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

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

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Interleukin 15 (IL-15) controls both the homeostasis and the peripheral activation of natural killer (NK) cells. The molecular basis for this duality of action remains unknown. Here we found that the metabolic checkpoint kinase mTOR was activated and boosted bioenergetic metabolism after exposure of NK cells to high concentrations of IL-15, whereas low doses of IL-15 triggered only phosphorylation of the transcription factor STAT5. mTOR stimulated the growth and nutrient uptake of NK cells and positively fed back on the receptor for IL-15. This process was essential for sustaining NK cell proliferation during development and the acquisition of cytolytic potential during inflammation or viral infection. The mTORC1 inhibitor rapamycin inhibited NK cell cytotoxicity both in mice and humans; this probably contributes to the immunosuppressive activity of this drug in different clinical settings.
nutrient transporters, and also takes part in the control of lipid synthesis by activating the transcription factor SREBP, and in the control of autophagy. mTORC2 phosphorylates the kinase Akt at Ser473, which completes activation initiated by phosphorylation of Akt at Thr308 by the phosphoinositide-dependent kinase PDK1 and allows export of members of the Foxo family of transcription factors from the nucleus. mTORC2 also controls cytoskeletal organization. Moreover, mTOR phosphorylates itself at Ser2481. Many studies have explored the role of mTOR and downstream effector molecules in T cell differentiation. In contrast, there is a dearth of information on the metabolic regulation of NK cells and the role of mTOR in their physiology.

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

RESULTS
Development and activation regulate NK cell metabolism

We monitored metabolic changes during the differentiation and activation of NK cells. We detected substantial contraction of cell size and granularity as the cells terminally differentiated (Supplementary Fig. 1a). Conversely, when NK cells were activated in vitro with IL-15 (Supplementary Fig. 1b) or were activated in vivo in mice given injection of the synthetic RNA duplex poly(I:C) (the ligand of Toll-like receptor3 and RIG-I) (Supplementary Fig. 1c) or in mice infected with influenza virus strain A/WSN/33 H1N1 (Supplementary Fig. 1d), the size and granularity of NK cells increased substantially. In metastasis, a cell’s ability to access nutrients depends on chain of the system L transporter), as well as glucose uptake (estimated by measurement of uptake of the fluorescent glucose analog 2-NBDG), decreased by two- to threefold following the transition of mTOR and downstream effector molecules in T cell differentiation.

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

![Figure 1](image-url)  
Figure 1: NK cell metabolism is regulated developmentally and after activation. (a) Expression of CD71 and CD98 and incorporation of 2-NBDG by CD11b+ and CD27+ NK cell subsets from the BM or spleen, assessed by flow cytometry. Right, results for CD11b+CD27+ (DP) and CD27+ NK cells, presented as mean fluorescent intensity (MFI) relative to the synthetic RNA duplex poly(I:C) (the ligand of Toll-like receptor3 and RIG-I) (Supplementary Fig. 1c) or in mice infected with influenza virus strain A/WSN/33 H1N1 (Supplementary Fig. 1d), the size and granularity of NK cells increased substantially. In metastasis, a cell’s ability to access nutrients depends on chain of the system L transporter), as well as glucose uptake (estimated by measurement of uptake of the fluorescent glucose analog 2-NBDG), decreased by two- to threefold following the transition from CD11b+CD27+ to CD27+ in the BM (Fig. 1a). There was lower expression of those markers (CD71 and CD98) in the spleen, but their expression also decreased upon differentiation (Fig. 1a). In vivo activation in mice following injection of poly(I:C) resulted in higher expression of CD98 and CD71, as well as in more uptake of glucose, in splenic NK cells than that in resting NK cells from uninjected mice (Fig. 1b).

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

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

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

mTOR regulation upon differentiation and activation

The observation of metabolic changes during NK cell differentiation prompted us to assess the phosphorylation status of mTOR targets. We analyzed the phosphorylation status of mTOR itself (at Ser2448 and Ser2481), of downstream targets of mTORC1 (4EBP1, at Thr37 and Thr46; and S6, at Ser235 and Ser236) and of a target of mTORC2 (Akt, at Ser473). In parallel, we assessed the phosphorylation of STAT5 at Tyr694 by the kinase Jak3, downstream of the receptor for IL-15, and the phosphorylation of Akt at Thr308 by the kinase PDK1. We first measured the phosphorylation status of these proteins in BM and splenic NK cells at steady state and correlated it to the expression of CD11b and CD27. Phosphorylation decreased in a coordinated fashion as the cells matured (Fig. 2a), indicative of a progressive shutdown in mTOR activity both in the BM and in the spleen. We observed a similar pattern for the phosphorylation of STAT5 (Fig. 2a). In contrast, the phosphorylation of Akt at Thr308 decreased only in the BM (Fig. 2a). Direct comparison of phosphorylation in BM and splenic NK cells showed significantly greater phosphorylation of Akt at Ser473 and of mTOR at Ser2448 and Ser2481 on BM NK cells, especially in the CD11b<sup>hi</sup> subset (Supplementary Fig. 2). We then set out to measure phosphorylation events induced in vivo after stimulation with poly(I:C). The extent of the various phosphorylation events was upregulated in a coordinated fashion (Fig. 2b), which indicated an overall increase in the activity of the pathway. As expected, since injection of poly(I:C) increases the availability of IL-15, phosphorylation of STAT5 was significantly upregulated (Fig. 2b). In contrast, phosphorylation of Akt at Thr308, which is dependent on the activity of phosphatidylinositol-3-OH kinase (PI3K) via PDK1, was not significantly changed (Fig. 2b). Together these results demonstrated that mTOR activity was under the control of developmental and inflammatory signals in NK cells. Moreover, there was a close parallel between the metabolic status of NK cells and mTOR activity.

mTOR activity is mainly under the control of IL-15

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

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

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

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

To establish the physiological relevance of mTOR signaling in NK cells, we deleted mTOR in NK cells by crossing mice with Mtor floxed (lox) mice with mice expressing Cre recombinase from the gene encoding the NK cell–specific activating receptor Nkp46 (Ncr1; called ‘Nkp46-iCre’ here)27. This cross resulted in Mtor flox/loxNkp46-iCre mice (called ‘NK-Mtor−/−’ mice here). We analyzed the consequences of this deletion in NK cells in various organs (Supplementary Fig. 3). We confirmed that in NK-Mtor−/− mice, mTOR was indeed absent from NK cells but was present in normal amounts in the surrounding T cells (Supplementary Fig. 4a). In addition, NK cells were also smaller, and phosphorylation of mTOR targets was reproducibly diminished (Supplementary Fig. 4b,c), which demonstrated that mTOR was functionally absent. Despite that finding, the frequency and number of BM NK cells were normal in NK-Mtor−/− mice (Fig. 4a). In contrast, NK cells almost completely disappeared from the peripheral organs of NK-Mtor−/− mice (Fig. 4a and Supplementary Fig. 4d). Phenotyping of the remaining NK cells in NK-Mtor−/− mice revealed a substantial block in differentiation in the BM at the CD11blo-to-CD11bhiCD27hi stage (Fig. 4b and Supplementary Fig. 5a). This resulted in a shift in the distribution among the various NK cell subsets in the spleen (Fig. 4b and Supplementary Fig. 5a). Consistent with that observation, expression of the maturation and senescence marker KLRG1 on NK-Mtor−/− NK cells almost completely disappeared (Fig. 4c).

Next we conducted a broad phenotypic analysis of splenic Mtor+/+ Nkp46-iCre (called ‘NK-MtorWT/WT’ here) NK cells and NK-Mtor−/− NK cells (Fig. 4d). To compensate for the differentiation bias that occurred in the absence of mTOR, we focused our analysis on the CD11blo subset. The expression of markers such as CXCR4, CD127 and NK1.1 was upregulated on splenic NK-Mtor−/− NK cells relative to their expression on NK-MtorWT/WT NK cells (Fig. 4d). In contrast, NK-Mtor−/− NK cells had lower expression than did NK-MtorWT/WT NK cells of activating receptors acquired during maturation, such as 2B4, NKG2D, Ly49G2 and Ly49H (Fig. 4d). The frequency of cells expressing receptors of the Ly49 family, however, was similar in the presence or absence of mTOR, with the exception of Ly49H (Supplementary Fig. 5b). Expression of the T-box transcription factors Eomes and T-bet, which are responsible for the maturation of NK cells28, was also downregulated in NK-Mtor−/− NK cells relative to such expression in NK-MtorWT/WT NK cells (Fig. 4d). As expected, expression of the nutrient receptor CD71 and glucose uptake were downregulated in the absence of mTOR (Fig. 4d). Expression of CD122 and CD132, molecules that occur in the absence of mTOR, we focused our analysis on the CD11blo subset. The expression of markers such as CXCR4, CD127 and NK1.1 was upregulated on splenic NK-Mtor−/− NK cells relative to their expression on NK-MtorWT/WT NK cells (Fig. 4d). In contrast, NK-Mtor−/− NK cells had lower expression than did NK-MtorWT/WT NK cells of activating receptors acquired during maturation, such as 2B4, NKG2D, Ly49G2 and Ly49H (Fig. 4d). The frequency of cells expressing receptors of the Ly49 family, however, was similar in the presence or absence of mTOR, with the exception of Ly49H (Supplementary Fig. 5b). Expression of the T-box transcription factors Eomes and T-bet, which are responsible for the maturation of NK cells28, was also downregulated in NK-Mtor−/− NK cells relative to such expression in NK-MtorWT/WT NK cells (Fig. 4d). As expected, expression of the nutrient receptor CD71 and glucose uptake were downregulated in the absence of mTOR (Fig. 4d). Expression of CD122 and CD132, molecules that constitute the IL-15Rβc heterodimer of the receptor for IL-15, was halved in the absence of mTOR (Fig. 4d). The functional relevance of that last result is explored below. Expression of the other markers analyzed was not altered (Fig. 4d), which indicated that deletion of mTOR selectively affected part of the NK cell gene-expression program. We observed a similar pattern for the BM (Supplementary Fig. 5c).

The Nkp46 promoter also drives Cre expression in IL-22-producing NCR+ ILC3 cells in the gut. We thus investigated whether deletion of mTOR affected this cell subset. Indeed, Nkp46+ ILC3 cells were absent from the gut of NK-Mtor−/− mice (Supplementary Fig. 4d), which indicated a nonredundant role for mTOR in the generation of this population. Overall, these results demonstrated that functional mTOR was required for the presence of mature NK cells in peripheral organs and selectively affected part of the NK cell gene-expression program.
Optimal response to IL-15 depends on mTOR

We next investigated whether the absence of mature NK cells in the periphery of NK-Mtor−/− mice was a result of diminished survival, decreased generation of NK cells or both. The viability of NK-Mtor−/− splenic NK cells was identical to that of their NK-MtorWT/WT counterparts in the CD11blo subset and CD11bihCD27hi subset and was only slightly lower than that of NK-MtorWT/WT cells in the mature CD27lo subset (Fig. 5a). Moreover, ‘acute’ deletion of mTOR in Mtorlox/lox NK cells treated in vitro with the cell-permeable Cre recombinase fusion protein TATCre and then transferred back into wild-type mice in vivo did not impair their grafting capacity compared with that Mtor+/+ cells treated with TATCre and transferred together into the mice (Supplementary Fig. 6a). Thus, diminished viability probably had only a minor role in the defect observed. The CD11blo−,to-CD11bihCD27hi transition is preceded in the BM by a proliferation phase<sup>39</sup>. Deletion of mTOR resulted in a threefold decrease in the frequency of proliferating cells, as determined by incorporation of the thymidine analog BrdU (Fig. 5b) or staining with the proliferation marker Ki67 (Supplementary Fig. 6b). This probably profoundly affected the output from the BM and

![Figure 4](https://example.com/figure4.png)

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

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

As for effector functions, poly(I:C)-induced expression of granzyme B was halved in NK cells rendered deficient in mTOR (Fig. 6d). We obtained similar results when we compared the responses of NK-MtorWT/WT mice and NK-Mtorlox/lox mice to poly(I:C) (Supplementary Fig. 7a–c). We also monitored the degradation of CD11b⁺ NK cells and secretion of IFN-γ from NK cells following stimulation in vitro with a combination of IL-12 and IL-18 or plate-bound agonistic anti-CD48 antibodies specific for the activating NK cell receptors NKp46, NK1.1 and

blocked subsequent differentiation of NK cells. NK cell proliferation is controlled by IL-15. As noted above, NK-Mtorlox/lox NK cells had around half the surface expression of the β-chain (CD122) and γ-chain (CD132) of the receptor for IL-15 that NK-MtorWT/WT cells had (Fig. 5c and Supplementary Fig. 6c). That ‘translated’ into a lower steady-state amount of phosphorylated STAT5 in the BM of NK-Mtorlox/lox mice than in that of NK-MtorWT/WT mice (Fig. 5c and Supplementary Fig. 6c). In support of the proposal of a role for mTOR in the maintenance of optimal IL-15R expression, ‘acute’ deletion of mTOR induced by treatment with TATCre led to a reproducible decrease in the surface expression of CD122 (Supplementary Fig. 6d). Quantification of the phosphorylation of STAT5 in response to in vitro treatment with increasing concentrations of IL-15 led to the finding that NK-Mtorlox/lox cells properly sensed low concentrations of IL-15 and become defective only at concentrations able to induce proliferation (Fig. 5d and data not shown). This suggested that IL-15R was limiting when NK-Mtorlox/lox NK cells were exposed to an IL-15-rich environment that dictated NK cell proliferation. Overall, these results showed that mTOR deficiency had a substantial effect on NK cell proliferation in the BM due to direct and indirect effects through the regulation of IL-15R expression. The maturation block observed in NK-Mtorlox/lox NK cells was probably a consequence of this proliferation defect.
Ly49D. For these analyses, we used resting splenic NK cells or splenic NK cells preactivated with poly(I:C). As expected, preactivation with poly(I:C) led to a threefold increase in the proportion of cells that degranulated in response to cytokines or antibodies (Supplementary Fig. 7d). In contrast, although basal degranulation was fairly normal in the absence of mTOR, stimulation with poly(I:C) was unable to enhance it (Supplementary Fig. 7d). IFN-γ production followed a similar pattern when we assessed the response to stimulation of activating receptors on NK cells (Supplementary Fig. 7d). However, stimulation with IL-12 plus IL-18 drove normal IFN-γ responses, in terms of the frequency of IFN-γ+ cells (Supplementary Fig. 7d) and quantity of IFN-γ per cell (data not shown). The last finding demonstrated that NK–Mtor−/− cells were reactive to IL-12 plus IL-18 and were not generally insensitive to stimuli.

To assess the effect of mTOR deficiency on a more physiological NK cell response, we infected NK–MtorWT/WT mice and NK–Mtor−/− mice with MCMV. During MCMV infection, it is well described that early activation of NK cells is mediated by a combination of various cytokines30; while later during the infection, the interaction between Ly49H and the microbe-encoded protein m157 drives the proliferation and sustains the activation of Ly49H+ NK cells31. We measured the proliferation of Ly49H+ and Ly49H− NK cell subsets and the expression of granzyme B, IFN-γ, KLRG1 and the chemokine CCL3 by these cells, as well as degranulation of these cells, during the cytokine-driven (early) and m157- and Ly49H-dependent (late) phases of the response. The cytokine-driven phase of the response was severely impaired in terms of expression of granzyme B (Fig. 6e, middle) and CCL3 (Supplementary Fig. 7e, middle) as well as degranulation (Supplementary Fig. 7e, right), in agreement with data reported above (Fig. 6d and Supplementary Fig. 7c,d). In contrast, we confirmed that IFN-γ secretion was unaffected by mTOR deficiency (Fig. 6e, right). Unexpectedly, at day 6.5, the mTOR-deficient Ly49H+ NK cell subset expanded 12-fold (Fig. 6e). However, that proliferation was less than the proliferation of mTOR-sufficient NK cells (25-fold), and the number of mTOR-deficient Ly49H+ NK cells never reached that of mTOR-sufficient Ly49H+ NK cells (data not shown). Notably, the induction of KLRG1+ NK cells following infection with MCMV was impaired in the absence of mTOR in both the Ly49H+ subset and the Ly49H− subset (Supplementary Fig. 7e, left).

Overall these results demonstrated that mTOR deficiency profoundly impaired the early cytokine-driven activation of NK cells at multiple levels. In contrast, proliferation induced by m157 and Ly49H was partly independent of mTOR. The last result correlated with the reported independence of IL-15 of Ly49H-mediated NK cell proliferation during MCMV infection32.

Inhibition of mTOR abrogates inflammation-induced priming

The results reported above suggested that mTOR deficiency might affect the cytotoxicity of NK cells. To test this hypothesis further and to eliminate confounding developmental effects, we first stimulated wild-type NK cells with IL-15 in vitro and ‘acutely’ inhibited mTOR with three pharmacological inhibitors: rapamycin, an inhibitor of mTORC1 that is used clinically33; and two competitors of ATP, Ku-0063794 and PP242, that inhibit mTORC1 and mTORC2 (these inhibitors have been tested ‘preclinically’)34,35. To evaluate inhibitor efficacy, we measured phosphorylation of S6. All three inhibitors consistently impaired the increase in the phosphorylation of S6 and granzyme B in response to IL-15, with Ku-0063794 and PP242 showing higher potency (Fig. 7a). As rapamycin is an inhibitor that is already in use clinically, we fed mice an orally administered form of rapamycin for 2 d before treatment with poly(I:C) to test whether in vivo inhibition could be achieved. Indeed, treatment with rapamycin resulted in a decrease in the abundance of phosphorylated S6 and granzyme B (Fig. 7b). Treatment with rapamycin did not seem to affect the ‘trans-presentation’ of IL-15 to NK cells, since the abundance of Akt phosphorylated at Ser473 and upregulation of CD69 expression were normal after such treatment (Supplementary Fig. 8a,b).

We then directly investigated whether treatment with rapamycin impaired the reactivity of NK cells to ‘missing-self’ targets. For this we transferred a mixture of wild-type target cells and target cells sensitive to NK cells and lacking major histocompatibility complex class I (deficient in the gene encoding β2-microglobulin) into untreated (control) mice or mice given injection of poly(I:C), previously fed rapamycin orally or not given rapamycin. The killing of β2-microglobulin deficient target cells was significantly less efficient in rapamycin-treated mice than in their control counterparts (Fig. 7c), which emphasized the importance of mTOR activity in NK cell function.
Rapamycin is used in various therapeutic settings. We thus thought it important to investigate whether the priming of human NK cells was sensitive to inhibitors of mTOR. To address this, we stimulated human NK cells in vitro with IL-2 in the presence of mTOR inhibitors and measured phosphorylation of S6 and the abundance of granzyme B. IL-2 induced an increase in phosphorylated S6, which, as expected, was suppressed by pharmacological inhibitors of mTOR (Fig. 7d). The inhibitors of mTOR also prevented IL-2-induced expression of granzyme B (Fig. 7d), with little effect on the expression of perforin (Supplementary Fig. 8c). These results showed that, as in the mouse, the mTOR pathway controlled the cytotoxicity of human NK cells downstream of IL-15R. Overall, these results showed that mTOR activity in NK cells was inhibited in vitro and in vivo by pharmacological inhibitors and that this inhibition resulted in diminished priming and cytolytic functionality of NK cells.

DISCUSSION

mTOR is an integrator of various extracellular cues\(^\text{31}\). Among all the signals we tested in vitro, we found that IL-15 and IL-18, with or without IL-12, were the only inducers of mTORC1 and mTORC2 activity in NK cells. In contrast, none of the other homeostatic cytokines or agonistic antibodies known to stimulate activating receptors on NK cells had any effect on mTOR activity. This was unexpected, since ligation of the T cell antigen receptor plus the coreceptor CD28 is a potent inductor of mTOR activity\(^\text{36–38}\), and the pathways induced by ligation of activating receptors on NK cells or ligation of the T cell antigen receptor plus CD28 are similar\(^\text{29}\). Our in vitro findings were confirmed by the finding that following infection with MCMV, Ly49H-dependent responses were affected less than were cytokine-dependent responses of the Ly49H- NK cell subset. It thus seems that the Ly49H-dependent signal is able to bypass the need for mTOR signaling. Proliferation in particular was still important in the absence of mTOR. Ly49H-mediated proliferation can be independent of IL-15 (ref. 32); this may explain the nonessential role of mTOR in this context. We hypothesize that Ly49H-dependent signals compensate in part for the lack of IL-15 responsiveness in the induction of NK cell proliferation. In CD8\(^+\) T cells, several pathways converge at the level of the phosphorylation of S6 to control metabolic signaling\(^\text{40}\). In particular, pathways involving the mitogen-activated protein kinase MEK and Erk contribute to the phosphorylation of S6, and this might compensate for mTOR deficiency in NK cells\(^\text{40}\).

How mTOR is activated downstream of IL-15 receptor in NK cells remains unsolved. So far, mTORC2’s mode of activation remains elusive but involves PI(3)K\(^\text{41}\). As for mTORC1, the exact sequence of events that lead to its activation in T cells and in particular the involvement of PI(3)K signal are still debated\(^\text{42}\). However, a relevant fact is the observation that NK cells doubly deficient in both the p110\(\gamma\) and p110\(\delta\) catalytic subunits of PI(3)K\(^\text{45}\) or deficient in p110\(\delta\) alone\(^\text{44,45}\) have a phenotype partially convergent with that of NK-Mtor\(^-\text{−/−}\) NK cells, albeit milder. Indeed, a defect in PI(3)K signaling prevents the final maturation of NK cells\(^\text{43,45}\) and impairs their response to activating receptors on NK cells\(^\text{43,45}\). The importance of PI(3)K in the responsiveness of NK cells to IL-15 was not tested in those studies\(^\text{43,45}\). This suggests that mTOR activity is controlled partially but not exclusively by PI(3)K in NK cells.

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

The phenotype of NK-Mtor\(^-\text{−/−}\) NK cells correlates well with the metabolic requirements of NK cells during development and activation. Indeed, NK cell differentiation was associated with the onset of cell quiescence, as shown by the decrease in cell size and uptake of glucose concomitant with loss of the transmembrane nutrient receptors CD71 and CD98. These changes were regulated transcriptionally, since we observed higher expression of genes encoding molecules in categories of metabolic terms associated with decreased cell cycling and increased catabolism along differentiation. We propose that deletion of mTOR blocks subsequent differentiation by blocking early steps of NK cell development associated with proliferation. In this context, the developmental defect observed in NK cells obtained from mice under caloric restriction might be due to partial inhibition of mTOR\(^\text{47}\). Moreover, it has become evident that metabolic regulators control effector T cell function\(^\text{18}\). We made a similar observation with NK cells, since deletion of mTOR greatly diminished their ability to be activated in response to treatment with poly(I:C). Indeed, despite their normal upregulation of the expression of early markers such as CD69, NK-Mtor\(^-\text{−/−}\) NK cells had lower expression of granzyme A and granzyme B. Moreover, the responses of mTOR-deficient NK cells to the triggering of activating receptors on NK cells and in particular degranulation were completely insensitive to injection of poly(I:C). Therefore, mTOR controls a key checkpoint for the activation of NK cells. How mTOR mediates these effects remains a field for further investigation. We found that IL-15-mediated activation of NK cells induced a substantial increase in glycolysis and respiration. These essential bioenergetic pathways probably support the enhanced energetic demands associated with cellular activation and proliferation. Moreover, mTOR is known to increase mRNA translation both qualitatively and quantitatively through inactivation of 4EGBP1 (ref. 48). This could be particularly relevant for NK cells that contain large amounts of untranslated mRNA encoding efferector molecules such as granzyme B or perforin\(^\text{25}\). Finally, mTOR also regulates rearrangements of the cytoskeleton through the kinase PKC-\(\alpha\) (ref. 49), which could be important for cell–cell interactions required for proper activation of NK cells. For example, the role of the ‘trans-presentation’ of IL-15 by dendritic cells to NK cells during inflammation is well described in the literature\(^\text{50}\).

Rapamycin is a clinically approved inhibitor of mTORC1 that is used mainly in renal transplantation, in the treatment of renal and breast cancers and in the treatment of afflictions such as tuberous sclerosis\(^\text{33}\). Several derivatives of rapamycin and ATP-competitive inhibitors of the active site of mTOR have also been developed and tested clinically. mTOR is also viewed as a potential target for antiaging therapy, and some of the aforementioned inhibitors could be used on healthy patients\(^\text{33}\). Given our results obtained with mice, we tested the effects of some of these inhibitors on the cytotoxicity of NK cells. Such treatment resulted in inhibition of the induction of granzyme B following stimulation with IL-15 in vitro. In vivo treatment with Sirolimus (a clinical form of rapamycin) noticeably
decreased the cytotoxicity of NK cells toward missing-self target cells. Notably, these results could be ‘translated’ to humans, since activation of NK cells from healthy donors was inhibited by inhibitors of mTOR; this emphasizes the evolutionarily conserved role of this kinase in the control of NK cell cytotoxicity. These results could have broad implications in the design of therapies that target mTOR.

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

**METHODS**

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

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

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**AUTHOR CONTRIBUTIONS**

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

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Mice. This study was carried out in strict accordance with the French recommendations in the Guide for the ethical evaluation of experiments using laboratory animals and the European guidelines 86/609/CEE. All experimental studies were approved by the bioethic local committee CECAPP. Wild-type C57BL/6 mice were from Charles River Laboratories. NKp46-iCre mice were crossed with Mtorlox/lox mice and bred in animal facility of the T.W. laboratory. Female mice 8–24 weeks of age were used unless specified otherwise. For some experiments, mice were given intraperitoneal injection of 150 µg poly(I:C) (polyinosinic-polycytidylic acid; Invivogen) and were killed 4 or 18 h later. For infection with MCMV, mice were infected intraperitoneally with 50,000 plaque-forming units of MCMV, Smith strain.

Flow cytometry. Single-cell suspensions of BM, blood, spleen and liver were obtained and stained. Foxp3 Fixation/Permeabilization Concentrate and Diluent (BD) were used for intracellular staining of EF4BP4, T-bet, Eomes, K67, granyme A and granyme B. Cytofix/Cytoperm (BD Biosciences) was used for intracellular staining of mTOR. Lyse/fix and PermIII buffers (BD Biosciences) were used for intracellular staining of phosphorylated proteins. For analysis of the incorporation of BrdU, BrdU (2 mg per mouse) was injected intraperitoneally on days 0 and 1, mice were killed on day 2 and incorporation was measured with a kit (Becton-Dickinson). Cell viability was measured by staining with annexin V and 7-AAD (7-aminooctanoyclinomycin D; BD Biosciences). A FACScanto, LSR II or FACSFortessa (all from Becton-Dickinson) or a Navios 5 (Beckman Coulter) was used for flow cytometry. Data were analyzed with FlowJo software (Treestar).

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

Whole blood samples were collected from healthy human donors by venipuncture into heparin-containing vials. Peripheral blood mononuclear cells were then isolated by Ficoll gradient centrifugation and were stimulated for 36 h at 37 °C in 1,000 U/ml recombinant human IL-2 in the presence or absence of mTOR inhibitors. The Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience) was used for intracellular staining to assess phosphorylated S6, granyme B and perforin.

NK cell purification. NK cells were purified with biotinylated anti-CD3 (2C11), anti-CD19 (eBio1D3), anti-CD5 (53-7.3), anti-CD24 (M1/69), anti-F4/80 (BM8) and anti-Ly6G (1A8; all from eBioscience), which were then recognized by anti-biotin beads (Miltenyi) before passage onto an AutoMACS (Miltenyi) with the Deplete5 program.

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

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

In vivo cytotoxicity assay. Splenocytes from C57BL/6 or β2-microglobulin-deficient mice were labeled with CellTraceViolet (1 µM; Invitrogen) or CFSE (carboxyfluorescein diacetate succinimidyl ester; 5 µM; Invitrogen), respectively, and 5 × 10⁶ to 10 × 10⁶ cells were transferred into recipient mice by intravenous injection. 14 h after transfer, splenocytes were isolated and analyzed by flow cytometry. The abundance of the remaining β2-microglobulin-deficient cells was calculated by the following formula: % remaining cells = 100 × (β2-microglobulin-deficient cells/wild-type cells) at 14 h/ (β2-microglobulin-deficient cells/wild-type cells) at 14 h. For statistical analysis, Student’s t-test was used.

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

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