciences) using anti-CD3 FITC (145-2C11 Biolegend), anti-CD19 FITC (1D3 BD), anti-NKp46 APC (29A1.4 Biolegend), anti-NK1.1 APC (PK136 BD) and a viability dye LIVE/DEAD fixable Near-IR (Invitrogen).

Generation of in vitro Activated NK cells

Purified NK cells were cultured in a medium containing 10% supernatant of IL-2 producing cells (muIL2, (56)). These activated cells (>98% NK cells) were used after 5 days for CRISPR/Cas9 RNP electroporation and after 9 days for mTOR affinity purification.

Culture and Cell Reagent

Unless otherwise stated, cells were cultured in RPMI 1640 GlutaMAX™ medium (Gibco, Thermo Fisher) supplemented with 10% FCS, 1,000 U/ml of penicillin/streptomycin, 1mM sodium pyruvate, 20mM Hepes and 500µM 2-mercaptoethanol (all from Gibco). Cells were cultured with rmIL-15 (100ng/ml, Peprotech), rmIL-18 (5ng/ml, Sinobiological) or rmIL-12 (25ng/ml, Peprotech). The following blocking antibodies and inhibitors were used: anti-CD122 (100µg/ml, TMb1, BioXcell), anti-IL-18 (20µg/ml, MBL Life science), Torin 2 (10nM, MedChemExpress), Rapamycin (25nM, Sigma), Tofacitinib (500nM, Sigma), Wortmaninn (60nM, Sigma), IC-87114 (10µM, Cayman), AKTi VIII (670nM, Cayman), PD98059 (25µM, CST), SB203580 (2700nM, Bio-Techne), BIRB796 (220nM, Bio-Techne), PF-3644022 (830nM, Sigma).

For functional assay, cells were stimulated for 4h in Immulon 2HB plates (Corning) coated with anti-NK1.1 antibody (40µg/ml, PK136, BioXcell) or co-cultured with RMA-KR at a 5:1 Target/Effector ratio in the presence of Golgi Stop (BD Biosciences) and anti-CD107a. Cells were then stained for flow-cytometry analysis.

For puromycin staining, cells were incubated for 15min at 37°C with 10µg/ml of puromycin (Sigma) following by staining for flow cytometry analysis.

Flow Cytometry Analysis

Single cell suspension of spleen was obtained and stained with the antibodies detailed in Table S1. Cell surface stainings were performed for 15 min at 4°C in PBS, 2% FBS, 2mM EDTA. Intracellular staining for Ki67 was performed using Foxp3 Fixation/Permeabilization buffer kit (eBioscience). Intracellular staining for IFN- γ and puromycin was performed using Cytotfix/Cytoperm kit (BD Biosciences). Intracellular staining of phosphorylated proteins was performed with Lyse/Fix and Perm III buffers (BD Biosciences). Flow cytometry analysis was performed on a FACS Fortessa (BD). For in vitro NK cell proliferation assay, purified NK cells were stained with 2 μ M of CTV (Thermo-Fischer) for 15min at 37°C, and then cultured for 3 days (murine experiments) or 5 days (human experiments) as indicated. Cell numbers and CTV staining were measured on a MACSQuant (Miltenyi). Proliferation score represents the mean number of cell divisions of each condition. Data were analyzed with FlowJo 10.8.2 software (BD).

Metabolic Analysis

Sorted NK cells were resuspended in Seahorse XF RPMI medium (Agilent) containing 1g/l glucose, 1mM pyruvate, 2mM glutamine. Cells were distributed at 200,000 cells/well in Agilent Seahorse XF HS PDL Miniplates (Agilent). Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were assessed using Agilent Seahorse XF HS Mini Analyzer (Agilent Technologies). OCR and ECAR were measured under basal conditions and after the sequential addition of 1.5 μ M oligomycin, 2 μ M carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), 0.5 μ M antimycin A + 0.5M rotenone, and 50mM 2-deoxyglucose (all from Sigma-Aldrich).

In vitro Cytotoxic Assay

Purified NK cells were co-cultured with 2,500 RMA-KR cells expressing NanoLuciferase at different Effector to Target (E/T) ratios in round-bottom 96-well plates in a final volume of

180µl. Cytotoxicity was measured by quantification of the NanoLuc activity in the supernatant as previously described (57).

Quantitative Automated Capillary Western immunoblotting

Briefly, cells were lysed in RIPA lysis buffer supplemented with Phosphatase Inhibitor Cocktail 2 et 3 (Sigma) for 30 min at 4°C. Protein concentration was quantified using the µBCA Protein Assay kit (Thermo Fisher Scientific). Immunoblots were performed using JESS Simple Western™ instrument (ProteinSimple®, Bio-Techne), according to the manufacturer's instructions. Primary antibody used are listed in Table S1 and followed by chemiluminescence detection (ProteinSimple®, Bio-Techne). Total proteins were quantify using Protein Normalization™ or RePlex™/Total Protein™ Kit (ProteinSimple®, Bio-Techne). Analysis were performed using Compass software (Protein Simple).

CRISPR/Cas9 Genome Editing

crRNA guides were chosen among predesigned Alt-R CRISPR-Cas9 RNA from Integrated DNA Technologies (IDT) (Table S2). To prepare crRNA-tracrRNA duplex, Alt-R crRNA and Alt-R tracrRNA were reconstituted to 100 µM with Nuclease-Free Duplex Buffer (IDT) and mixed at equimolar concentration, annealed at 95°C for 5 minutes, and slowly cooled down to room temperature. 180pmol of crRNA-tracrRNA duplex and 60pmol of S.p. Cas9 Nuclease V3 (IDT) were gently mixed and incubated at room temperature for 10min. 2×10^6 in vitro activated NK cells were washed in PBS and resuspended in P3 Buffer from P3 Primary Cell 4D-Nucleofector™ X Kit S (Lonza). Cells were then mixed with the RNA duplex/Cas9 RNP and electroporated (nucleofector 4D Amaxa, CM150 program). For multiple KO, several RNA duplex/Cas9 RNP were added to the cells. After electroporation, cells were cultured for 3 days. On the day of analysis, cells were washed 3 times with PBS and incubated for 2h without IL-2, followed by 1h incubation with IL-18.

Affinity Purification of mTOR Complexes

In vitro activated NK cells from MTOR^{OST} mice were washed 3 times with PBS and incubated for 2h without IL-2. Cells were then stimulated with the indicated cytokines for 15min. After stimulation, NK cells were washed with PBS and lysed in lysis buffer (Tris 50 mM pH 7.4, EDTA 0.5 mM, 0.3 % CHAPS, NaCl 137mM, 10 % Glycerol) complemented with cOmplete™ ULTRA Tablets, Mini, EDTA-free, EASYpack Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail 2 et 3 (Sigma) on ice for 10min. Lysates were then centrifuged for 10min at 16,000 g at 4°C. Postnuclear lysates were used for affinity purification. 2mg of protein were incubated with 80µl of prewashed Strep-Tactin Sepharose beads (IBA GmbH) for 1.5h at 4 °C on a rotary wheel. Beads were then washed 3 times with 1ml of lysis buffer, plus 2 times in the absence of detergent and of protease, glycerol and phosphatase inhibitors. Proteins were eluted from the Strep-Tactin Sepharose beads with 2.5 mM biotin.

MS analysis of mTOR complexes

Following affinity purification, protein samples were dried in a speed-vac concentrator and reconstituted in Tris 50 mM buffer, pH 8, containing 1% sodium deoxycholate. Cysteines were reduced and alkylated for 5 min at 95°C with 10mM Tris(2-carboxyethyl)phosphine and 40mM chloroacetamide. Proteins were then captured and washed on hydrophilic, carboxylate-coated paramagnetic beads (Sera-Mag carboxylate-modified magnetic beads, GE Healthcare) and digested with 0.75µg of trypsin as described in (58). Tryptic peptides were dried and resuspended in 17 µl of 2% acetonitrile, 0.05% trifluoroacetic acid, and analyzed by nano-liquid chromatography (LC) coupled to tandem MS, using an UltiMate 3000 system (NCS-3500RS Nano/Cap System; Thermo Fisher Scientific) coupled to an Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). Five microliters of each sample were loaded on a C18 precolumn (300 µm inner diameter × 5 mm, Thermo Fisher Scientific) in a solvent made of 2% acetonitrile and 0.05% trifluoroacetic acid, at a flow rate of 20 µl/min. After 5 min of desalting,

the precolumn was switched online with the analytical C18 column (75 μm inner diameter \times 50 cm, in-house packed with Reprosil C18) equilibrated in 95% solvent A (5% acetonitrile, 0.2% formic acid) and 5% solvent B (80% acetonitrile, 0.2% formic acid). Peptides were eluted using a 5%-50% gradient of solvent B over 60min at a flow rate of 300 nl/min. The mass spectrometer was operated in data-dependent acquisition mode with the Xcalibur software. MS survey scans were acquired with a resolution of 70,000 and an AGC target of $3\text{e}6$. The 10 most intense ions were selected for fragmentation by high-energy collision induced dissociation, and the resulting fragments were analyzed at a resolution of 17500, using an AGC target of $1\text{e}5$ and a maximum fill time of 50ms. Dynamic exclusion was used within 30 s to prevent repetitive selection of the same peptide.

Raw MS files were processed with MaxQuant software (v.1.5.2.8) for database search with the Andromeda search engine and quantitative analysis. Data were searched against *M. musculus* entries of the UniProt KB protein database (release UniProtKB/Swiss-Prot+TrEMBL 2017_01, 89,297 entries including isoforms), plus the One-Strep-tag peptide sequence, and the set of common contaminants provided by MaxQuant. Carbamidomethylation of cysteines was set as a fixed modification, whereas oxidation of methionine, protein N-terminal acetylation, and phosphorylation of serine, threonine and tyrosine were set as variable modifications. Specificity of trypsin digestion was set for cleavage after K or R, and two missed trypsin cleavage sites were allowed. The precursor mass tolerance was set to 20 ppm for the first search and 4.5 ppm for the main Andromeda database search. The mass tolerance in tandem MS mode was set to 0.5 Da. Minimum peptide length was set to seven amino acids, and minimum number of unique or razor peptides was set to one for validation. The I = L option of MaxQuant was enabled to avoid erroneous assignation of undistinguishable peptides belonging to very homologous proteins. Andromeda results were validated by the target decoy approach using a reverse database, with an FDR set at 1% at both peptide spectrum match and protein level. For label-

free relative quantification of the samples, the match between runs option of MaxQuant was enabled with a match time window of 1 min, to allow cross-assignment of MS features detected in the different runs, after alignment of the runs with a time window of 20 min. Protein quantification was based on unique and razor peptides. The minimum ratio count was set to one for label-free quantification calculation, and computation of the iBAQ metric was also enabled.

Data processing and identification of specific mTOR interactors.

From the 'proteinGroups.txt' files generated by MaxQuant with the options described above, protein groups with negative identification scores were filtered out as well as proteins identified as contaminants. In situations where protein groups corresponded to the same gene name, protein intensities in a given sample were summed over the redundant protein groups. Protein intensities were normalized across all samples by the median intensity. Normalized intensities corresponding to different technical replicates were averaged (geometric mean) and missing values were replaced after estimating background binding from WT intensities. For each bait and each condition of stimulation, we used a two-tailed Welch t-test to compare normalized log-transformed protein intensities detected in OST-tagged samples across all biological replicates to WT intensities pooled from all conditions of stimulation. To avoid spurious identification of interactors due to missing value imputation, we repeated this process (missing value imputation followed by a two-tailed Welch t-test) five times and estimated fold changes and P values as their respective average (geometric mean) across all five tests. Specific interactors were identified as preys showing a greater than fourfold enrichment with an p-value below or equal to 0.05 in at least two conditions of stimulation.

Global proteomic analysis of NK cells

Primary NK cells were lysed in 5% SDS, 50mM ammonium bicarbonate and sonicated on a Bioruptor (Diagenode). Proteins were reduced and alkylated for 5 min at 95°C with 10mM

Tris(2-carboxyethyl)phosphine and 40mM chloroacetamide, then digested with trypsin on S-trap micro devices (Protifi) according to the manufacture's protocol. Around 50ng of the resulting peptides were analyzed by nanoLC-MS/MS using an UltiMate 3000 RS nanoLC system (ThermoFisher Scientific) coupled to a TIMS-TOF SCP mass spectrometer (Bruker). Peptides were separated on a C18 Aurora column (25cm x 75µm ID, IonOpticks) using a gradient ramping from 2% to 20% of B in 30 min, then to 37% of B in 3min and to 85% of B in 2min (solvent A: 0.1% FA in H₂O; solvent B: 0.1% FA in acetonitrile), with a flow rate of 150nL/min. MS acquisition was performed in DIA-PASEF mode on the precursor mass range [400-1000] m/z and ion mobility 1/K₀ [0.64-1.37]. The acquisition scheme was composed of 8 consecutive TIMS ramps using an accumulation time of 100ms, with 3 MS/MS acquisition windows of 25 Th for each of them. The resulting cycle time was 0.96s. The collision energy was ramped linearly as a function of the ion mobility from 59 eV at 1/K₀=1.6Vs cm⁻² to 20 eV at 1/K₀=0.6Vs cm⁻². The raw data was searched and quantified with DIA-NN 1.8.1, using a predicted library generated by the software from the Uniprot mouse reference proteome. Validation was performed at 1% precursor and protein FDR. Protein abundance data were extracted from the "report.pg_matrix" DIANN output. Protein entries from the DIANN output were first filtered to eliminate entries from reverse and contaminant databases. In situations where protein groups corresponded to the same gene name, protein intensities in a given sample were summed over the redundant protein groups. Cellular protein abundances were determined from raw intensities using the protein ruler methodology(59). Cellular protein abundances were averaged over biological replicates. Overall, the cellular protein abundance could be estimated for 7,222 protein groups.

Statistical Analysis

Statistical analysis was performed using Prism 10 (Graph-Pad Software) and specific tests are indicated in figures' legends.

Supplementary Materials

Supplementary Materials and Methods: Table S1 and S2.

Supplementary Figures: Fig. S1 to S3

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Author Contributions:

AM and TW came up with the general approach.

LF, AM and TW designed the experiments and LF performed them with the help of KP, LA, NR, AD, ALM, LP, AN, SB, EmB, SD.

EM, PM, RR and BM contributed critical reagents.

EIB, KC, AGdP and RR performed the proteomics analysis.

LF and AM wrote the paper with inputs from TW. All authors critically read the manuscript.

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Data and materials availability: All data are available in the main text of the supplementary materials.

Figures:

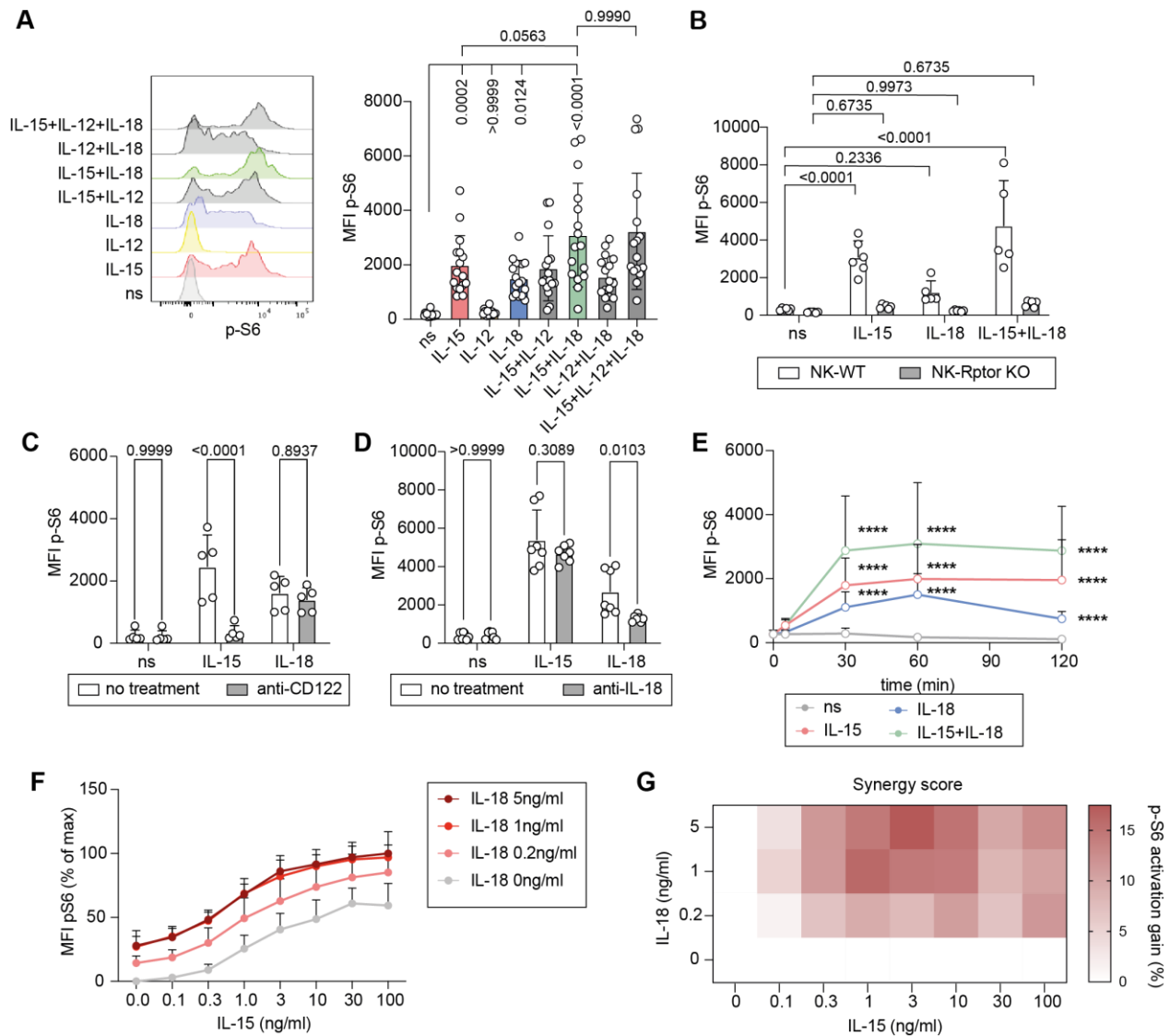


Fig. 1. IL-15 and IL-18 activate mTORC1 synergically in primary NK cells. (A) Splenic NK cells were treated with the indicated combination of cytokines for 1h (ns: no stimulation). Phosphorylation (p-) of S6 (Ser 235-236) was measured by flow cytometry. A representative histogram overlay is shown on the left and the mean (\pm SD) of all experiments is shown on the right (n=17 mice). (B) p-S6 in NK cell from NK-WT and NK-Rptor KO mice treated for 1h with IL-15/IL-18 (n=5/6 mice). (C) p-S6 in NK cell treated for 1h with anti-CD122 antibody and IL-15/IL-18 (n=5 mice). (D) p-S6 in NK cells treated for 1h with IL-15/IL-18 with or w/o anti-IL-18 antibody (n=7 mice). (E) Kinetic of p-S6 in NK cells following IL-15/IL-18

stimulation (n=17mice). (F) p-S6 after 1h of culture with the indicated concentrations of IL-15 and IL-18. (G) A synergic score was calculated from the data present in F. Synergy is defined here as the difference between IL-15/IL-18 combined effect and the sum of IL-15 and IL-18 taken separately, and expressed as p-S6 activation gain upon combined treatment (n=10 mice). Data are displayed as mean \pm SD with individual mice indicated. P-values were calculated using one-way ANOVA with Šídák's multiple comparisons test (A) or two-way ANOVA followed by Dunnett's multiple comparisons test compared to ns (B and E) or Šídák's multiple comparisons test (C-D). **** p<0.0001.

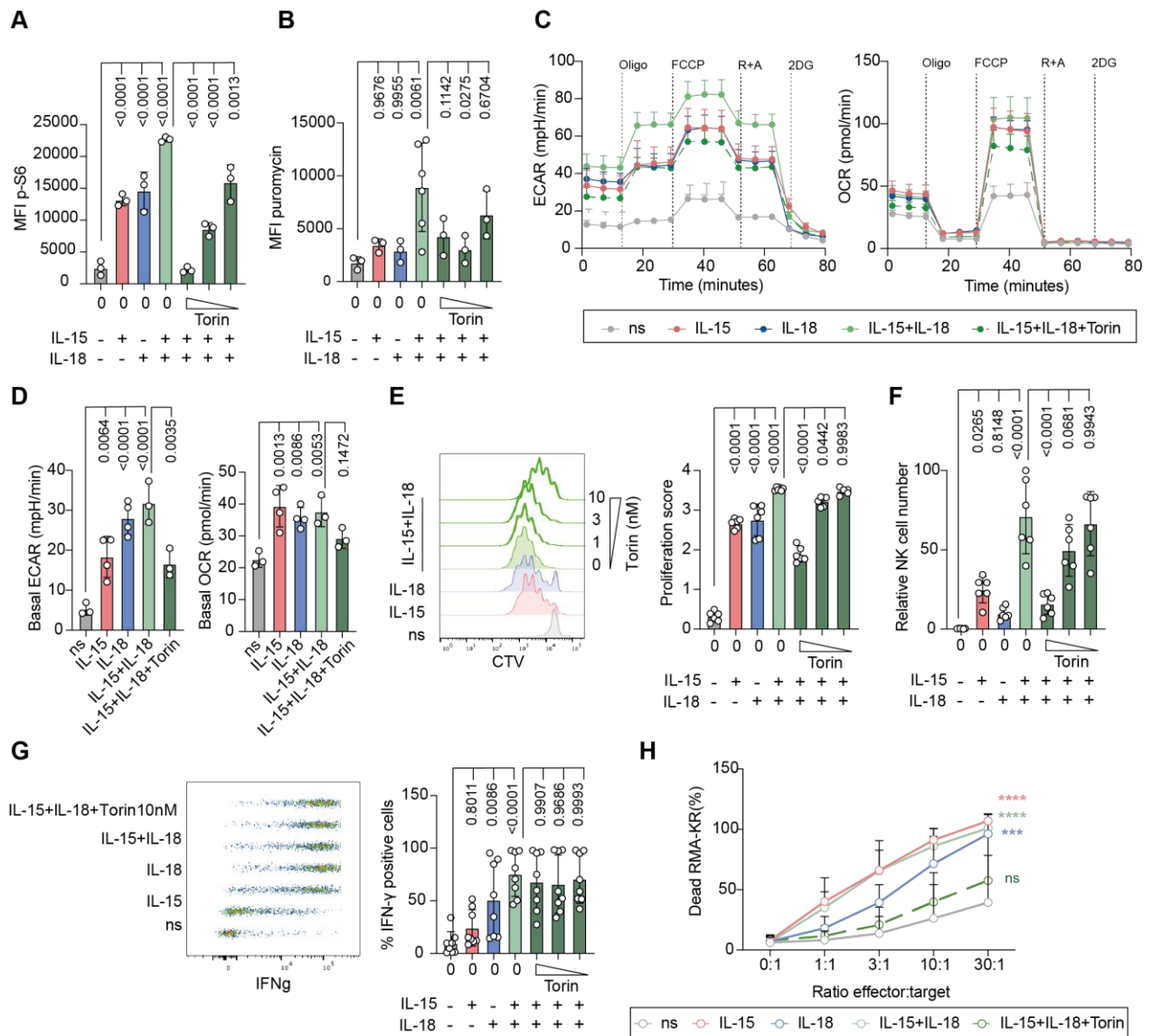


Fig. 2. IL-15 and IL-18 induce NK cells proliferation, metabolism and function which are in part dependent on mTORC1. (A) Splenic murine NK cells were purified and stimulated 16h with IL-15 (100ng/ml) and IL-18(5ng/ml) and increasing doses of Torin (1nM, 3nM, 10nM). p-S6 in NK cells was measured by flow cytometry (n=3 mice). (B) Puromycin incorporation after 16h of treatment with IL-15/IL-18, +/- increasing doses of Torin as indicated (n=3 mice). (C) Splenic murine NK cells were purified and stimulated 16h with IL-15/ IL-18 and 10nM of Torin. After 16h, NK cells were sorted and their ECAR and OCR were measured using Seahorse technology. (D) Basal ECAR and OCR calculated from C is shown (n=3-4 mice). (E-F) Splenic NK cells were labeled with CTV and stimulated with the indicated

cytokines +/- increasing doses of Torin for 3 days (n=6 mice). (E) A representative histogram overlay and the calculated proliferation scores are shown. (F) NK cell numbers are indicated. (G) Percentage of IFN- γ + NK cells after 16h of treatment with IL-15/IL-18 +/-Torin (n=8 mice). (H) Splenic NK cells were stimulated with IL-15/IL-18 +/- Torin for 16h and then co-cultured with RMA-KR target cells for 4h at the indicated effector/target (E/T) ratio. The percentage of dead RMA-KR for different ratios of effector/target is indicated (n=4 mice). Data are displayed as mean \pm SD with individual mice indicated. P-values were calculated compared to the condition IL-15+IL-18 without Torin or ns, using one-way ANOVA with Šídák's multiple comparisons test (A-G) or two-way ANOVA with Dunnett's multiple comparisons test compared to ns (H). *** p<0.001, **** p<0.0001.

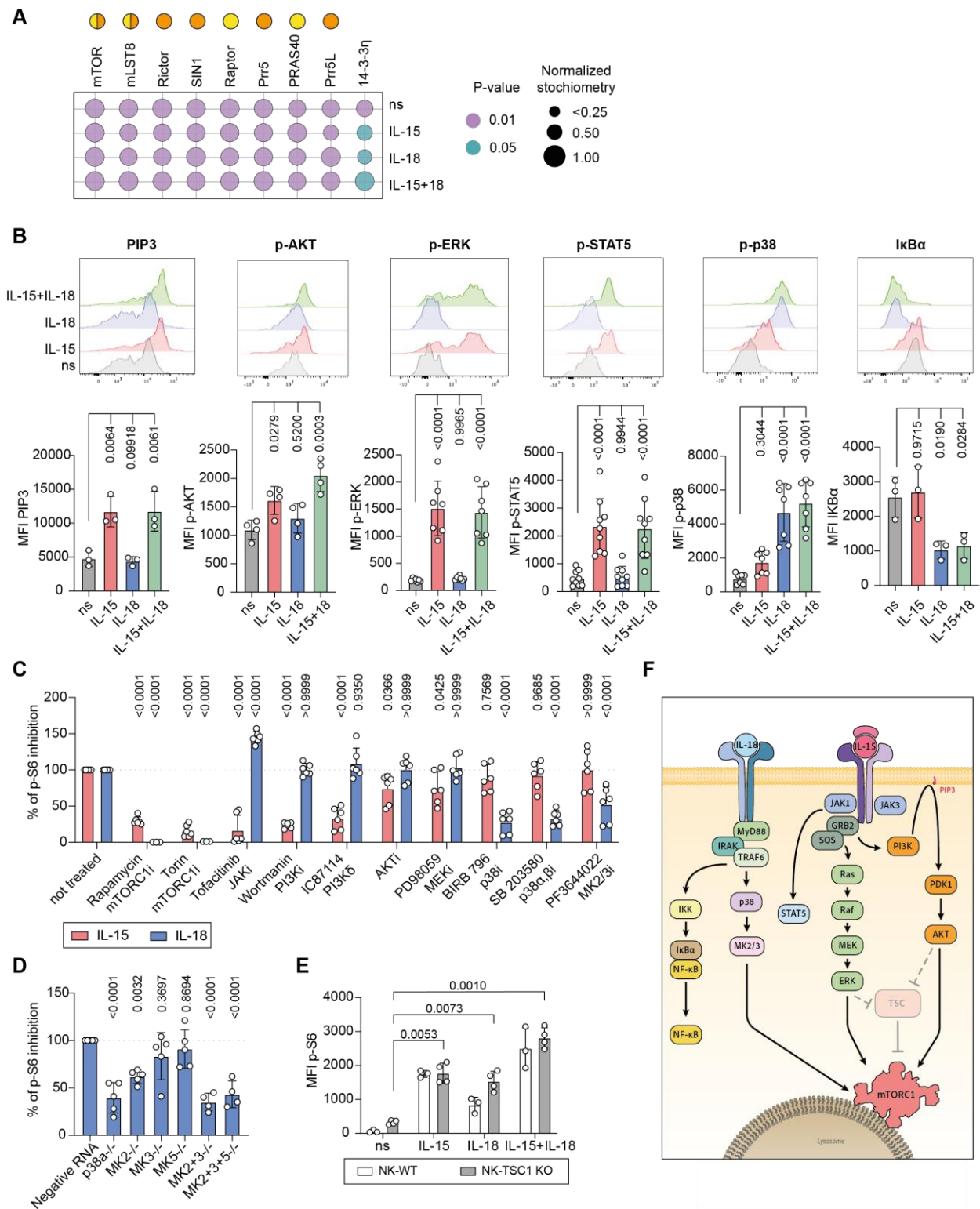


Fig. 3. IL-15 and IL-18 activate mTORC1 through non-canonical pathways. (A) Dot plot showing the interaction stoichiometry of proteins significantly associated with $MTOR^{OST}$ in non-stimulated (ns) or stimulated NK cells for 15min with IL-15/IL-18. Yellow and orange dots indicate proteins belonging to mTORC1 and mTORC2 complexes respectively (n=4

independent experiments). **(B)** Splenic murine NK cells were treated with the indicated combination of cytokines. The phosphorylation status of AKT (T308), ERK1/2 (T202/Y204), STAT5 (Y694), p38 (T180/Y182) or the PIP3 and I κ B α levels were assessed by flow cytometry at 5min (PIP3, p-ERK) or 30min (p-AKT, p-STAT5, p-p38, I κ B α) (n=3-7 mice). **(C)** Splenic NK cells were treated for 1h with IL-15 or IL-18 and the indicated inhibitor. Relative p-S6 MFI compared to the condition without inhibitor is indicated (n=6 mice). **(D)** NK cells KO for the indicated proteins were generated using CRISPR-Cas9 and treated with IL-18 for 1h. Level of p-S6 compared to control guide is indicated (n=5 mice). **(E)** p-S6 in splenic NK cell from NK-WT and NK-TSC1 KO mice treated for 1h with IL-15/IL-18 (n=3/4 mice). **(F)** Summary of mTORC1 activation in primary NK cells by IL-15 and IL-18. Data are displayed as mean \pm SD with individual mice indicated. P-values were calculated using one-way ANOVA followed by Dunnett's multiple comparisons test compared to ns condition (B and D) or two-way ANOVA with Dunnett's multiple comparisons test (C and E).

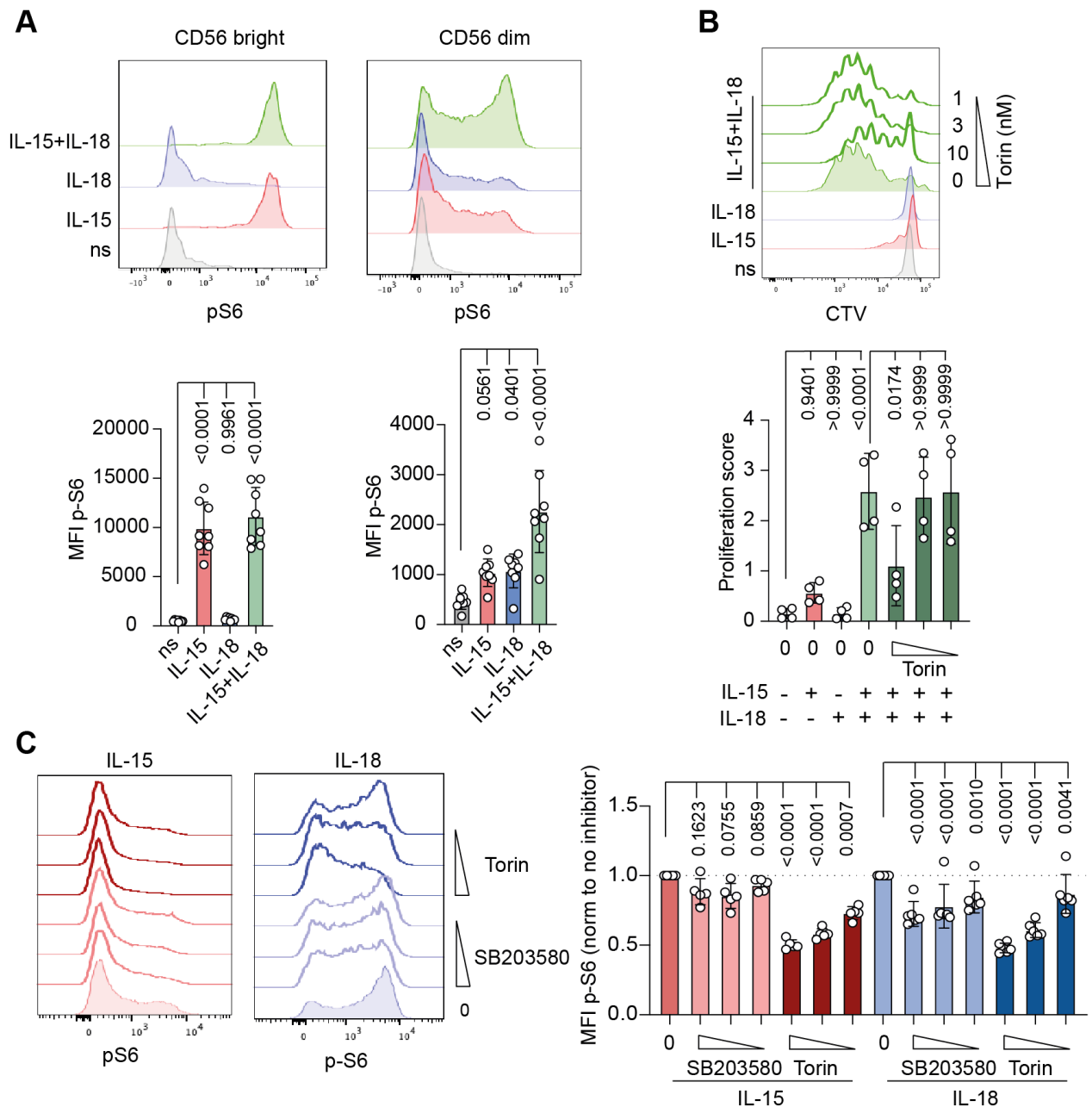


Fig. 4. p38 is required for human CD56^{Dim} NK cell activation of mTORC1 by IL-18. (A) CD56^{bright} and CD56^{Dim} NK cells from the peripheral blood of healthy donors were treated for 1h with IL-15/IL-18 and p-S6 was measured (n=8 healthy donors). **(B)** Purified human peripheral blood NK cells were labeled with CTV and treated for 5 days with IL-15/IL-18 in the presence of Torin at the indicated dose. A representative histogram overlay and proliferation scores are shown (n=4 healthy donors). **(C)** Peripheral blood NK cells from healthy donor were treated for 1h with IL-15 or IL-18 in the presence or the absence of Torin. p-S6 level in CD56^{Dim}

NK cells is shown (n=5 healthy donors). Data are displayed as mean \pm SD with individual mice indicated. P-values were calculating using one-way ANOVA with Dunnett's multiple comparisons test compared to ns (A), or with Šídák's multiple comparisons test (B), and two-way ANOVA with Dunnett's multiple comparisons test compared to ns (C).

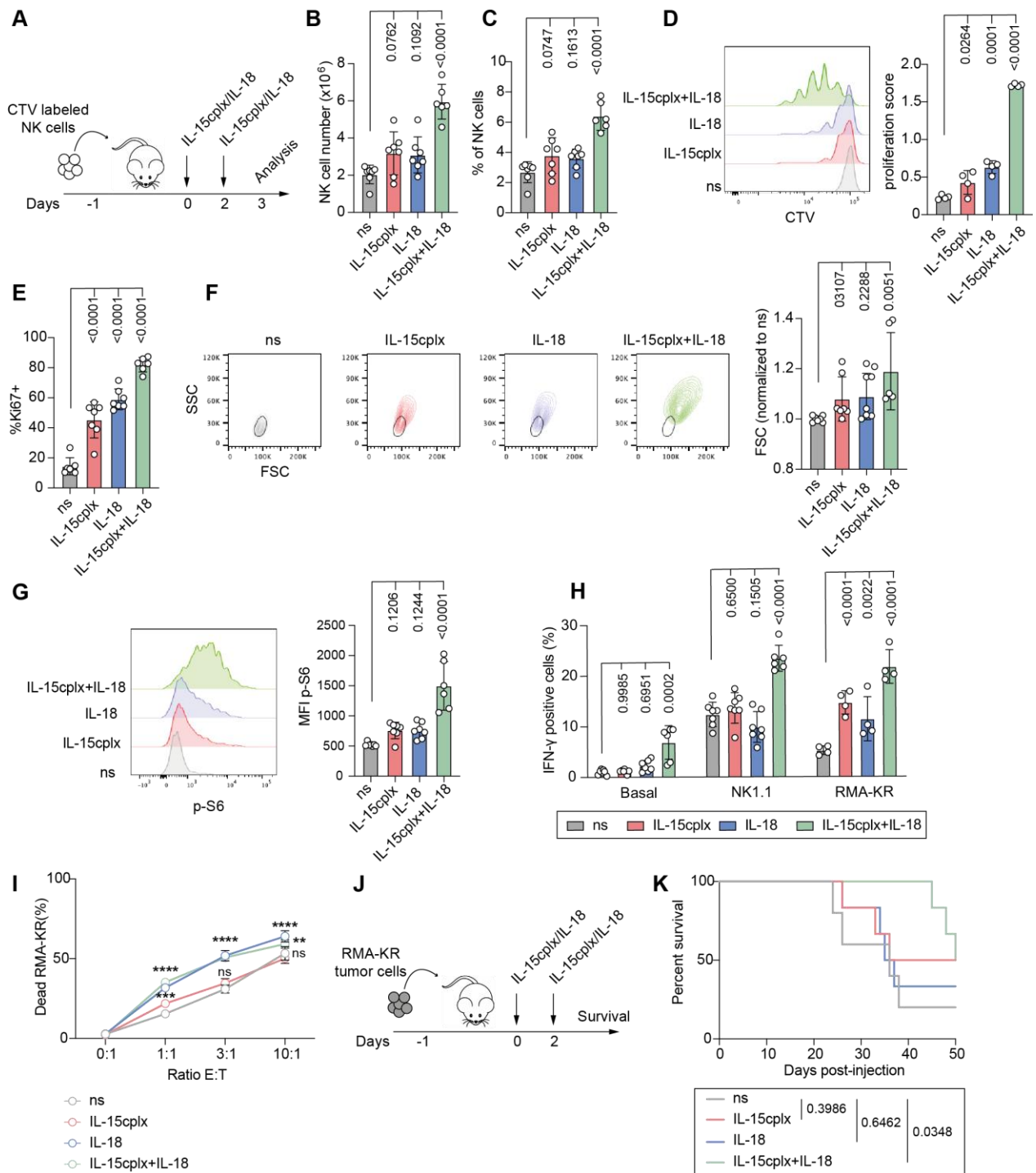


Fig. 5. IL-15 and IL-18 co-injection enhances NK cell proliferation and anti-tumor function in vivo. (A) Experimental design. Mice received CTV labeled NK cells and were treated with HBSS, IL-15/IL-15R α complex (IL-15cplx), IL-18 or IL-15/IL-15R α complex+IL-18 the following day (day 0) and two days later (day 2). Analyses were performed at day 3. (B) NK cell number and (C) frequency in the spleen is shown. (D) A representative

histogram overlay showing CTV dilution in CTV labeled NK cells and proliferation scores are shown. (E) Percentage of Ki67+ NK cells are shown. (F) FSC and SSC analysis of splenic NK cells of the indicated mice were measured. Representative FACS-plots and the mean (\pm SD) are shown. The dark gate on the plot represents the position of non-treated control NK cells. (G) *Ex vivo* S6 phosphorylation level in splenic NK cells (representative histogram overlay and quantification) is shown. (H) Splenic NK cells from mice treated as indicated were co-cultured for 4h with media (basal), NK1.1 or RMA-KR cells. The percentage of IFN- γ + NK cells was measured and is shown. (I) Splenic NK cells from the mice treated as indicated were co-cultured with RMA-KR for 4h. The percentage of dead RMA-KR for the different ratios of effector/target is indicated. (J) Experimental design. Mice were injected with RMA-KR cells and then treated with HBSS, IL-15/IL-15R α complex, IL-18 or IL-15/IL-R α complex+IL-18 the following day (day 0) and two days later (day 2) (K). The survival curves of these mice are presented. Data are displayed as mean \pm SD from n=7 mice/group from two independent experiments (A-I), and from 5-6 mice/group (J-K). P-values were calculated compared to ns using one-way ANOVA with Dunnett's multiple comparisons test (B-G), two-way ANOVA with Dunnett's multiple comparisons test (H-I) or Gehan-Breslow-Wilcoxon test (K). **p<0.01, *** p<0.001, **** p<0.0001.

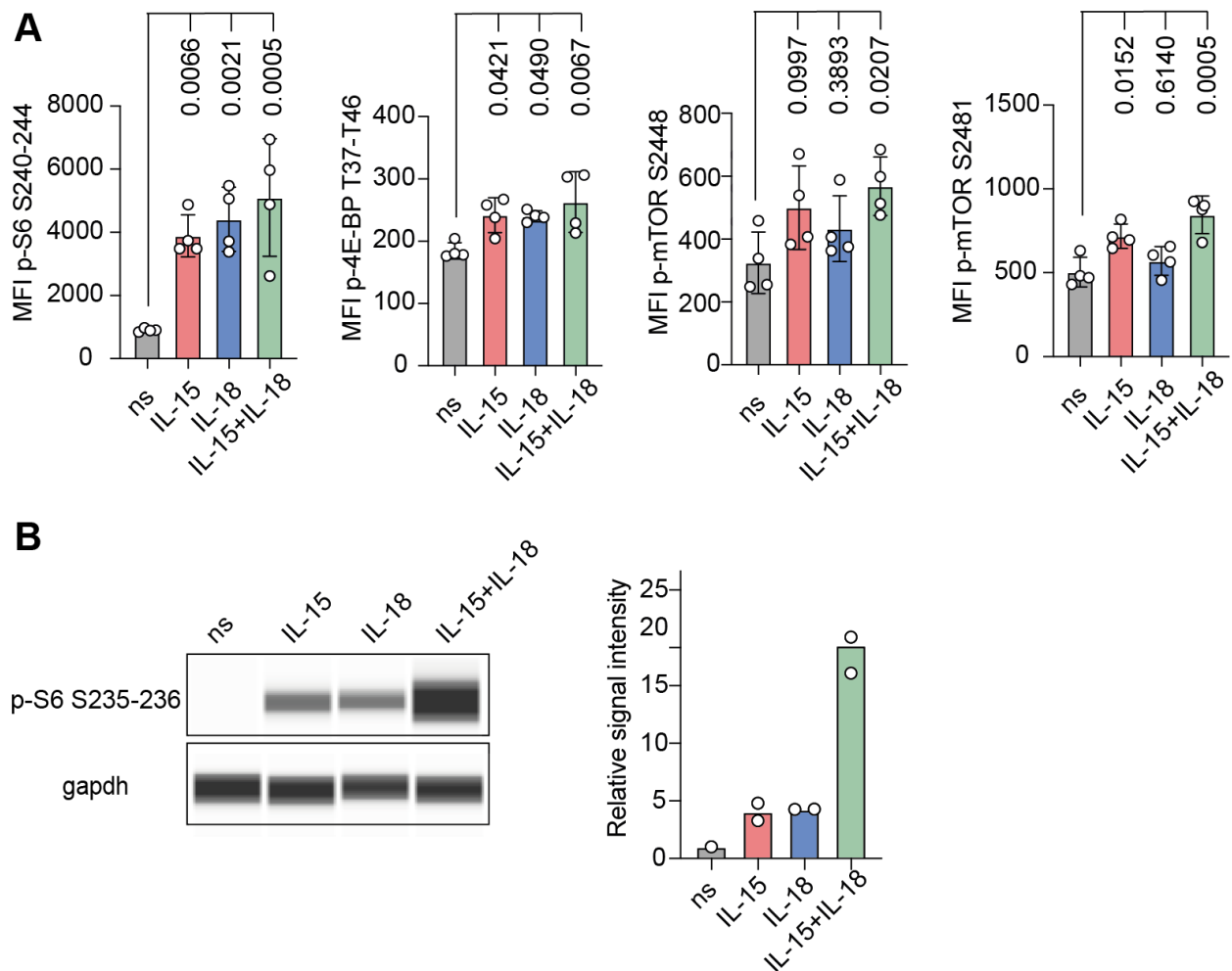


Fig. S1. mTORC1 activation following IL-15 and IL-18 treatment. (A) Phosphorylation of the indicated protein in splenic NK cells treated with the indicated combination of cytokines for 1h (ns: no stimulation). Data are displayed as mean \pm SD from n=3 mice with individual mice indicated. P-values were calculated using one-way ANOVA with Dunnett's multiple comparisons test, compared to ns. (B) p-S6 Ser235-236 in IL-2 expanded NK cells treated for 1h with the indicated cytokines measured using capillary western blot (ProteinSimple, Bio-Techne). A blot representative of 2 independent experiments and quantification.

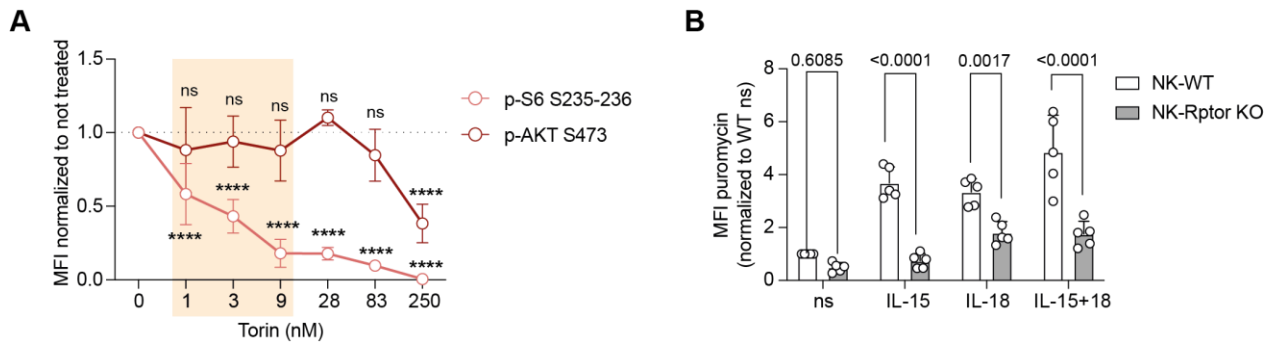


Fig. S2. Impact of Torin treatment on mTOR activity and Raptor deletion on translation.

(A) Splenic NK cells were treated with IL-15 and increasing doses of Torin. p-S6 S235-236 and p-AKT S473, as a readout of mTORC1 and mTORC2 activity respectively, were measured by flow cytometry. Concentrations of Torin below 10nM were selected for the rest of the study as indicated (n=6 mice). (B) Puromycin incorporation after 16h of treatment with IL-15/IL-18 in NK cells from NK-WT and NK-Raptor KO mice (n=5 mice). Data are displayed as mean \pm SD with individual mice indicated. P-values were calculated using two-way ANOVA with Dunnett's multiple comparisons test (A) or Šídák's multiple comparisons test (B). **** p<0.0001.

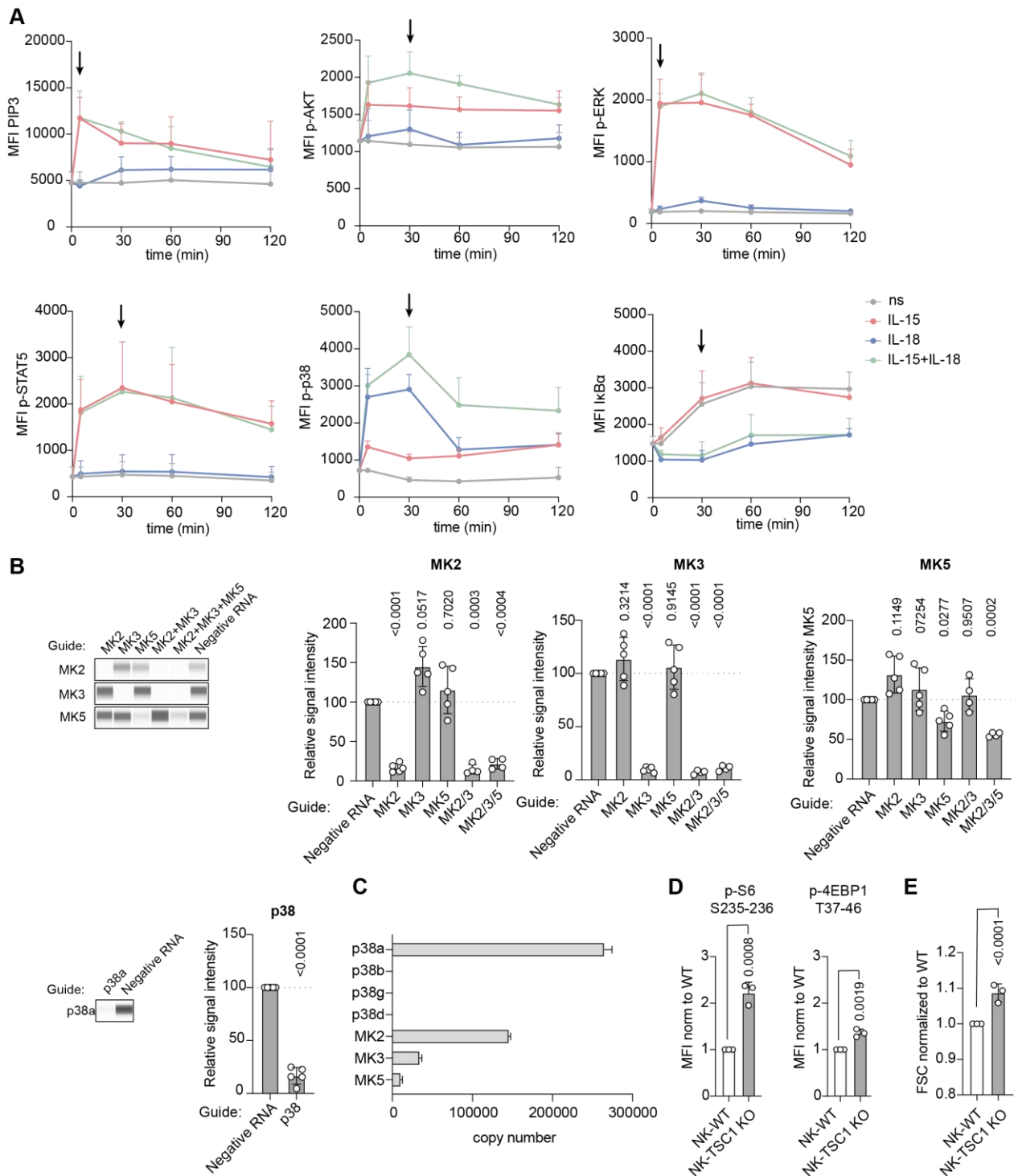


Fig. S3. Analysis of signaling pathways downstream IL-15 and IL-18. (A) Kinetic of the phosphorylation status of AKT (T308), ERK1/2 (T202/Y204), STAT5 (Y694), p38 (T180/Y182), PIP3 and $\text{I}\kappa\text{B}\alpha$ level in splenic murine NK cells treated with the indicated combination of cytokines. Arrows indicated the time point showed in Figure 3 ($n=3-7$ mice). **(B)** Expression of MK2, MK3, MK5 and p38 in NK cells electroporated with crRNA targeting

the indicated proteins. Measures were performed using capillary western blot. A representative blot and quantification from 5 independent experiments are presented. (C) Copy numbers per cell of indicated proteins, extracted from total proteomes of murine splenic NK cells quantified by MS analysis (n=3 mice). (D) Basal phosphorylation level of the indicated protein in splenic NK cells from $Ncr1^{iCre}$ (NK-WT) and $Ncr1^{iCre+/-} \times Tsc1^{lox/lox}$ (NK-TSC1 KO). (E) FSC of splenic NK cells from the indicated genotype (n=3 mice). Data are displayed as mean \pm SD with individual mice indicated. P-values were calculated using one-way ANOVA with Dunnett's multiple comparisons test compared to Negative RNA (B) or t-test (B (p38) and D-E).

Table S1. List of the antibodies used in this study.

Specificity	Fluorophore	Dilutions	Used	Suppliers	Clones	References	Used against
CD56	BUV737	1/200	Flow cytometry	BD	NCAM16.2	564447	Human
CD3	BUV496	1/50	Flow cytometry	BD	UCHT1	564810	Human
CD3	FITC	1/100	Flow cytometry	Biologend	145-2C11	100306	Mouse
CD19	FITC	1/100	Flow cytometry	BD	1D3	553785	Mouse
NK1.1	APC	1/100	Flow cytometry	BD	PK136	550627	Mouse
CD49b	APC	1/100	Flow cytometry	Biologend	DX5	108910	Mouse
NKp46	AF647	1/100	Flow cytometry	BioLegend	29A1.4	137628	Mouse
CD3	BV605	1/100	Flow cytometry	BD	145-2C11	563004	Mouse
CD19	BV605	1/100	Flow cytometry	BD	1D3	563148	Mouse
NKp46	PerCP-eF710	1/100	Flow cytometry	eBioscience	29A1.4	46-3351-80	Mouse
CD49b	PerCP-eF710	1/100	Flow cytometry	eBioscience	DX5	46-5971-82	Mouse
IFN γ	PE-Dazzle594	1/300	Flow cytometry	BioLegend	XMG1.2	505845	Mouse
CD107a	eF660	1/100	Flow cytometry	eBioscience	eBio1D4B	50-1071-82	Mouse
NK1.1	BV650	1/100	Flow cytometry	BD	PK136	564143	Mouse
CD49a	PE	1/400	Flow cytometry	BD	Ha31/8	562115	Mouse
CD27	BUV737	1/400	Flow cytometry	BD	LG.3A10	612831	Mouse
CD11b	PE-Dazzle594	1/800	Flow cytometry	Biologend	M1/70	101255	Mouse
KLRG1	BUV395	1/100	Flow cytometry	BD	2F1	740279	Mouse
KI-67	PerCP-eF710	1/800	Flow cytometry	eBioscience	soIA15	46-5698	Mouse
CD3	APC-Cy7	1/100	Flow cytometry	Biologend	17A2	100222	Mouse
CD19	APCeF780	1/100	Flow cytometry	e-bioscience	eBio1D3	7-0193	Mouse
puromycin	AF647	1/400	Flow cytometry	Millipore	12D10	MABE343-AF647	Mouse
Phospho-S6 ^{S235-236}	PE	1/400	Flow cytometry	CST	D57.2.2E	5316	Mouse/Human
Phospho-STAT5 ^{Y694}	AF488	1/40	Flow cytometry	BD	47/stat5(pY694)	562075	Mouse
Phospho-STAT5 ^{Y694}	PE-CF594	1/100	Flow cytometry	BD	47/stat5(pY694)	562501	Mouse
Phospho-p44/42 ^{MAPK/Erk1/2/T202/Y204}	PE	1/50	Flow cytometry	CST	197G2	14095	Mouse
Phospho-S6 ^{S240/244}	PE	1/400	Flow cytometry	CST	D68F8	14236	Mouse
Phospho-4E-BP1 ^{T37/T46}	PE	1/50	Flow cytometry	CST	236B4	7547	Mouse
Phospho-mTOR ^{S2448}	Purified		Flow cytometry	CST	D9C2	5536	Mouse
Phospho-mTOR ^{S2481}	Purified		Flow cytometry	CST	polyclonal	2974	Mouse
Phospho-Akt ^{T308}	Purified	1/400	Flow cytometry	CST	C31E5E	2965	Mouse
Phospho-p38 ^{MAPK/Thr180/Tyr182}	Purified	1/200	Flow cytometry	CST	D3F9	4511	Mouse
PtdIns(3,4,5)P3	Purified	1/50	Flow cytometry	echelonBiosciences		Z-P345B	Mouse
I κ B α	Purified	1/400	Flow cytometry	CST	L35A5	4814	Mouse
Anti-Rabbit	PE	1/50	Flow cytometry	JacksonImmunoResearch	polyclonal	711-116-152	Mouse
Anti-Mouse α G1	PE-CF594	1/400	Flow cytometry	BD	A85-1	562559	Mouse
Phospho-S6 ^{RibosomalProtein/S235/S236}	Purified	1/100	Immunoblot	CST	D57.2.2E	4858	Rabbit
Gapdh	Purified	1/1000	Immunoblot	CST	4C10	2118	Rabbit
P38 α	Purified	5 μ g/ml	Immunoblot	R&D	polyclonal	AF8691	Rabbit
MAPKAPK-2	Purified	1/25	Immunoblot	CST	polyclonal	3042	Rabbit
MAPKAPK-3	Purified	1/50	Immunoblot	CST	D54E4	7421	Rabbit
PRAK	Purified	1/50	Immunoblot	SantaCruzBiotechnology	A-7	46667	Mouse

Table S2. List of the crRNA guides used in this study and their sequence.

Name	Sequence
Mm.Cas9.MAPKAPK2.1.AB	CGCCATCACCGACGACTACA
Mm.Cas9.MAPKAPK3.1.AA	ACGGGAACCCAAGAAGTACG
Mm.Cas9.MAPKAPK5.1.AB	CAGTGCAAACCGTTCTTGAG
Mm.Cas9.MAPK14.1.AF	ATTCGGCTGACATAATTCAC
Negative Control crRNA #1 (IDT 1072544)	

PART III : DISCUSSION AND COMPLEMENTARY RESULTS

This PhD work was focused on the mechanisms underlying mTOR activation in primary NK cells by cytokines. We show that IL-15 and IL-18, two positive regulators of NK cells, synergistically activate mTORC1 in both murine and human primary NK cells. IL-15 and IL-18 activation of mTORC1 does not rely on the canonical inhibition of the major mTORC1 inhibitor TSC, in contrast to what has been extensively reported in various cell lines and primary cell types (H. Zhang et al. 2003; Dunkerly-Eyring et al. 2022; Menon et al. 2012; Bentzinger et al. 2013; Alesi et al. 2024). We show that mTORC1 activation by IL-15 relies on PI3K-AKT and ERK pathway, whereas IL-18 signals through a unique pathway depending on the MAPK p38. IL-15 and IL-18 combined treatment *in vitro* results in NK cell proliferation, metabolism and translation activation which are in part mTORC1 dependent. *In vivo*, IL-15 and IL-18 co-injection increases NK cell number and functionality which led to better tumor control in a mouse lymphoma model.

In this part, I will first discuss our findings on the signaling pathways and the molecular mechanisms leading to mTORC1 activation in primary NK cells. I will then comment on the importance of mTORC1 downstream IL-15 and IL-18 in NK cell priming *in vitro* and *in vivo*.

I. IL-15 and IL-18 activate complementary pathways in primary NK cells

1. mTORC1 activation downstream IL-15

Our results indicate the importance of PI3K, and in particular PI3K δ downstream IL-15 for mTORC1 activation. As mentioned previously, NK cell deficiency in p110 δ alone or in combination with p110 γ , results in a decrease in NK cell number and a defect in NK cell maturation (Tassi et al. 2007; H. Guo et al. 2008; N. Kim et al. 2007). This phenotype is reminiscent of the one of mTOR deficient NK cells, although mTOR activation status was not assessed in these studies. In addition, another group showed that NK cells deficient in PDK1, the kinase directly downstream PI3K and upstream AKT, display a decrease in S6 phosphorylation following IL-15 stimulation (M. Yang et al. 2015; J. He et al. 2019), therefore confirming the importance of the PI3K/PDK1 module to mediate mTORC1 activation downstream IL-15. We also observed that AKT inhibition reduces mTORC1 activation downstream IL-15, although to a lesser extent than PI3K δ inhibition, suggesting the existence of at least another parallel pathway. In this sense, we show that ERK pathway inhibition also alters S6 phosphorylation following IL-15 stimulation. This redundancy participates in the signaling robustness and may also explain that earlier study did not observed any impact of AKT inhibition on p-S6 following IL-2/IL-12 stimulation *in vitro* (Loftus et al. 2018). In accordance with our findings, several other groups have reported that AKT or ERK inhibition negatively affect IL-15 priming in both human and murine NK cell, corroborating our findings (Nandagopal et al. 2014; Wagner et al. 2017; Niogret et al. 2019). Whether the pathway originating from PI3K is strictly parallel to ERK signaling or whether PI3K could also be upstream of ERK activation is also unclear. Indeed, PI3K-AKT and ERK pathway are classically presented as parallel pathways (L. Ma et al. 2005; Winter, Jefferson, et Kimball 2011) although a previous study

indicates that PI3K acts upstream ERK in NK cells following target cell recognition (K. Jiang et al. 2000). The impact of combined treatment with both AKT and ERK inhibitors on p-S6 also remained to be tested. Finally, our analysis of IL-15 signaling is mainly based on the use of pharmacological inhibitors which present different drawbacks such as off-target activity at high doses or on the contrary incomplete target inhibition. Unfortunately, our use of CRISPR-Cas9 approach was limited regarding analysis of the IL-15 signaling pathway since NK cells required to be expanded and maintained in IL-2, a cytokine that shares its signaling with IL-15.

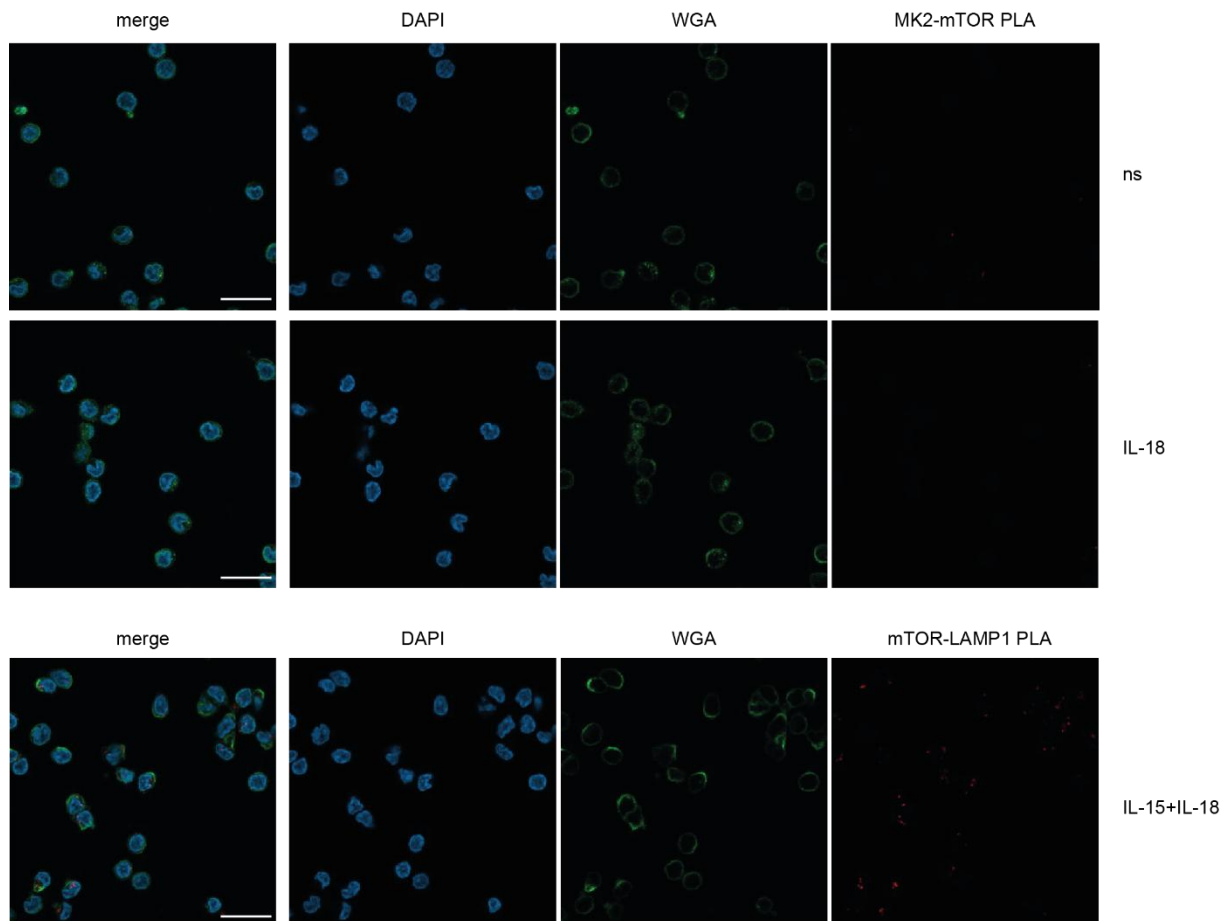
2. mTORC1 activation downstream IL-18

We also show that mTORC1 activation following IL-18 stimulation is not impaired by the inhibition of any of the classic mTORC1 upstream regulators such as PI3K, AKT or ERK. On the contrary, mTORC1 activation by IL-18 is severely impacted by the inhibition of the MAPK p38, a classic component of IL-18 signaling but whose role on mTORC1 regulation is just starting to emerge. p38 α is the most expressed isoform of p38 in eukaryotic cells in general and in NK cells in particular. Accordingly, our data indicate that deficiency in p38 α in particular greatly reduce p-S6 following IL-18 stimulation. p38 kinases are classically considered as stress-activated protein kinases, as they are activated by a large set of stimuli, including oxidative stress, chemical stress, inflammation or cytokines. They are highly versatile proteins that have been shown to be involved in numerous biological process, including stress response, cell cycle, immune response, or cell differentiation (Canovas et Nebreda 2021). Indeed, a multitude of protein potentially phosphorylated by p38 have been reported and p38 α alone has been shown to directly phosphorylate over 100 different proteins (Trempelec, Dave-Coll, et Nebreda 2013). Among them, the kinase MK2 and MK3 are consider as *bona fide* substrates of p38. NK cells mainly express MK2, and we show that MK2 along with MK3 participates in mTORC1 activation by IL-18.

p38 has long been consider as a cellular stress response kinase, and therefore its activating role on mTORC1 signaling was quite surprising. Few studies have reported regulation of mTORC1 by the p38 pathway, with both activating (Yong Li et al. 2003; Cully et al. 2010; X.-N. Wu et al. 2011; Linares et al. 2015) and inhibitory role for p38 (M. Zheng et al. 2011). Several reports are indeed in favor of the context-dependency of p38 signaling which may explained this dual activating and inhibitory roles on mTORC1, depending on the stimulus and the cell type. Indeed, different stimuli are likely to engage different signaling pathways which could modulate the potential substrate and regulators of p38. In this sense, IL-18 activates JNK which activity has been shown to be a source of heterogeneity in p38 response (Miura et al. 2018). Different stimuli such as IL-18, could also engage different sets of MAP3Ks and MAP2Ks which could modulate p38 activation level and post-translational modifications. The cellular context has also been shown to influence p38 signaling. In particular a study showed that p38 inhibitor have opposite effect on mTORC1 activation in colorectal cancer cells depending on their level of Protein phosphatase 2A C expression (Y. Zhang et al. 2015). Therefore the specific stimulus and cell type investigated may explain the apparently contradictory results observed in the studies deciphering mTOR regulation by p38. Of particular relevance, a study indicates

that p38 α / β or MK2/3 deficiency in CD4⁺ T cells resulted in a decrease in p-S6 following TCR stimulation, suggesting a decrease in mTORC1 activity (M. Hayakawa et al. 2017). This was also associated with an enhanced CD4⁺ T cell capacity to differentiate into Treg, a phenotype similar to the phenotype described for mTOR deficient CD4⁺ T cells (M. Hayakawa et al. 2017). Therefore, these results are in favor of an underappreciated role of p38-MK2/3 signaling in mTORC1 regulation in primary lymphocytes.

Our analysis also indicates that MK2, and MK3 to a lesser extent, mediates p38 α activation of mTORC1 downstream IL-18. Classically, MK2 has been shown to bind to p38 α at steady state and to be phosphorylate by p38 α upon activation. Phosphorylated MK2 leads to p38 α /MK2 translocation to the cytoplasm, where MK2 can activate its downstream targets (Ben-Levy et al. 1998; Engel, Kotlyarov, et Gaestel 1998; White et al. 2007). Whether IL-18 promotes this translocation in NK cell remains to be tested. We did test however the potential recruitment of mTOR by MK2. Indeed, mTOR sequence analysis reveals a consensus MK2 phosphorylation site on at serine 1893, suggesting that MK2 may potentially phosphorylate mTOR directly. This phosphorylation of mTOR has also been reported in a study of tissue response to tumor hypoxia, suggesting that this site may be accessible for MK2 (Mertins et al. 2014). In order to identify a potential direct interaction between MK2 and mTORC1, we performed a proximity ligation assay (PLA) between MK2 and mTOR using confocal microscopy (**Extended Figure 1**). Our analysis indicates no proximity between these two molecules under cytokine stimulation. As a positive control, we observed PLA signal between mTOR and the lysosome marker LAMP-1 upon stimulation validating the technique in our hands. This result suggest the existence of other intermediates between MK2 and mTOR, that remains to be identified.



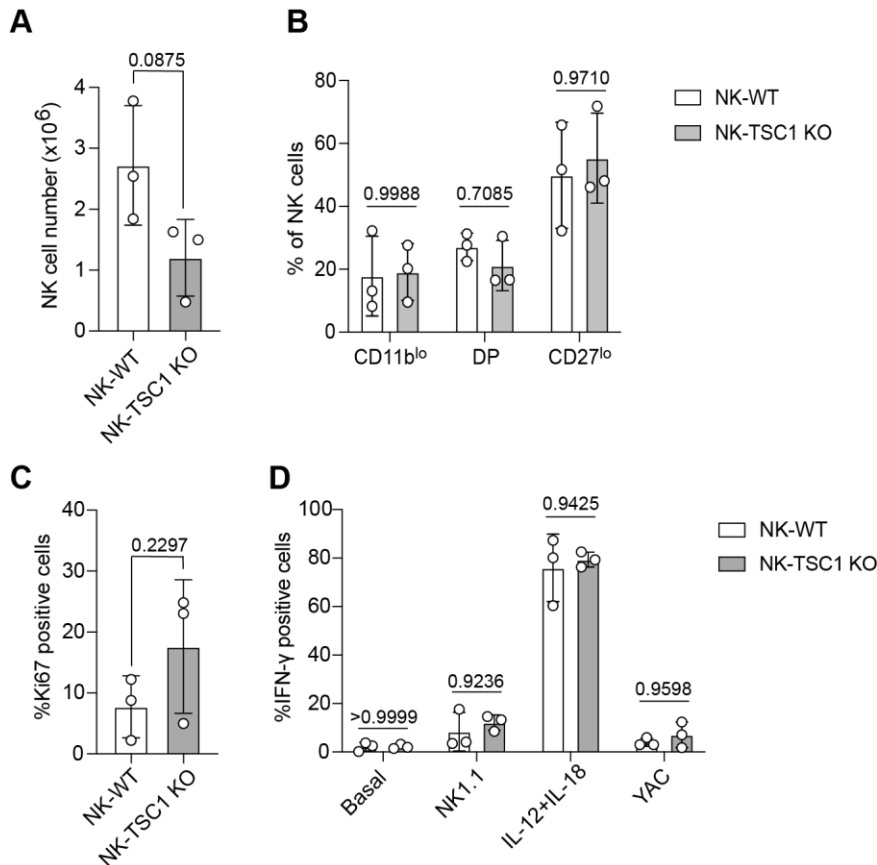
Extended Figure 1: Proximity Ligation Assay (PLA) between mTOR and MK2. (A) Human NK cells were stimulated for 30min with the indicated cytokines. Cells were then stained using Naveniflex kit (Naveni) according to manufacturer's instructions. Nuclei were stained with DAPI and cytoplasmic membrane with Wheat Germ Agglutinin (WGA). (B) As a control, PLA between mTOR and the lysosomal marker LAMP-1 is shown. Scale bar: 20 μ m. Image representative from two independent experiments.

3. IL-15 and IL-18 activation of mTORC1 is TSC-independent

3.1. Phenotype resulting from TSC deletion in NK cells

We show that IL-15 and IL-18 activation of mTORC1 in NK cell is largely independent of the canonical TSC inhibition. This result was surprising given the major role ascribed to TSC in mTORC1 regulation. Indeed in cell lines, TSC deficiency is associated with maximal mTORC1 activity, to the same extent as insulin-treated conditions (H. Zhang et al. 2003; Dunkerly-Eyring et al. 2022). Similarly TSC deletion in various primary cells such as muscle cells (Bentzinger et al. 2013), hepatocytes (Menon et al. 2012), or kidney cells (Alesi et al. 2024), results in increase mTORC1 activity. On the contrary we observed only a minor increase in mTORC1 activity at basal state in TSC1 deficient NK cell, far below the level of p-S6 measure after IL-15 or IL-18 treatment. Similarly, we and other did not observe any phenotype in mice bearing TSC deficient NK cells at steady state (M. Yang et al. 2016) (**Extended Figure 2**). Mice deficient in TSC1 in NK cells display the same number of NK cell in the spleen, the same maturation profile, KI67 expression and IFN- γ production in response to the stimuli tested. In contrast, TSC deletion has been

associated with severe phenotype in other cell types: for instances TSC1 deletion in hepatocyte results in hepatocellular carcinoma (Menon et al. 2012) while TSC2 deletion in kidney is associated with severe cystic kidney disease (Alesi et al. 2024). Moreover in human TSC1/2 mutation is linked to the TSC syndrome, characterized by the development of non-cancerous-tumor in various organ, in particular in the brains, skin, heart, lungs and kidneys (Henske et al. 2016). As mentioned in introduction, in T cells, loss of TSC is associated with quiescence exit and increased apoptosis, a rather mild phenotype compared to the one observed in hepatocyte or kidney but still more severe than in NK cells.



Extended Figure 2: Phenotype of $NCR1^{Cre} \times TSC1^{lox/lox}$ mice (NK-TSC1 KO). (A) NK cell numbers in the spleen of the indicated mice were measured. (B) Percentage of CD11b^{lo}, double positive (DP) and CD27^{lo} in each genotype is indicated. (C) Percentage of Ki67 positive cell was measured. (D) Percentage of IFN- γ positive cells following NK1.1, IL-12+IL-18 or YAC tumor cell line stimulation. Data are displayed as mean \pm SD from 3 mice/group. P-value were calculated using t-test (A and C) or two-way ANOVA followed by Šidák's multiple comparisons test (B and D).

Although TSC expression decreases with NK cell maturation, its expression remains higher in NK cells than B or T cells (M. Yang et al. 2016). Therefore, the lack of phenotype of TSC deficient NK cells could not be explained by a lack of TSC expression. A hypothesis to explain the difference in phenotype of NK-TSC KO vs other TSC deficient cells may arise from the different metabolic state of the cell type considered. Indeed, NK cells display a lower basal metabolic rate compared to previously analyzed cell types. We can thus

hypothesize that in cell types displaying a high basal metabolic rate or in cell lines cultured in optimal nutrient conditions, the nutrients axis of mTORC1 regulation is fully primed, with the Rag arm of mTORC1 regulation already in an “ON” state. Therefore, one can propose that TSC inhibition in this context results in a higher response than in cells with a low basal metabolic rate, where the Rag are probably not fully activated. In this sense, deletion of TSC1 or SZT2, a negative regulator of the mTORC1 nutrient axis, in hematopoietic cells (Vav1-Cre), results only in a slight increase in mTORC1 activation at basal state (Yin et al. 2022). In contrast, the double KO of SZT2 and TSC1 is associated with a strong mTORC1 activity, a rapid HSC exhaustion and early mouse death (Yin et al. 2022). This data are in favor of a stronger effect of TSC deletion when the nutrient arm of mTORC1 regulation is not repressed, a situation that could mimic high metabolic cells. This of course would require further direct testing.

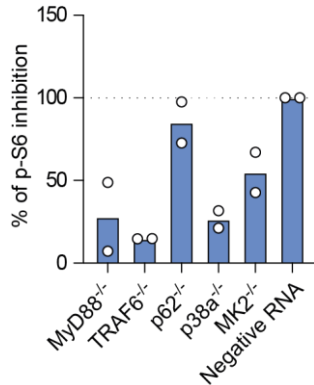
A recent report shed a new light on the drivers of TSC phenotype in TSC syndrome (Alesi et al. 2024). Focusing on the mechanism associated with kidney disease in TSC, the authors show that TFEB is surprisingly hypo-phosphorylated at mTORC1-dependent site in TSC deficient kidney cells which results in its nuclear localization and its hyper-activation. Deletion of TFEB rescue the kidney pathology in TSC KO mice indicating that the increase in TFEB activity is the main driver of the phenotype in this system. In addition, the increase in the phosphorylation of the classic target of mTORC1, 4E-PB and S6, was normalized upon genetic ablation of TFEB, confirming previous study indicating that TFEB is a positive regulator of mTORC1, notably through the transcriptional induction of RagC/D (Alesi et al. 2024). These data support the qualification of TSC disease as a “TFE-opathy”, where TFEB is the driver of the phenotype associated with the disease. We can hypothesize that the absence of phenotype in TSC deficient NK cell may arise from different importance of TFEB versus classic mTORC1 target or different mode of regulation of TFEB and lysosome biology in NK cell compared to other cell type such as kidney cell. In this sense, prior analysis of TFEB localization in different TSC deficient cell lines have reported different results, with some study showing nuclear localization (Alesi et al. 2021; 2024) and other cytoplasmic localization, depending on the cell type and the culture conditions used (Magini et al. 2017; Napolitano et al. 2020).

3.2. Molecular mechanisms of mTORC1 activation independently of TSC

a. Post translational modifications

What are the molecular mechanism allowing mTORC1 activation independently of TSC? Previous studies report direct phosphorylation of Raptor on serine residues by ERK (Carriere et al. 2011) and by its down target RSK (Carrière et al. 2008), which results in an increase of mTORC1 kinase activity. Similarly, p38 β has also been shown to directly phosphorylate Raptor on different serine residues resulting in enhanced mTORC1 activity (X.-N. Wu et al. 2011). Besides phosphorylation, another study has reported that K63 polyubiquitination of mTOR itself increases mTORC1 activity. The mechanism proposed involves TRAF6 activation by p38 δ and the adaptor p62 in response to amino acid in HEK393T (Linares 2013, 2015). We took advantage of the CRISPR-Cas9 system to test the importance of this pathway downstream IL-18 in NK cells, as described previously.

Deletion of MyD88 or TRAF6 reduced p-S6 downstream IL-18 stimulation to the same extent as p38 deletion, indicating the importance of these two proteins for mTORC1 activation downstream IL-18 (**Extended Figure 3**). However, p62 deletion does not impact p-S6 level in IL-18 treated NK cell which suggests that another mechanism of activation different from the one proposed by Linares and colleagues is involved.

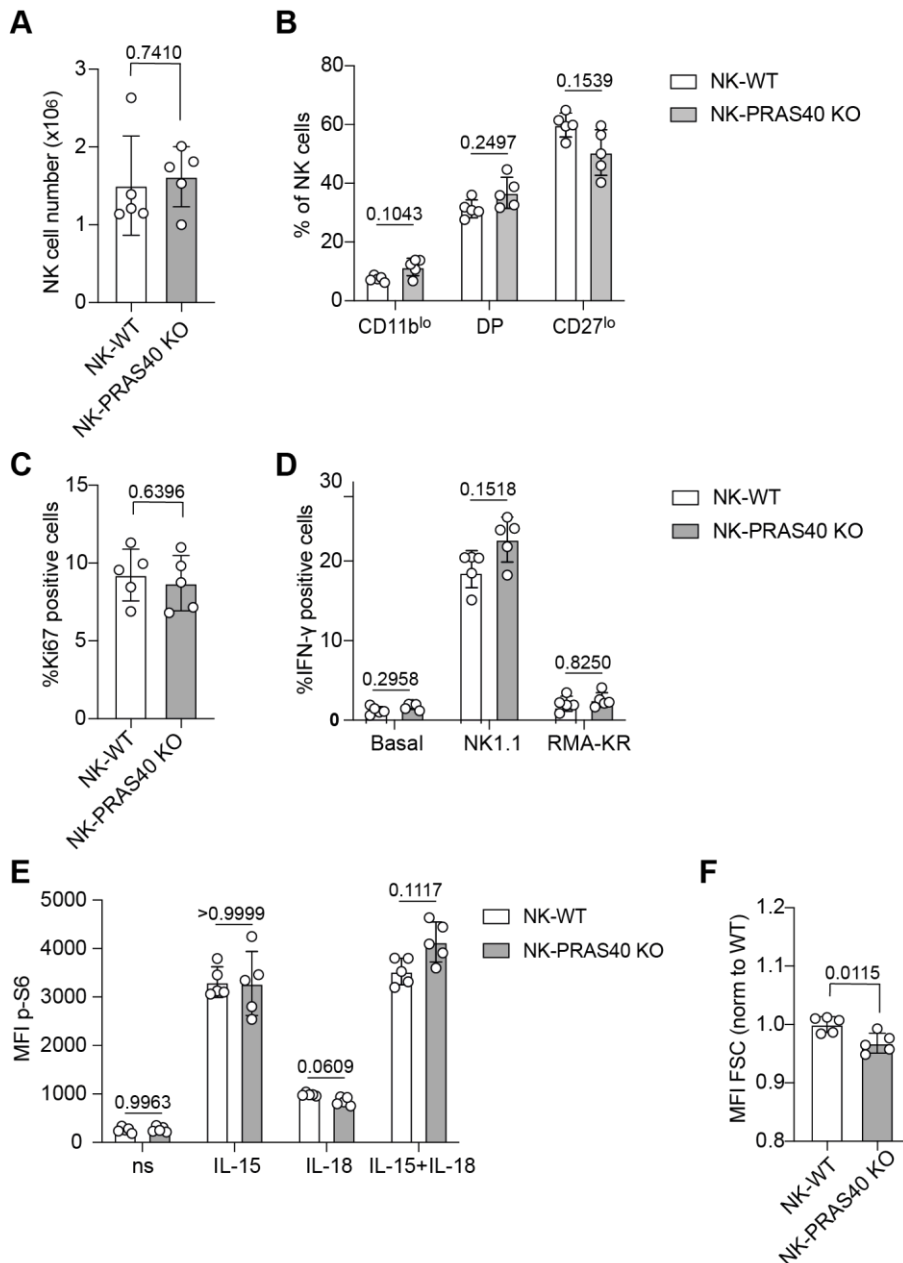


Extended Figure 3: mTORC1 activation by IL-18 is independent of p62. NK cells KO for the indicated protein were generated using CRISPR-Cas9 and treated with IL-18 for 1h. Level of p-S6 compared to control guide is indicated (n=2 mice).

Post translational modification of mTOR or components of mTORC1 could be a mechanism regulation of mTORC1 that would explain the partial independence to TSC1 but also the synergy observed between the cytokines on mTORC1 activation. The techniques deployed in our study also did not allow us to identify any change in mTORC1 proximal interactome and was not suited for analysis of brief interaction such as the one involving a kinase and its substrate. Analysis of post translational modifications by mass spectrometry using protocols adapted to phosphorylated or ubiquitinated protein as well as the use of cross-linker reagents to stabilize kinase/substrate interaction may provide a better understanding of mTOR activation by cytokines.

b. Other negative regulators of mTORC1

The importance of other negative regulators of mTORC1 in NK cell could also provide an explanation for the lack of phenotype of TSC null cells and a mechanism of regulation for TORC1 activation. Apart from TSC, AKT is also known to directly regulate another inhibitor of mTORC1: PRAS40. As mentioned in the introduction, it was reported that AKT phosphorylates PRAS40, counter-acting its inhibitory activity toward mTOR, through a mechanism dependent on the protein 14-3-3 (Sancak et al. 2007; Vander Haar et al. 2007). This mechanism is sometime mentioned as a TSC independent negative regulation of mTORC1. Our labs also generated mice bearing NK cells deficient for PRAS40 (**Extended Figure 4**). However, we observed no impact of this deletion on NK cell numbers, maturation, Ki67 expression or IFN- γ production. In addition, p-S6 level was identical in NK-WT and NK-PRAS40 KO cells at basal state and after cytokine stimulation. We even observed a slight decrease in NK cell size in PRAS40-KO mice compared to WT. Therefore, complementary studies are required to provide a molecular mechanisms controlling mTORC1 activity in a TSC and PRAS40 independent way.

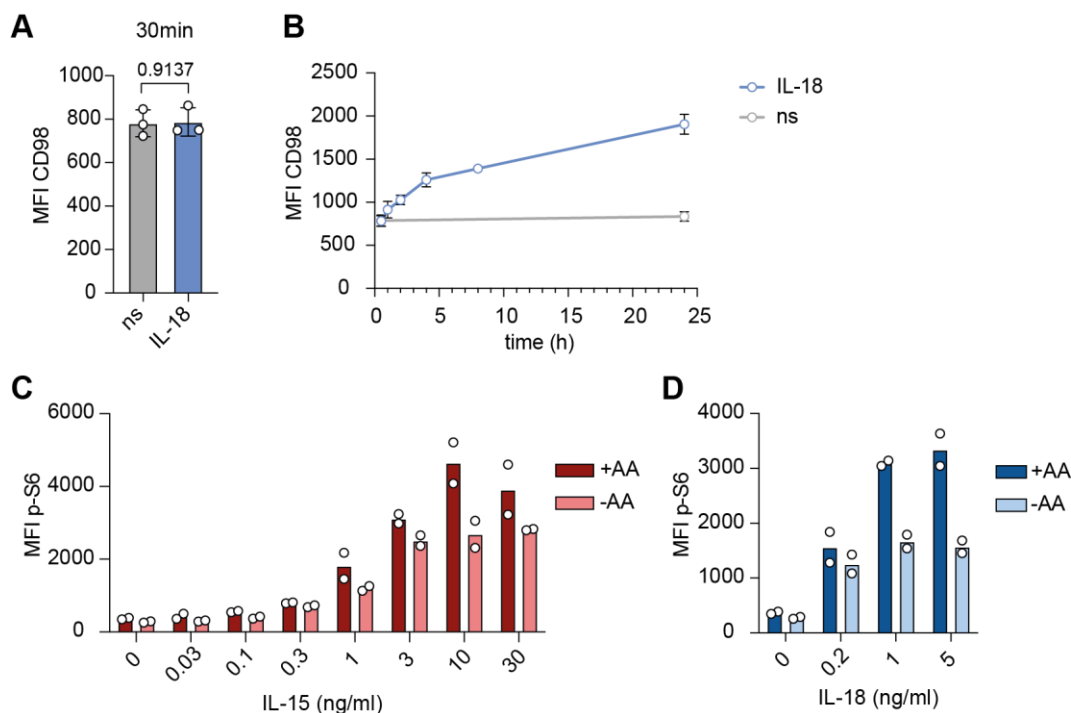


Extended Figure 4: Phenotype of $NCR1^{Cre} \times PRAS40^{lox/lox}$ mice (NK-PRAS40 KO). (A) NK cell numbers in the spleen of the indicated mice were measured. (B) Percentage of CD11b^{lo}, double positive (DP) and CD27^{lo} in each phenotype is indicated. (C) Percentage of Ki67 positive cell was measured. (D) Percentage of IFN- γ positive cells following NK1.1 or tumor cell line RMA-KR stimulation. (E) Splenic NK cells were treated with the indicated cytokine for 1h (ns: no stimulation). Phosphorylation (p-) of S6 (Ser 235-236) was measured by flow cytometry. (F) FSC of splenic NK cells of the indicated mice. Data are displayed as mean \pm SD from 5 mice/group. P-values were calculated using t-test (A, C and F) or two-way ANOVA followed by Šidák's multiple comparisons test (B, D and E).

c. Regulation of the nutrient axis

In addition to growth factors, nutrients, and amino acid in particular, are also strong activators of mTORC1. Previous studies from our lab and other indicated that another effect of IL-15 and IL-18 was to increase nutrients transporters (Marçais et al. 2014;

Almutairi et al. 2019). In particular, a study from Almutairi et al. showed that IL-18 treatment increased the expression of the glutamine transporter SLC1A5 and the leucine transporter CD98 (SLC3A2) which sustains mTORC1 activation through amino acids import, an event that will induce the recruitment of mTORC1 to the lysosome as exposed in the Introduction chapter (Almutairi et al. 2019). In this study however, mTORC1 activation is measured after 24h treatment which is sufficient to induce an increase in amino acid transporters expression, whereas in our study we observed an increase in p-S6 in the first minutes following IL-15 or IL-18 treatment which is not compatible with the kinetic of induction of CD98 expression (**Extended Figure 5A and B**). We additionally tested the capacity of IL-15 and IL-18 to induce p-S6 in the absence of amino acids. We observed that IL-15 and IL-18 were still able to induce mTORC1 activation in a medium depleted in amino acid when measuring mTORC1 activity at 1h. However, this activation was decreased compared to the one observed in complete medium in particular at higher cytokine concentrations (**Extended Figure 5C and D**). These data suggest a two step-mechanism: at low cytokine concentrations mTORC1 is activated independently from the Rag and the amino acid pathway, whereas higher concentrations of cytokine also trigger mTORC1 activation through amino acids dependent mechanisms. Amino acids dependent pathways are therefore not the only mechanism explaining IL-15 or IL-18 activation of mTORC1 independently of TSC, but are important to sustain long term mTORC1 activation and maximal mTORC1 activity.



Extended Figure 5: IL-15 and IL-18 activate mTORC1 independently of amino acid import. (A) Splenic murine NK cell were treated for 30min with 5ng/ml of IL-18 and the expression of CD98 was measured by flow cytometry. (B) Expression of CD98 was measure for 24h with or without 5ng/ml of IL-18. (C-D) Splenic murine NK cell were treated for 1h with the indicated concentration of cytokine in a media containing (+AA) or depleted (-AA) in amino acid. Level of p-S6 (Ser235-236)

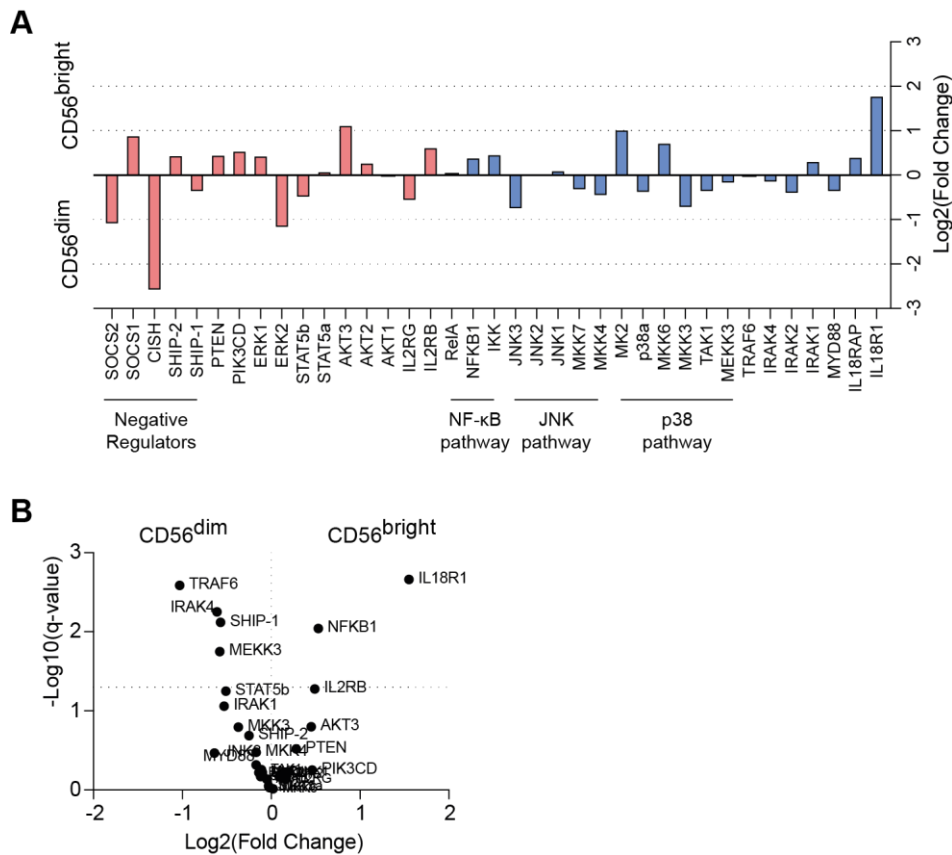
is indicated. Data are displayed as mean \pm SD from 3 mice/group (A-B) or 2 mice per group (C-D). In (B) P-value, was calculating using t-test.

4. mTORC1 activation in human NK cell

We also assessed the importance of cytokines for mTORC1 activation in human primary NK cells. Our data reveal that IL-15 treatment activates mTORC1 in both CD56^{dim} and CD56^{bright} NK cell whereas IL-18 increases p-S6 only in CD56^{dim} NK cells. This result was puzzling since CD56^{bright} NK cells are able to respond to IL-18 and produce IFN- γ in response to IL-12+IL-18, even to a higher extend than CD56^{dim} ((Romee et al. 2012) and unpublished data from our lab). In addition, CD56^{dim} and CD56^{bright} NK cell both express IL-18 receptor, with CD56^{bright} even expressing more IL-18R1 according to RNA sequencing data available on Human Cell Atlas Immune system and proteomics analysis performed by our team (**Extended Figure 6**). Therefore, the lack of mTORC1 activation following IL-18 treatment in CD56^{bright} cell is not explain by a lack of IL-18 receptor expression. Our analysis also shows that p38 is required for the activation of mTORC1 in CD56^{dim} NK cells, as for mouse NK cells. Proteomic data generated by our team indicates a slightly higher expression of TRAF6, IRAK4 and MEKK3 in CD56^{dim} whereas CD56^{bright} express slightly more NFKB1, the p105 subunit of NF- κ B (**Extended Figure 6B**). This could argue for an increase in the signaling leading to NF- κ B and IFN- γ production at the expense of mTORC1 activation in CD56^{bright} NK cells. However, whether these differences are sufficient to explain the lack of mTORC1 activation downstream IL-18 in CD56^{bright} is unknown and remains to be tested.

CD56^{bright} NK cells also display a stronger activation of mTORC1 in response to IL-15, with p-S6 level 5 time superior than the one measured in CD56^{dim} cells. This difference in responsiveness is unclear, but echoes a previous study indicating that IL-15 more robustly primes CD56^{bright} than CD56^{dim} cell, as measured by cytokine and killing capacity enhancement (Wagner et al. 2017). This study also highlighted that p-STAT5, p-AKT and p-ERK are further increased in term of fold change following IL-15 treatment in CD56^{bright} than CD56^{dim} NK cells, and that PI3K or ERK inhibition impaired CD56^{bright} functional priming by IL-15 (Wagner et al. 2017). We did not test the impact of PI3K-AKT and ERK inhibition on mTORC1 activation by IL-15 in human NK cells, but this higher activation of PI3K-AKT and ERK signaling is consistent with the stronger activation of mTORC1 in CD56^{bright} in our study. What are the molecular mechanism responsible for the higher mTORC1 activation following IL-15 stimulation in CD56^{bright}? RNA sequencing and proteomic data do not indicate major difference in expression of AKT, ERK or STAT5 pathway members (**Extended Figure 6**). We can however note a higher expression of the RNA of the negative regulator CISH in CD56^{dim} although its expression was not found at the protein level in our proteomic analysis. More convincing perhaps, the proteomic analysis indicates a higher expression of SHIP-1, a phosphatase that antagonize PI3K signaling, in CD56^{dim} NK cells (**Extended Figure 6B**). This echoes previous work suggesting that the higher expression of SHIP-1 in CD56^{dim} results in a stronger negative control of PI3K signaling in CD56^{dim} than CD56^{bright}(Trotta et al. 2005). Therefore the

differential expression of negative regulators of IL-15 signaling between CD56^{dim} and CD56^{bright} NK cells may be an argument for the stronger response of CD56^{bright} NK cells.



Extended Figure 6: Expression of IL-15 and IL-18 signaling molecules in human CD56^{dim} and CD56^{bright} NK cells. (A) Expression of the indicated RNA in CD56^{bright} compared to CD56^{dim} NK cells. RNA sequencing data are from the Human Cell Atlas Immune system databank. (B) Expression of the indicated protein in CD56^{bright} compared to CD56^{dim} NK cell. Data are from proteomic analysis performed by our lab.

Taken together, our data indicate that IL-15 and IL-18 activates mTORC1 by both canonic and non-canonic mechanisms in primary NK cell. In one hand, IL-15 activation of mTOR relies on PI3K-AKT and ERK, both known as strong regulators of mTORC1 and extensively review in introduction. In the other hand, mTORC1 regulation in mature NK cell is largely independent of the known mTORC1 inhibitors TSC and PRAS40. We also show that IL-18 activation of mTORC1 relies on p38-MK2, an emerging pathway in the landscape of mTOR regulators, and confirm its importance in both mouse and human primary NK cells. In a second time, we assess the importance of these pathways on NK cell biology.

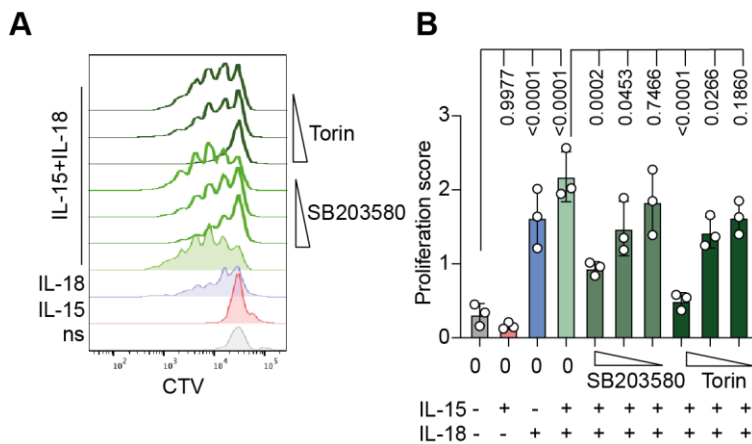
II. Impact of IL-15 and IL-18 on NK cell biology

1. IL-15 and IL-18 synergistically increase NK cell proliferation

The main consequence of IL-15 and IL-18 combination on NK cell biology is the synergic activation of NK cell proliferation, both *in vitro* and *in vivo*, and both in human and mouse NK cells. Beside is well characterized role on NK cell survival, we confirmed that high doses of IL-15 also trigger NK cell division. On the contrary, IL-18 impact on survival is

minor. However, our results indicate that IL-18 promotes NK cell division to the same extent as IL-15. Consequently, NK cell cotreatment with both cytokines results in proliferation and cell accumulation, probably due to the pro-survival effect of IL-15. We show that proliferation of IL-15+IL-18 treated NK cells is decreased by Torin treatment at doses that inhibit mTORC1 but not mTORC2 as measured by S6 and AKT phosphorylation respectively, indicating the importance of this complex in this process. Of note, IL-15 and IL-18 also engage other pathways besides mTORC1 that can promote NK cell survival and/or proliferation. As described in introduction, IL-15 also triggers STAT5 activation which has been shown to promote NK cell survival (Gotthardt et al. 2016; Wiedemann et al. 2020). Similarly, IL-18 activates the transcription factor NF- κ B which targets a multitude of genes, including anti-apoptotic and cell cycle ones (Karin et Lin 2002).

IL-15 displays higher capacity to activate mTORC1 than IL-18 when the cytokines are used alone, which may mask some aspects of mTORC1 regulation by IL-18. This last point is reminiscent of a study from Heberle et al., which takes advantage of both computational modelling and *in vitro* assay, and shows that PI3K and p38 act in a hierarchical manner toward mTORC1 activation (Heberle et al. 2019). Studying the regulation of stress granule formation by mTORC1, the authors point out that the activation of mTORC1 by p38 is visible only when PI3K signaling is reduced by at least 40%. Similarly, using low doses of IL-15 to unmask IL-18 proliferation effect, we observed a decrease in mouse NK cell division in the presence of a p38a/b inhibitor or an mTORC1 inhibitor, which suggests the importance of p38/mTORC1 activation downstream IL-18 for NK cell proliferation (**Extended Figure 7**). These non-canonical activation pathways, unmasked when stimulation levels are limiting, could be of particular relevance to understand mTORC1 regulation in physiological settings, where stimuli and cytokines may be limiting.



Extended Figure 7: p38 is required for mouse NK cell proliferation resulting from low dose of IL-15 and IL-18 treatment. Murine splenic NK cells were labeled with CTV and stimulated with low dose of IL-15 (1ng/ml) and same dose of IL-18 (5ng/ml) +/- increasing doses of Torin and SB203580 for 3 days. **(A)** Representative histogram overlay and **(B)** calculated proliferation score are shown. Data are displayed as mean \pm SD from 3 mice/group and p-value, were calculating using one-way ANOVA followed by Šidák's multiple comparisons test.

2. IL-15 and IL-18 enhance NK cell translation, metabolism and functions

Beside proliferation, we show that IL-15 and IL-18 increase NK cell translation capacity and metabolism which both sustain NK cell growth and division. Translation and glycolysis induction by IL-15+IL-18 cotreatment are partly mTORC1 dependent, in line with the described known role of mTORC1 in these process (Thoreen et al. 2012; Hsieh et al. 2012; Düvel et al. 2010, 1). Interestingly, S6 phosphorylation and some biological parameters such as protein translation are not strictly correlated. Indeed, cells treated 24h with IL-15 or IL-18 alone show robust mTORC1 activation measured by p-S6 level, which is not associated with a significant increase in protein translation measured by puromycin incorporation. This suggests the existence of threshold, where energy demanding process such as translation are activated only in response to higher stimulation.

On the contrary, IFN- γ production induced by IL-15+IL-18 is independent of mTORC1. This result corroborates previous studies indicating that IL-18 control of IFN- γ production is not impaired by metabolism inhibition but instead relies on IL-18 transcriptional activation, notably through the NF- κ B pathway (Keppel et al. 2015). IL-15+IL-18 priming also results in an increase in NK cell killing capacity, to the same extent as IL-15 alone for our *in vitro* results. This increase is impaired by mTORC1 inhibition. This corroborates already mentioned studies demonstrating the importance of mTORC1 in IL-15 priming of cytotoxic capacity (Marçais et al. 2014; F. Wang et al. 2018).

3. IL-15 and IL-18 enhance NK cell anti-tumor function *in vivo*

In the last part of our study, we assess the outcomes of the synergy between IL-15 and IL-18 *in vivo*. We confirm that IL-15 and IL-18 co-injection results in an increase in NK cell proliferation and function *in vivo*, which leads to a better tumor control in a model of RMA cell line sensitive to NK cells. Surprisingly, IL-15 treatment *in vivo* does not increase NK cells cytotoxicity in contrast to what we observed *in vitro*. Considering that we tested only one dose of cytokine in the two conditions, the dose used *in vivo* may have been insufficient to induced an increase in killing against the RMA-KR cell line used in this setting. We also show that IL-15+IL-18 combination increases p-S6 and cell size, two readouts of mTORC1 activity. However, the importance of mTORC1 in this gain of function and proliferation remain to be tested. We could take advantage of the Raptor KO mice to evaluate the importance of mTORC1 in cytokine response *in vivo*. However, the severe impact of Raptor deficiency on NK cell development and number at steady state may render difficult the drawing of a firm conclusion. Another approach could consist in treating mice with mTORC1 inhibitors such as rapamycin at the time of cytokine injection to avoid such a bias.

PERSPECTIVES

Improving immune cell expansion and activation is attracting interest with the development of cellular therapies based on the infusion of these cells. mTOR being a key molecule for cell proliferation and metabolism, understanding its regulation in primary cells can have direct application in this matter. Our analysis sheds a new light on mTOR regulation in primary lymphocytes and unmasks the importance of alternative pathways such as p38 in primary NK cell, and the limited importance of classical negative mTOR regulators such as TSC or PRAS40 (**Figure 25**). These alternative pathways, triggered by weaker stimuli such as IL-18, may not be essential for transformed activated cell line, but may be of particular importance in physiological contexts. Our results also indicate a synergy between IL-15 and IL-18 on both mTORC1 activation and the priming of NK cell responses. The knowledge gained on IL-15 and IL-18 signaling could also be used to improve the design of CAR molecules using cytokine receptor intracellular signaling domains but also the development of new cytokine agonist fusing several cytokines at ones (Cubitt et al. 2022; Becker-Hapak et al. 2021). Finally, our study points out the limits of transformed cell lines as working models and illustrates the importance of the development of new technologies and assays sensitive enough for the study of small cell populations of primary cells.

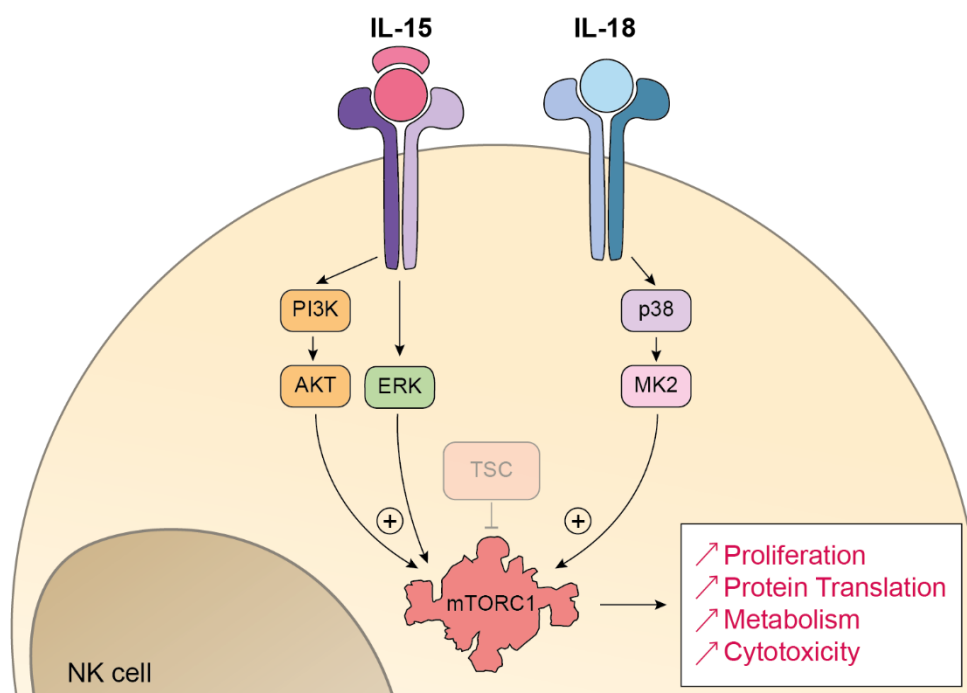


Figure 25: IL-15 and IL-18 regulation of mTORC1 in primary NK cells. Graphical representation of the conclusions from our study on cytokines signaling toward mTOR in primary NK cells.

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ANNEXES

Review: **Signaling pathways leading to mTOR activation downstream cytokine receptors in lymphocytes in health and disease.** Lucie Fallone, Thierry Walzer, Antoine Marçais. Int.J.Mol.Sci. 2023



Review

Signaling Pathways Leading to mTOR Activation Downstream Cytokine Receptors in Lymphocytes in Health and Disease

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Abstract: CD8+ T cells and Natural Killer (NK) cells are cytotoxic lymphocytes important in the response to intracellular pathogens and cancer. Their activity depends on the integration of a large set of intracellular and environmental cues, including antigenic signals, cytokine stimulation and nutrient availability. This integration is achieved by signaling hubs, such as the mechanistic target of rapamycin (mTOR). mTOR is a conserved protein kinase that controls cellular growth and metabolism in eukaryotic cells and, therefore, is essential for lymphocyte development and maturation. However, our current understanding of mTOR signaling comes mostly from studies performed in transformed cell lines, which constitute a poor model for comprehending metabolic pathway regulation. Therefore, it is only quite recently that the regulation of mTOR in primary cells has been assessed. Here, we review the signaling pathways leading to mTOR activation in CD8+ T and NK cells, focusing on activation by cytokines. We also discuss how this knowledge can contribute to immunotherapy development, particularly for cancer treatment.

Keywords: mTOR; CD8+ T cell; NK cell; cytokine signaling



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1. Introduction

The target of rapamycin (TOR) pathway orchestrates metabolic regulation from yeast to plants and humans. More specifically, the activation of the TOR kinase, the effector of the TOR pathway, promotes anabolic pathways, such as translation or synthesis of nucleotides and lipids, while it represses catabolic activities, such as autophagy. TOR kinase activity is regulated by intracellular (e.g., ATP) and extracellular cues (e.g., amino acids) so that anabolic pathways are activated when the building blocks are present while catabolic activities are switched on in less prosperous conditions. In multicellular organisms, however, the organism's homeostasis maintains nutrient availability [1], thus calling for a second control mechanism to prevent unrestricted growth, a hallmark of cancer [2]. During evolution, extracellular signals controlling cell activation and growth, referred to as growth factors, have thus been co-opted as inputs regulating the TOR signaling network. This explains why mechanistic TOR (mTOR) behaves as an integrator, being active only when both nutrients and growth signals are present. Importantly, the surface receptors that detect such growth signals are different from one cell type to another to ensure the independent regulation of different cell types. As a consequence, the classes of receptors involved in growth signal detection are varied, ranging from tyrosine kinase receptors to G-protein coupled receptors. This diversity suggests that the signaling pathways leading to mTOR activation can differ from one cell type to another and even inside a given cell type if two distinct receptors are triggered. Historically, the study of mTOR signaling has been conducted in vitro in transformed cell lines using the pathway triggered by insulin as a model. This pathway and its components are conserved in most cell types, making it a good model system. However, it does not reveal the whole breadth of the signaling network regulating mTOR activity. In addition, the complete rewiring of cellular metabolism in cultured transformed

cell lines makes them a poor model for specific regulatory steps taking place in primary resting cells. Here, we will review the signaling pathways leading to mTOR activation in lymphocytes, taking the example of CD8+ T cells and Natural Killer (NK) cells. Indeed, various steps of these cells' biology are regulated by cytokines, a subset of growth factors classically associated with hematopoietic or immune cells development, which stimulate the mTOR pathway. We also focused this review on mTORC1 activation, the complex primarily involved in metabolic regulation, since we and others have demonstrated the importance of this complex in the regulation of lymphocyte functions and activity.

2. CD8+ T and NK Cell Development and Functions: Common Themes and Divergences

CD8+ T lymphocytes and Natural Killer (NK) cells are cytotoxic lymphocytes specialized in the response to intracellular pathogens and cancer. They are able to kill infected or tumor cells through directed exocytosis of granules containing perforin and granzyme, and produce large quantities of pro-inflammatory cytokines, such as IFN- γ [3]. These capacities place them at the forefront of both anti-tumor and antiviral responses. However, they diverge in the conditions that lead to the acquisition of these effector functions: while naïve CD8+ T cells require clonal expansion and subsequent differentiation into cytotoxic T lymphocytes (CTL) to acquire these capacities, NK cells acquire them during their development. This translates into very different response kinetics during immune responses: while NK cells display their effector functions in a matter of hours, CD8+ T cells require a differentiation step of several days. Moreover, the signals leading to this acquisition also differ. Indeed, naïve T cell differentiation into CTL is triggered upon the engagement of the T cell receptor (TCR), and this first signal is complemented by the help of Interleukin (IL)-2, secreted by helper CD4+ T cells or, to a lesser extent, by CD8+ T cells themselves. In contrast, NK cell development and acquisition of effector potential is principally driven by the cytokine IL-15. IL-2 and IL-15 bind to partially overlapping receptors comprised of IL2R β and the γ chain for the common part, while the alpha chains IL2R α and IL15R α bring the specificity. Since the α chains are unable to signal, the signal arising from IL-2 or IL-15 receptors is almost indistinguishable [4]. Other cytokines, such as cytokines of the IL-1 family, also take part in the activation of certain functions of CD8+ and NK cells. Indeed, cytokines from the IL-1 family are important for terminal differentiation and acquisition of effector functions, such as IL-1 β , which increases CD8+ T cell cytotoxic capacity [5], or IL-18, which, in association with IL-12, is the main driver of IFN- γ production by both CD8+ T cells and NK cells [6]. In addition, it becomes clear that additional factors, such as the presence of metabolites in the extracellular milieu or the bioenergetic state of the cell, strongly influence lymphocyte fate decisions. Therefore, lymphocyte activation depends on the integration of a large set of intracellular and environmental cues, including antigenic signal, cytokine stimulation and nutrient availability. This integration is achieved by signaling hubs, such as mTORC1.

3. A Brief Overview of the mTOR Pathway

3.1. mTOR Complexes and Targets

mTOR was identified thanks to its sensitivity to rapamycin, a macrolide compound isolated from the bacteria *Streptomyces hygroscopicus* found in a soil sample of Easter Island (Rapa Nui being the native name of the island). It is the core kinase of two complexes, the mTOR complexes 1 (mTORC1) and 2 (mTORC2). mTORC1 is composed of the kinase mTOR associated with mammalian lethal with SEC13 protein 8 (mLST8) and the scaffold regulatory-associated protein of mTOR (RAPTOR). Two endogenous inhibitors also belong to the complex, the proline-rich AKT substrate of 40 kDa (PRAS40) and the DEP domain-containing mTOR-interacting protein (DEPTOR). mTORC2 is organized around mTOR, the protein mLST8 and the scaffold protein rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) in lieu of RAPTOR, along with DEPTOR (as in mTORC1), mammalian stress-activated protein kinase-interacting protein 1 (mSIN1) and protein

observed with RICTOR 1 and 2 (PROTOR $\frac{1}{2}$). The two complexes differ by their sensitivity to rapamycin, mTORC1 being sensitive and mTORC2 not being directly sensitive to it [7], but also by their substrates and functions. mTORC1 plays a well-known role in the control of protein synthesis through the phosphorylation of 4E-BP and S6K, which are classical hallmarks of mTORC1 activation. In addition, mTORC1 also activates glycolysis, lipids and nucleotide synthesis and represses autophagy, therefore supporting anabolism and energy synthesis. Therefore, mTORC1 activation is usually associated with cell growth and proliferation. The role of mTORC2 is less understood; it phosphorylates AKT on Ser473, thereby potentiating its kinase activity, which represses transcription factors FOXO1/3 in lymphocytes [8]. Such factors regulate the expression of multiple genes associated with lymphocyte quiescence or trafficking. Moreover, in some systems, mTORC2 regulates cytoskeleton dynamics. As stated above, this review will mainly discuss mTORC1 signaling and control.

3.2. The Importance of mTOR in Lymphocytes

The notion that mTORC1 activity is essential for lymphocyte activation and functions stems from earlier studies that discovered the immunosuppressive activity of rapamycin [9]. mTORC1 inhibitors have thus been used in clinics, notably in the context of lymphoproliferative diseases [10–12] or organ transplantation [13,14]. Therefore, the essential role of mTOR for immune cell development and function is now widely recognized, especially in lymphocytes [15]. Immune signals delivered by the TCR and co-stimulatory molecules in T cells, NK-activating receptors in NK cells or cytokine receptors in both T and NK cells trigger mTORC1 activation, which switches on the metabolic pathways required to support cellular activation. mTOR also has a crucial role during NK cell development by controlling their maturation and maintaining their reactivity [16,17]. Furthermore, memory CD8+ T cell differentiation also relies on tight regulation of mTOR activity [18–20].

3.3. mTOR Upstream Regulation

In eukaryotic cells, mTORC1 senses two broad categories of signals: growth factors and nutrients. The concomitance of these two signals is required for mTORC1 activation as it regulates both mTORC1 kinase activity and localization (Figure 1).

3.3.1. Linking Growth Factors to mTOR Activation: The Example of Insulin Signaling

Growth factors, which we will refer to here as molecules capable of stimulating cell proliferation and survival, are a first set of signals that trigger mTORC1 activation. In 1998, insulin was found to activate mTOR [21] and has since become the best-documented example of mTOR activation by a growth factor. The insulin receptor is a receptor tyrosine kinase expressed by virtually all cells. Insulin binding to its receptor triggers receptor autophosphorylation on several cytoplasmic tyrosines, driving the recruitment and phosphorylation of the adaptor protein Insulin Receptor Substrate (IRS). Mammals express four IRS isoforms, the main ones being IRS1 and IRS2 [22]. Phosphorylated IRSs then serve as binding sites to recruit proteins with SH2 domains, such as phosphatidylinositol 3-kinases (PI3K). Class I PI3K is formed by a 110 kDa catalytic subunit (p110) among the isoforms α , β , γ and δ , associated with a regulatory subunit among p85 α , p85 β or p55 γ . The four p110 catalytic subunits can act upstream of mTOR; however, they are recruited downstream of different receptors, p110 γ only being recruited downstream of G-protein-coupled receptors. In mature lymphocytes, the most expressed subunit is p110 δ ; it associates with p85 α or p85 β [23]. Class I PI3K catalyzes the phosphorylation of phosphatidylinositol (4,5) biphosphate (PI(4,5)P2) into PI(3,4,5)P3, which acts as a docking site for proteins containing pleckstrin homology (PH) domains, such as the protein kinase B (PKB also named AKT) and 3-phosphoinositide-dependent protein kinase-1 (PDK1). This allows PDK1 to phosphorylate the activation loop of AKT at Thr-308, an essential event for AKT activation. In addition, AKT is also phosphorylated on Ser-473, primarily by mTORC2, even though this modification can be added by other kinases such as TBK1 [24–26]. This

second phosphorylation event stabilizes the Thr308 phosphorylation and increases AKT kinase activity. IRS also recruits the adaptor GRB2, which binds SOS, a guanine nucleotide exchange factor for RAS. Stimulation of RAS constitutes the starting point of the activation of the ERK mitogen-activated protein kinases (MAPK) cascade.

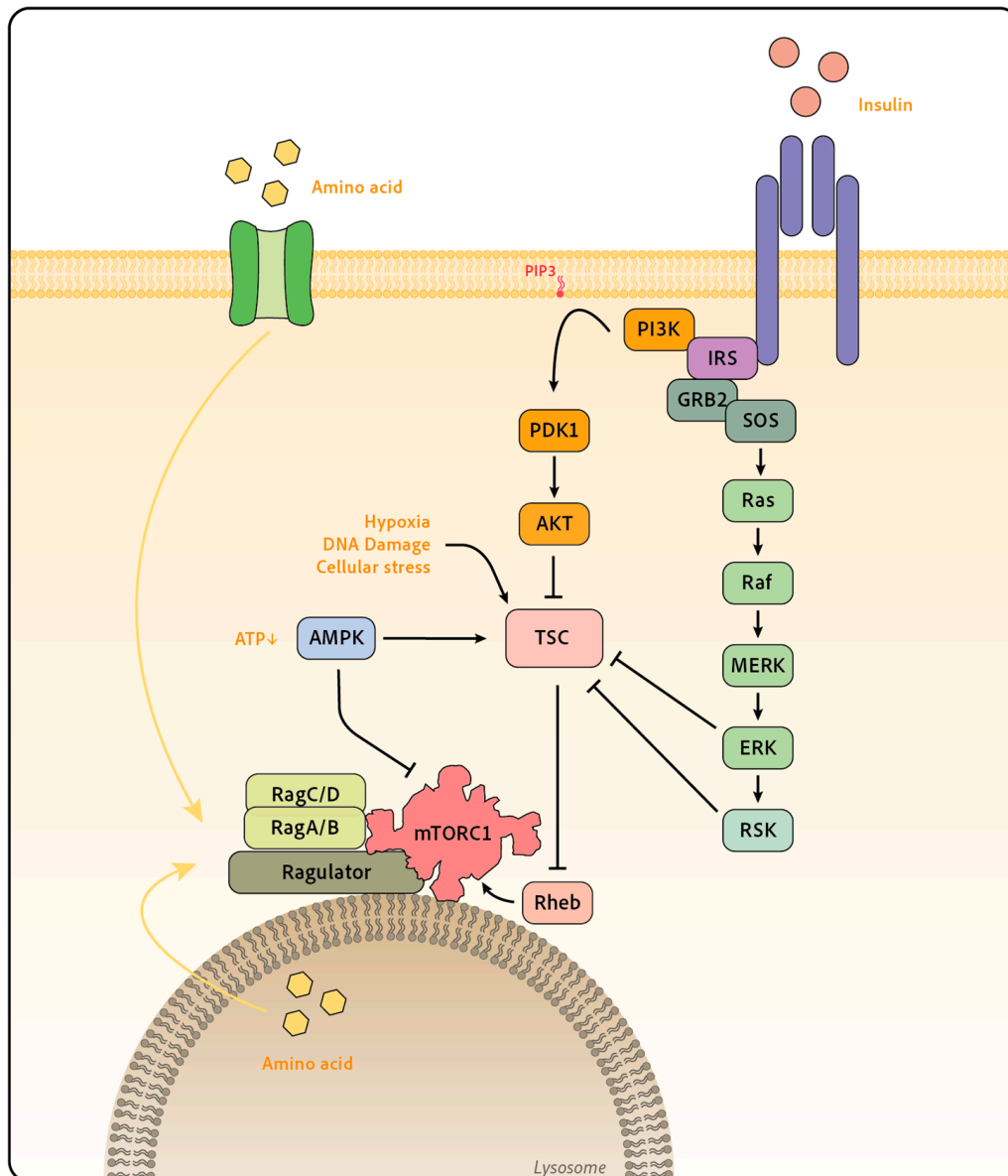


Figure 1. Overview of mTORC1 upstream regulation. mTORC1 activation is controlled by both growth factors and nutrients. Growth factors such as insulin activate both the PI3K-AKT signaling and the MAPK ERK pathway, which converge on TSC inhibition. TSC acts as a GAP for RHEB, which boosts mTORC1 kinase activity. Among nutrients, amino acids are essential for mTORC1 activation as they control mTORC1 translocation to the lysosome through the activation of the RAG GTPase A/B and C/D. At the lysosome, mTORC1 is in close vicinity to its activator RHEB. This two-step mechanism allows mTORC1 to integrate both growth factors and nutrient presence. Other parameters, such as energy level or hypoxia, further regulate mTORC1.

Regarding the mTOR pathway, a major convergence point of the AKT and ERK pathways is the inhibition of the tuberous sclerosis complex (TSC), a key inhibitor of mTORC1. TSC is composed of three proteins, TSC1, TSC2 and TBC1D7 [27], and acts as a GTPase-activating protein (GAP) for the small GTPase RHEB [28,29], an essential

mTORC1 activator. By inhibiting TSC, growth factors such as insulin thus allow mTOR to be fully activated by RHEB. Structural studies show that RHEB loaded with GTP binds to mTOR distally from the kinase site and causes a global conformational change of the complex [30]. This molecular mechanism explains that GTP-loaded RHEB behaves as an allosteric activator increasing mTORC1 activity by several orders of magnitude. On note, RHEB has unusually slow intrinsic GTPase activity. As a result, TSC, which stimulates RHEB GTPase activity and therefore inhibits this small GTPase, behaves as a gatekeeper of mTORC1 activity. In addition to this well-described canonical pathway, some studies also suggest that the mTORC1 complex itself can be post-translationally modified following growth factor stimulation [31,32]. The relevance of such modifications is unclear.

The activity of TSC is tightly regulated by multiple proteins. AKT phosphorylates TSC2 at different sites, thus suppressing its control of RHEB and mTOR [33]. TSC is also inhibited by phosphorylation from ERK [34] and the p90 ribosomal S6 kinase (RSK) [35], another downstream kinase of the Ras-ERK pathway. Other studies described a regulation of TSC by growth factors through the control of its recruitment to the lysosome [36–39]. The regulation of TSC through the disruption of the complex itself or through its degradation is still debated (reviewed in [40]). Overall, these results define TSC as a major hub collecting inputs that negatively regulate mTORC1.

3.3.2. mTOR Control by Nutrients and Cellular Stress

In addition to growth factors, nutrients are essential for mTORC1 activation. In particular, the role of amino acids has long been recognized and involves a set of four GTPases, RAG GTPases A, B, C and D, that act as heterodimers combining RAG A or B and RAG C or D [41,42]. In conditions of amino acids sufficiency, the RAG GTPases are locked in an “on-state”, with RAG A/B bound to GTP and RAG C/D bound to GDP, and vice versa in case of amino acid scarcity. In their “on-state”, RAG GTPases mediate mTORC1 attachment to the lysosome surface via a multi-proteic complex called the Ragulator [43,44], bringing mTORC1 in close vicinity to its activator RHEB, which also lies on the external membrane of the lysosome. Thus, when nutrients are abundant, mTORC1 is tethered to the lysosome surface, where it can be fully activated by mitogen signals, such as insulin or other growth factors. On the contrary, when nutrients are limiting, mTORC1 is released from the lysosome surface and thus inactivated. This two-step mechanism explains the importance of the coincidence of both nutrients and growth signals for mTORC1 activation and supports its role as a signal integrator. Several proteins are involved in amino-acid sensing both inside the lysosome and the cytoplasm and constitute today a topic of active research (review in [45]). Of note, not all amino acids seem to have the same potency. Arginine, Leucine, and Methionine have been described as direct regulators of mTORC1, each one possessing its dedicated sensor [46]. Other nutrients, such as glucose [47] or cholesterol [48–50], have also been shown to impinge directly onto the mTORC1 pathway in a manner partially overlapping the amino acid pathway.

In addition to this direct control, the lack of nutrients can also be sensed via the decrease of ATP. Indeed, energy drop or cellular stress also negatively regulate mTOR activity. Glucose depletion activates the AMP-activated protein kinase (AMPK), which senses the decrease in ATP/ADP ratio and inhibits mTORC1 either directly through phosphorylation of RAPTOR on inhibitory sites [51] or indirectly by activating the phosphorylation of TSC2 [52]. Independently of AMPK, hypoxia also negatively regulates mTORC1 through TSC activation [53,54]. Several mTORC1 negative regulators, including TSC or AMPK, are targets of p53, which plays a central role in DNA damage sensing [55]. Finally, endoplasmic reticulum stress or oxidative stress also negatively regulate mTORC1 function (review in [56]).

4. mTOR Activation Downstream of γ Cytokine Receptors in Lymphocytes

Cytokines are soluble proteins with signaling functions in the immune system, and some of them have a growth factor activity. Physiologically, γ chain cytokines, such as

IL-2 and IL-15, are potent mitogens for T and NK cells. IL-1 family cytokines increase cytotoxic functions and cytokine production of lymphocytes, two processes that require energy. Therefore, it is no surprise that these cytokines activate the mTORC1 pathway. The next two sections discuss our current knowledge of mTORC1 regulation in T and NK cells, focusing on γ chain and IL-1 type cytokines.

4.1. Overview of the γ c Cytokines Family

The γ chain (γ c) family regroups cytokines that signal through the common receptor γ chain, namely IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. γ c was first discovered as a component of the IL-2 receptor, making IL-2 the archetypal member of this family [57,58]. The IL-2 receptor is composed of three proteins: CD25, the α chain of the IL2R (IL-2R α); CD122, the β chain of the IL2R (IL-2R β); and the γ chain CD132. As mentioned before, IL-15 signals through two of the three IL-2 receptor chains, γ c and IL-2R β , while its unique IL-15R α chain serves to transpresent IL-15 from neighboring cells [59,60]. In terms of signaling, most of our knowledge comes from studies on the IL-2 receptor. Contrary to the insulin receptor, the IL-2 receptor lacks intrinsic kinase activity. IL-2R β and γ c are associated with the kinases JAK1 and JAK3, respectively, and the binding of IL-2 results in JAK1/3 activation and phosphorylation of tyrosine residues on IL-2R β [61–63]. These residues serve as docking sites for proteins containing SH2 domains. Phosphorylated IL-2R β binds the p85 subunit of PI3K [64], which triggers the activation of the PI3K-AKT pathway. Phosphorylated IL-2R β also serves as a docking site for the GRB2-SOS complex through the adaptor SHC, which constitutes the starting point of the Ras-ERK MAPK cascade. The transcription factor STAT5 is also recruited to the activated IL-2R and is of particular importance for IL-2 signaling [65].

4.2. mTOR Regulation by γ c Cytokines in T Cells

The first evidence of mTOR activation by γ c cytokines comes from studies using IL-2 [66,67]. In T cells, IL-2 treatment triggers ribosomal protein S6 phosphorylation, a marker of S6K activity downstream of mTORC1. IL-2 treatment is also associated with an increase in cell size and metabolism, other indirect hallmarks of mTORC1 activation [68–71]. Several studies show an increase in AKT phosphorylation a few minutes after IL-2 treatment, confirming that IL-2 activates this pathway in T cells [69,70,72]. However, the importance of PI3K-AKT in mTORC1 activation in T cells is debated. Studies from Cantrell's group show that the inhibition of AKT or PI3K δ does not decrease mTORC1 activity following IL-2 treatment in CTL [69]. The same group, however, showed that PDK1 is required for mTORC1 activity, since CTL deleted for this protein display decreased S6 phosphorylation, glucose uptake and cell proliferation [68]. Conversely, the deletion of PTEN, a negative regulator of PI3K signaling that can drive the activation of AKT, is not sufficient to activate mTORC1 and mTORC1-dependent glucose metabolism [73]. This evidence suggests that the PI3K-AKT pathway is activated by IL-2 but is dispensable for mTORC1 activation. How PDK1 controls mTORC1 independently of the PI3K-AKT pathway remains to be deciphered. One hypothesis would be that its activity is not required extemporaneously but more upstream to phosphorylate and license the neosynthesized S6K [74].

How can IL-2 activate mTORC1 if not through the well-described PI3K-AKT axis? One possibility could be through the JAK-STAT pathway. STAT5-deficient CD4⁺ T cells treated with IL-2 show decreased mTORC1 activity, as measured by S6 phosphorylation, associated with a reduction in cell size, proliferation and metabolism compared to their WT counterpart. However, STAT5-deficient T cells have also decreased levels of AKT phosphorylation at Thr308, suggesting that STAT5 may act upstream from AKT. To directly test the relationship between STAT5 and AKT, T cells deleted for STAT5 were complemented with either constitutively activated STAT5 or constitutively activated AKT. Only STAT5 transduction rescued mTORC1 activation, whereas the transduction of AKT did not, suggesting a mechanism whereby mTOR activation relies on STAT5 while it is independent of AKT [70]. To explain the activation of AKT, Ross and Cantrell proposed a mechanism involving

pathways depending on the SRC family kinases LCK and FYN. This signaling, independent of STAT5, would participate in the context of “preorganized” signal transduction pathways, which integrates with IL-2-STAT5 signaling [72]. On note, STAT5 is a transcription factor and activates the transcription of several key proteins relevant for mTORC1 signaling, such as IL-2R chains, AKT, RHEB or amino acid transporters and sensors, which participate in longer-term activation of mTORC1 [70,71].

In T cells, the γ cytokine IL-7 is also known to activate mTORC1, although the molecular details are less documented than for IL-2. Indeed, IL-7 treatment enhances S6 phosphorylation [75] as well as cell size and survival [76] and glucose uptake [77]. This last metabolic change seems to require AKT and STAT5 activation [77], although no clear evidence has demonstrated a molecular link between these pathways and mTORC1 in IL-7-treated lymphocytes.

4.3. mTOR Regulation by γ Cytokines in NK Cells

NK cell maturation and survival is governed by the γ cytokine IL-15. IL-15 treatment activates mTORC1, which increases cellular metabolism [16]. IL-15 also activates STAT5, AKT [16], ERK [78] and its downstream target RSK [79]. Several studies have investigated the role of these different proteins in the activation of mTORC1. Using pharmacological inhibitors, Nandagopal and colleagues showed that PI3K or STAT5 inhibition following IL-15 treatment decreased NK cell production of IFN- γ and granzyme B as well as its proliferative capacity to the same extent as rapamycin. However, the status of mTORC1 activation itself was not measured in this study [80]. NK cells deficient in PTEN, an antagonist of PI3K, display increased AKT and S6 phosphorylation [81], while NK cells deficient in the catalytic subunits of PI3K p110 γ and p110 δ display a defective maturation and function [82]. Interestingly, the observed defect included a strong decrease in circulating NK cells and a nearly complete disappearance of the most mature CD11b⁺ subset, phenocopies of mTOR or Raptor deficiencies [16,83,84]. In addition, IL-15 activates the ERK pathway. The use of an MEK inhibitor suggests that ERK could participate in mTORC1 activation in NK cells, as ERK inhibition leads to decreased S6 phosphorylation following IL-15 treatment [78]. Overall, these data are indicative of a role of both the PI3K-AKT and ERK pathways in the control of mTORC1 in NK cells. On the contrary, a study conducted in IL-2/-12 in vitro-activated NK cells reached the opposite conclusion that the PI3K-AKT pathway is not involved in mTORC1 activation. Indeed, in this last study, NK cells treated with AKT inhibitors show no decrease in cell size, IFN- γ production, granzyme expression or S6 phosphorylation [85]. This suggests that mTORC1 regulation differs when comparing CD8⁺ T cells and resting or activated NK cells and that their regulation can be adapted to the cellular activation status. The reason for these differences is unknown at this point (Figure 2).

4.4. The Role of the TSC Axis in Lymphocytes

As described above, TSC constitutes a hub controlling mTORC1 activation thanks to its capacity to integrate both negative and positive inputs. As a result, TSC null cell lines show maximal mTORC1 activity to the same extent as insulin-treated ones [86,87], whereas TSC overexpression results in drastic decreases in mTORC1 activity [33,52,88,89]. A similar high activity of mTORC1 is observed in vivo in primary muscle cells [90] or in hepatocytes [91] upon TSC inactivation. For these reasons, different groups looked at the importance of the TSC hub in T and NK cells.

Four distinct studies have investigated the role of TSC in T cells and concluded that TSC is required to maintain cell quiescence in the absence of antigen stimulation [20,92–94]. TSC1-specific deletion in immature T cells, using the T cell-specific CD4-Cre deleter, leads to a decrease in CD4⁺ and CD8⁺ T cell numbers [92–94]. The remaining T cells show an increase in cell proliferation and display an activation of the intrinsic apoptotic pathway [93,94], characterized by a decrease in the anti-apoptotic protein Bcl-2 [93]. They also display elevated cellular ROS, which could be reversed by antioxidant treatments that restore T cell numbers [93,94]. This phenotype is associated with an increase in mTORC1

activity and a decrease in mTORC2 activity [93,94]. Such a decrease in mTORC2 activity is supposed to arise from the negative feedback loop leading from S6K to targets lying upstream of mTORC2 [45]. Focusing on CD8+ T cells, Pollizzi et al. show that TSC2-deficient T cells display characteristics of terminally differentiated effector T cells and are unable to transition to the memory state. Confirming these results, T cells deficient in the GTPase RHEB fail to differentiate into effector cells but retain memory characteristics [20].

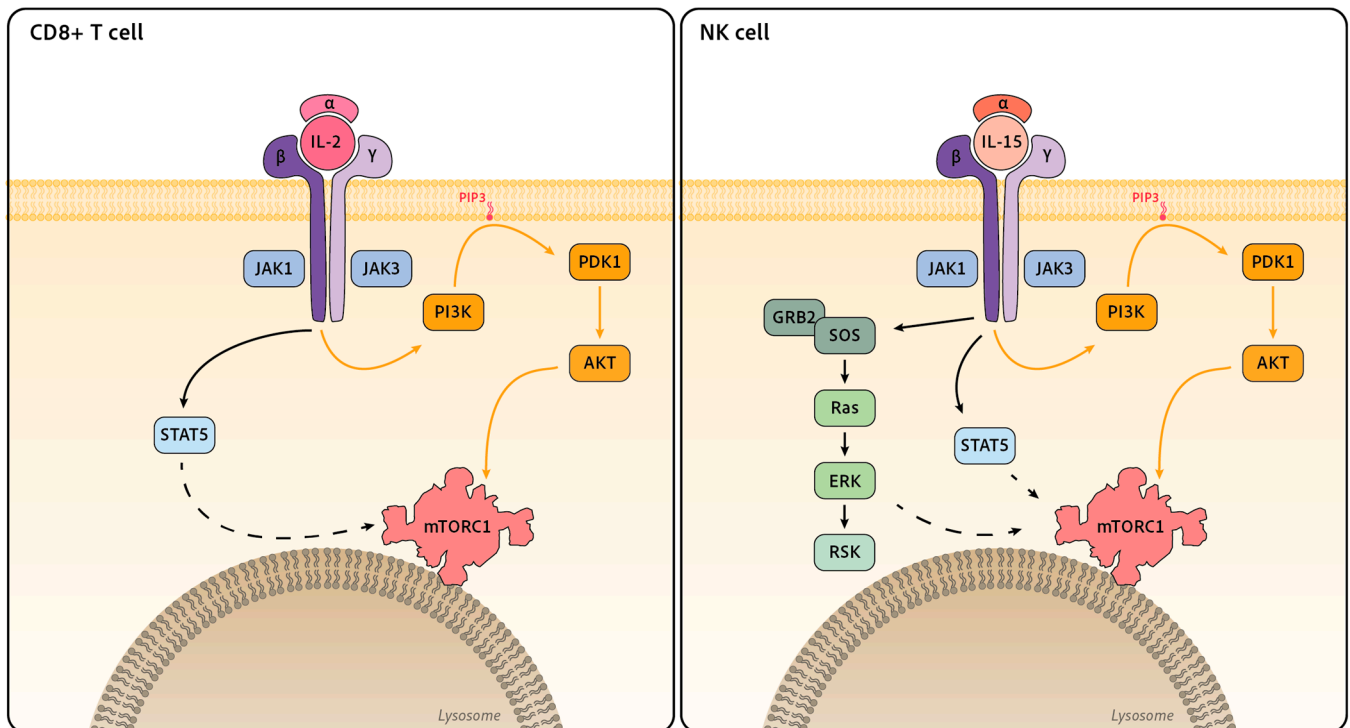


Figure 2. Signaling downstream of IL-2 and IL-15 receptors toward mTORC1 in CD8+ T cell and NK cells. IL-2 and IL-15 receptors share both their IL-2R β and γ chain (γ c) associated with a unique IL-2R α or IL-15R α chain depending on the receptor. IL-2R β and γ c are associated with the kinases JAK1 and JAK3, respectively. The binding of the cytokine results in JAK1/3 activation and phosphorylation of tyrosine residues on IL-2R β , which serves as an activating platform for STAT5, PI3K and GRB2-SOS. Even though the PI3K-AKT pathway is engaged by the IL-2 receptor (in orange), its importance in mTORC1 activation remains under debate.

In NK cells, TSC1 deletion at an early stage produces a similar phenotype as TSC1 deletion in immature T cells, with a decrease in NK cell number associated with an increase in cell proliferation and apoptosis and a constitutive mTORC1 activation [95]. However, the deletion of TSC1 in mature cells does not have any effect on NK cell phenotype, function or response to cytokines [95]. In the same line, inducible inhibition of TSC1 at latter stages in T cells using an ER-Tamoxifen construct has only a mild effect on the T cell population [93]. On note, TSC is mainly expressed by progenitors, and its expression decreases during the maturation process, both in NK and T cells [95], which could account for the more important phenotype of TSC deletion in immature cells compared to mature ones. These results also echo those from Cantrell's group, suggesting that PI3K-AKT signaling is not mandatory for mTOR regulation by γ c chain cytokines. In fact, T and NK cell homeostasis mainly depends on the γ c cytokines IL-2 and IL-15, respectively, which, as discussed earlier, may not depend on the classical pathway involving AKT and, therefore, TSC, being one of the main targets of AKT, for their signaling in lymphocyte. On note, even if these studies show the importance of TSC in the maintenance of cell quiescence, TSC1 mutations in lymphocytes result in a mild phenotype compared to other cell types. For instance, mice bearing liver-specific TSC1 ablation developed hepatocellular carcinoma [91]. This

suggests other mechanisms of regulation involving TSC-independent control of mTOR in mature lymphocytes.

5. mTOR Activation Downstream of IL-1 β Family Receptor in Lymphocytes

5.1. Overview of the IL-1 β Cytokines Family

The IL-1 family is a large group of cytokines composed of agonist molecules, such as IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β , IL-36 γ and IL-37, and antagonist ones, such as IL-1R α . They all signal through receptors composed of two different chains, which are all characterized by the presence of a conserved cytosolic region containing a signaling domain called the Toll/IL-1 Receptor (TIR) domain. The binding of the cytokine to the first receptor chain triggers the recruitment of the second one and the juxtaposition of their TIR domains. This induces the recruitment of the protein MyD88 and the kinase IRAK4. The latter is activated by autophosphorylation, leading to the subsequent recruitment and phosphorylation of IRAK1 and 2, two adaptors and kinase proteins. MyD88 and its IRAK partners then form a protein platform that recruits the E3 ubiquitin ligase TRAF6, which triggers the activation of different pathways, such as the MAPK and NF- κ B pathways [96].

IL-1 family cytokines typically engage the MAPK p38. There are four isoforms of p38 (p38 α , β , γ , δ), the most expressed in mammals being p38 α . As for other MAPKs, p38 activation results from phosphorylation cascades involving different MAP3Ks, such as TAK1, which is classically engaged by IL-1. Furthermore, as described for other MAPKs, p38 has the potential to phosphorylate a multitude of targets, including other kinases such as the MAPK-activated protein kinases (MAPKAPK) MK2, MK3 and MK5/PRAK [97]. Several studies report the control of mTORC1 by p38 in different cell lines, mentioning both an activator [98–101] or inhibitor [102] role for p38 depending on the stimulus involved. In addition to the p38 MAPK pathway, IL-1 family cytokines also engage the NF- κ B pathway. NF- κ B activation requires activation of the IKK complex through its binding to polyubiquitin chains on several molecules downstream of the IL-1 receptor, such as IRAK1 or TAK1. The activated IKK phosphorylates I κ B α , which promotes its degradation and the release and nuclear translocation of NF- κ B, which promotes the pathway activation. IKK has also been involved in mTORC1 activation. Indeed, in one study, cancer cell lines treated with TNF α activate IKK β , a component of the IKK complex that interacts with and phosphorylates TSC1, resulting in TSC1 inhibition and mTORC1 activation [103].

5.2. mTOR Regulation by IL-1 β Cytokine Family in T and NK Cells

In T and NK cells, different cytokines of the IL-1 family have been shown to activate mTORC1, although the molecular pathways involved are not fully understood yet. mTORC1 has been shown to be activated by IL-18 [16,104] or by IL-33 [105] in NK cells, and by IL-1 in Th17 [106,107] and IL-36b in CD8+ T cells [108,109]. The most complete mechanistic study comes from Gulen et al. [107]. Working on Th17, the authors showed that IL-1 induces the activation of mTORC1, measured by S6 and 4EBP1 phosphorylation. The authors also demonstrated that IL-1 signals through the IRAK1 and IRAK4 proteins to induce TSC disruption. The precise mechanism of TSC inactivation is still yet to be described. The authors proposed that inactivation of this complex is the event leading to mTORC1 activation. In the same study, IL-1 treatment is also shown to induce p38, JNK and I κ B α phosphorylation, although the implication of these pathways in TSC disruption was not clearly addressed [107] (Figure 3). Another study demonstrated that T cells deficient in p38 α / β or MK2/3 show decreased mTORC1 activity as measured by S6 phosphorylation upon TCR stimulation [110]. This defect correlates with increased regulatory T cell (Treg) numbers in vivo, which is reminiscent of the phenotype of mTOR-deficient T cells [111]. This suggests that p38 could control mTORC1 activation in lymphocytes; however, the significance of this pathway for cytokine signaling remains to be determined. As mentioned earlier, mTORC1 activity is also controlled by nutrient availability. In this context, Almutairi et al. showed that IL-18 treatment leads to enhanced surface expression of the leucine transporter CD98/LAT-1 on NK cells. This resulted in increased leucine import, thus

probably participating in mTORC1 activation [104]. It should, however, be noted that CD98 induction by IL-18 takes place after an overnight stimulation; it is thus unlikely that this mechanism is at play in short-term (<4 h) activation of mTORC1 upon IL-18 stimulation.

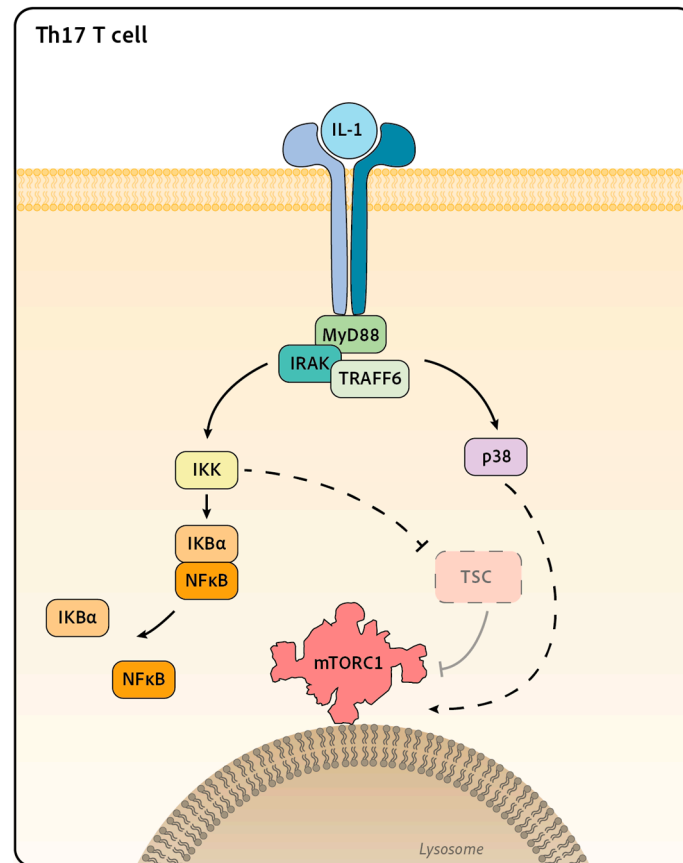


Figure 3. Signaling downstream of IL-1 receptor toward mTORC1 in T cell. IL-1 binding to its receptors induces the recruitment of the adaptors MyD88 and IRAKs. This activates the ubiquitin E3 ligase TRAF6, which triggers the activation of MAPK, such as p38 and the NFκB pathways. In Th17 T cell, Gulen et al. showed that IL-1 treatment drives the disruption of TSC and the activation of mTORC1. The role of the MAPK p38 or NFκB in this process in lymphocytes still remains to be determined, as p38 and IKK have been shown to activate mTOR in other cell types.

5.3. Signal Integration toward mTOR Control

Multiple signaling pathways modulate mTORC1 activity, as reviewed here. The outcome of co-stimulation with multiple cytokines will have to be deciphered. Indeed, we mostly focused our review on the effect of cytokines taken in isolation. However, cells generally encounter cytokines simultaneously. For instance, the generation of the so-called CIML (cytokine-induced memory-like cells) for therapeutic purposes implies the stimulation of NK cells with IL-15 and IL-18, both having the potential to activate mTORC1 with parallel pathways. Moreover, coincident stimulation with different cytokines can arguably lead to contradictory effects. For instance, T cell and NK cell proliferation and cytotoxicity induced in vitro with IL-2 can be inhibited by TGF-β treatment [112,113]. Interestingly, TGF-β antagonizes the IL-15 activation of mTOR in NK cells, resulting in a reduction in NK cell bioenergetic metabolism. This inhibition is detectable in the minutes following TGF-β treatment and is specific to mTOR since it does not impact STAT5 activation [114]. More recently, TGF-β has been shown to decrease mTOR activity in T cells [115]. In this study, early inhibition of mTOR in precursors of exhausted CD8+ T cells preserved their metabolic capacity and sustained the T cell response during chronic infection. In both studies, the molecular mechanism at play remains elusive but could

well involve some of the molecular players described above. Indeed, TGF- β has been shown to potentially activate a large set of pathways in a variety of cell types. This includes TAK1 [116], ERK [117], p38 [118] and PI3K [119]. Another possible mechanism to explain the inhibition of mTORC1 by TGF- β is the phosphatase PP2A. PP2A has been shown to inhibit mTORC1 in Tregs, and the ablation of PP2A in Tregs leads to severe autoimmunity [120]. The direct link between PP2A and the TGF- β signaling still remains to be determined. Whether activation of these pathways enters in competition with their involvement in mTOR signaling remains to be tested.

Some studies also highlight that several transduction pathways can be engaged for a given stimulus. For instance, in NK cells, a low dose of IL-15 only triggers STAT5 activation and cell survival, whereas higher doses engage mTORC1 and cell proliferation [16]. Similarly, in another model studying stress granule formation upon arsenite treatment, Heberle et al. addressed the respective importance of PI3K-AKT and p38 pathways in the control of mTORC1 activation in multiple cell lines. Using both *in silico* and *in vitro* approaches, the authors showed that PI3K-AKT and p38 pathways act in a hierarchical manner toward mTORC1 activation. Inhibition of PI3K results in residual mTORC1 activation, measured by p70-S6K phosphorylation, which is inhibited by an additional treatment with a p38 inhibitor. Based on these findings, the authors further show that the effect of p38 inhibition on mTORC1 activity is significant when PI3K activity is decreased to 40% or lower. From these results, it appears that moderate stress activates mTORC1 through p38, whereas increasing the stress signal also engages the PI3K-AKT pathway, leading to stronger mTORC1 activation [121]. Even though this study has not been performed on lymphocytes, we can hypothesize that similar mechanisms may take place in T and NK cells in response to different cytokine concentrations.

6. Therapeutic Targeting of mTOR

6.1. mTOR Inhibition in Cancer and Autoimmunity

As mTORC1 is essential for lymphocyte activation and function, the dysregulation of mTOR activity is associated with several lymphocyte defects leading to pathologies such as malignancies and autoimmune disorders. T cell acute lymphoblastic leukemia (T-ALL) is frequently characterized by activating mutation of NOTCH1 or loss-of-function mutation of PTEN [122,123]. Interestingly, these two mutations result in mTORC1 activation, and therefore, mTOR inhibitors have been proposed in T-ALL treatment. In a PTEN-deficient mouse model, rapamycin halts T-ALL initiation and development. However, most of these mice still developed T-ALL after rapamycin withdrawal. The author concluded that rapamycin effectively eliminates leukemia blast but fails to inhibit mTOR in leukemia stem cells, which could contribute to the limited clinical efficacy of rapamycin in T-ALL [124].

In parallel, mTOR is hyperactivated in up to 80% of cancers [45], founding the use of mTOR inhibitors as antiproliferative drugs in chemotherapies in other cancer types. This is not without side effects. Indeed, studies from our group have shown that patients with metastatic breast cancer treated with the mTOR inhibitor everolimus display lower levels of mTORC1 activation in NK cells as well as a decrease in NK cell number and maturation [125]. As NK cells have crucial antimetastatic activity [126], this side effect of mTOR inhibitors should be considered for the development of next-generation therapies. At the same time, mTOR inhibition may also benefit certain types of responses and promote certain differentiation pathways. Indeed, as discussed above, mTORC1 inhibition favors memory CD8⁺ T cell generation [18,20] and has a protective effect against T CD8⁺ exhaustion in chronic infection [115]. For these reasons, mTORC1 activation level in immune cells has to be well balanced to ensure a favorable anti-tumor effect.

Overactivation of the mTOR pathway in T cells is also associated with autoimmune disorders such as systemic lupus erythematosus [127]. mTOR inhibitors have been shown to reduce autoimmunity symptoms in both a mouse model [128] and in humans [129]. Indeed, in patients with systemic lupus erythematosus, rapamycin treatment has been

shown to improve the disease, associated with an increase of Treg and CD8+ memory T cells and a decrease of pro-inflammatory CD4+ and CD8+ T cells [130].

6.2. Targeting mTOR Activation in Lymphocytes for Immunotherapy

Controlling mTOR signaling can also constitute a promising strategy to modulate lymphocyte activity. In this context, a large set of therapies are now aiming at boosting lymphocyte functions, in particular for adoptive cell transfer therapies in cancer. Chimeric Antigen Receptor (CAR) T and NK cell-based immunotherapies have revolutionized the treatment of certain cancers [131]. The use of such therapies to treat viral infections (EBV and others) is also under assessment [132]. A better understanding of cytokine signaling toward mTOR could lead to improved T and NK cell expansion in vitro. The study of cytokine signaling also has the potential to benefit in tailoring better cytokine cocktails in order to prime lymphocytes in vitro. In the same line of idea, a better understanding of the signal transduction pathways involved in downstream cytokine receptors could inform the design of engineered receptors to improve CAR cell effector functions (review in [133]). Therefore, mTORC1 tight regulation is required to ensure a proper response to pathogens and cancer, which will benefit from a better understanding of its specific regulation in immune cells.

7. Concluding Remarks

The importance of the kinase mTOR for lymphocyte development and function is now widely recognized. Our knowledge of mTOR signaling comes mostly from early studies on the transformed cell line. If these studies allowed the discovery of the major molecular actors involved in mTOR control, such as the PI3K-AKT pathway or the TSC-Rheb axis, their relative importance in primary cells is now under debate. Therefore, further work focusing on mTOR regulation in primary cells should benefit our understanding of lymphocyte regulation, and could find a use in therapy design.

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