NK cell development requires Tsc1-dependent negative regulation of IL-15-triggered mTORC1 activation

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Activation of metabolic signalling by IL-15 is required for natural killer (NK) cell development. Here we show that Tsc1, a repressor of mTOR, is dispensable for the terminal maturation, survival and function of NK cells but is critical to restrict exhaustive proliferation of immature NK cells and activation downstream of IL-15 during NK cell development. Tsc1 is expressed in immature NK cells and is upregulated by IL-15. Haematopoietic-specific deletion of Tsc1 causes a marked decrease in the number of NK cells and compromises rejection of ‘missing-self’ haematopoietic tumours and allogeneic bone marrow. The residual Tsc1-null NK cells display activated, pro-apoptotic phenotype and elevated mTORC1 activity. Deletion of Raptor, a component of mTORC1, largely reverses these defects. Tsc1-deficient NK cells express increased levels of T-bet and downregulate Eomes and CD122, a subunit of IL-15 receptor. These results reveal a role for Tsc1-dependent inhibition of mTORC1 activation during immature NK cell development.
N atural killer (NK) cells have critical roles in innate immunity against ‘unwanted’ cells, such as transformed tumour cells and virally infected cells, as well as that against class I major histocompatibility complex (MHC-I)-mismatched allogeneic bone marrow. NK cell development occurs in the bone marrow of adults, and it is strictly dependent on signalling triggered by the pleiotropic cytokine interleukin-15 (IL-15). Thus, mice lacking either IL-15 or any one of the IL-15 receptor subunits, including the α, β and γ chains, display severely abnormal NK cell development1-2. IL-15 signalling is also thought to be involved in the maintenance of NK cell survival and homeostasis in peripheral immune niches. This cytokine supports NK cell development and survival mainly through the activation of a FOXO-dependent transcriptional programme and the prevention of Bim-mediated apoptosis3-5. IL-15-mediated signalling is also essential for NK cell homeostasis in vivo, primarily due to its regulation of the level of the anti-apoptotic protein Mcl-1 (ref. 6). In view of its importance to NK cell physiology, IL-15 signalling must be actively regulated to prevent its aberrant activation, which is likely detrimental to NK cell development, homeostasis and quiescence.

IL-15-responsive NK cell progenitors (NKp), characterized by the expression of IL-2Rβ (CD122), a subunit of IL-15 receptor, require the common gamma chain (γc/IL-2Rγ/CD132) to transduce activating signals. IL-15 stimulation primarily activates the kinase function of Janus kinase (JAK), which leads to phosphorylation and activation of signal transducer and activator of transcription 3 (STAT3) and STAT5. Thus, humans or mice lacking JAK and STAT5A/B display impaired NK cell development7. IL-15 dramatically induces the expression of suppressor of cytokine signalling 2 (SOCS2) during its late phase of stimulation, which negatively modulates the JAK-STAT pathway, suggesting that SOCS2 is likely required for NK cell function due to its downregulation of IL-15 signalling8.

IL-15 also has the ability to biochemically activate PI3K pathways10. Mice simultaneously lacking the PI3K subunits PI10 γ and δ have severe defects in early NK cell development11,12. Most recently, we have observed that PDK1 deficiency causes an almost 95% reduction in the number of NK cells13, comparable to p110 γ and δ double-mutant mice14 strongly suggesting that PI3K regulates NK cell development largely via the recruitment of PDK1. PI3K activity is negatively controlled by phosphorylation and tensin homologue deleted on chromosome ten and Src homology 2 domain-containing inositol-5-phosphatase-1. Mice lacking either phosphoryat and tensin homologue deleted on chromosome 10 or Src homology 2 domain-containing inositol-5-phosphatase-1 exhibit abnormal NK cell development and functionality, demonstrating the crucial roles of these two proteins in actively maintaining NK cell function15-17.

The distal signalling downstream of PI3K activation activates mammalian target of rapamycin (mTOR), which is a component of two active complexes, mTOR complex 1 (mTORC1) and mTORC2. mTOR kinase has been reported to play a crucial role as a key metabolic checkpoint in NK cell proliferation and activation18. The signalling mediated by mTOR regulates early NK cell development mainly via the induction of E4BP4, an NK cell-specific transcription factor, which maintains IL-15 responsiveness by upregulating CD122 expression. Therefore, the activity of IL-15 is amplified via a positive feedback loop19; however, the activation of mTOR must be tightly regulated according to this activity. One of the most indispensable proteins involved in this process is tuberous sclerosis 1 (Tsc1). This peripheral membrane protein has been implicated as a tumour suppressor. Tsc1 forms a complex with Tsc2, which regulates mTORC1 signalling, and this process is mediated by Akt. Human Tsc1 mutations may result in tuberous sclerosis by causing functional impairment of the hamartin-tuberin complex. Interestingly, Tsc1 plays critical roles in immune processes, such as T-cell differentiation20,21, peripheral T-cell homeostasis22, dendritic cell development23 and natural killer T (NKT) cell terminal differentiation24. Tsc1 is also required for the generation of memory CD8+ cells, a process that strictly requires IL-15 signalling25. However, it remains unknown whether Tsc1 is necessary for restraining IL-15/mTORC1 signalling during NK cell development, homeostasis and functioning.

To address how IL-15 signalling is negatively regulated in NK cells, in the current study, we examine dynamic changes in the expression of negative regulators of two of the above-mentioned signalling pathways, JAK-STAT and PI3K/mTOR, after IL-15 triggering. Tsc1 is found to be upregulated at the late time point of IL-15 stimulation. Thus, we generate Tsc1-deficient mice to dissect the roles of Tsc1 in IL-15 signalling and NK cell development. We identify a unique role of Tsc1-dependent inhibition of mTORC1 activity in the optimization of IL-15 signalling in the early stage of NK cell development.

**Results**

**IL-15 stimulation upregulates Tsc1 in NK cells.** To investigate how IL-15-mediated signalling is negatively controlled, we measured the messenger RNA levels of several potential suppressors involved in the JAK-STAT pathway and PI3K/mTOR pathway in NK cells stimulated with a high dose of IL-15 for 18 h (h). As expected, Socs2, a negative regulator of the JAK-STAT pathway, was found to be upregulated, as previously reported9. Notably, the expression of Tsc1 was increased by over twofold after IL-15 triggering (Fig. 1a). The stimulation of NK cells by a gradient concentration of IL-15 resulted in a dose-dependent increase in Tsc1 expression (Fig. 1b). An in-depth analysis demonstrated that Tsc1 expression was slightly suppressed 3 h after IL-15 stimulation but then gradually increased at the later time points (9–18 h; Fig. 1c). To examine whether the IL-15-induced changes in Tsc1 expression correlates with mTOR activity, the phosphorylation of S6K (pS6K), an indicator of mTORC1 activation, was measured. Tsc1 expression was decreased at the earliest time of IL-15 stimulation (3 h) whereas pS6K was upregulated. At 9–18 h after IL-15 stimulation, however, Tsc1 expression was increased whereas pS6K was downregulated to the baseline level (Fig. 1d). Taken together, these results indicate that Tsc1 likely acts as a negative regulator to prevent prolonged IL-15-induced mTORC1 activation.

To understand how the dynamic regulation of Tsc1 in response to IL-15 was achieved, Tsc1 expression was monitored after treatment with rapamycin, an inhibitor of mTORC1. This treatment significantly counteracted the upregulation of Tsc1 by IL-15 (Fig. 1e). Therefore, the increased expression of Tsc1 is dependent on mTORC1 activation.

**Tsc1 is mainly expressed in immature NK cells.** To understand the physiological role of Tsc1 in NK cell development, the expression levels of Tsc1 were compared among the three major types of lymphocytes. Compared with T and B lymphocytes, Tsc1 was highly expressed in common lymphoid progenitor (CLP) and NK cells (Fig. 1f,g), mainly NKp, as well as relatively immature NK cells (CD27-CD11b-). The Tsc1 level was found to gradually decrease with NK cell maturation (Fig. 1g). The dynamic expression of Tsc1 suggests that this protein might be involved in IL-15-regulated early NK cell differentiation.

**Tsc1 deletion affects the number of T and B cells.** We first generated haematopoietic-specific Tsc1-deleted mice, Tsc1fl/fl/Vav1-Cre+ (referred to as Tsc1−/− mice). The efficiency

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of Tsc1 deletion was confirmed by quantitative PCR (Supplementary Fig. 1a). Given that two previous studies have established that inducible knockdown of Tsc1 can lead to abnormal hematopoietic stem cell (HSC) numbers, we initially determined whether Tsc1 deletion affected the generation of HSCs and CLPs in our model. The total numbers of Tsc1-deleted bone marrow cells was much lower than in wild-type (WT) mice (Supplementary Fig. 1b); the percentage of HSCs in Tsc1-deleted mice were higher, but the absolute numbers of HSCs were slightly lower than in WT mice. Despite this observation, the absolute numbers of CLPs were comparable between the two genotypes (Supplementary Fig. 1c–e), which allowed us to study lymphocyte development in the Tsc1+/− mice.

The numbers of T and B cells were first measured. The Tsc1−/− mice had considerably fewer T cells (including CD1d-tetramer-reactive NKT cells) in the spleen (Supplementary Fig. 2a,b), and the residual Tsc1-deficient T cells had an expanded CD62LloCD44hi population with an activated or memory phenotype (Supplementary Fig. 1c); however, the CD4+ and CD8+ T-cell subsets displayed normal selection in the Tsc1−/− thymus and had comparable numbers of maturation-related markers, such as CD62L and CD69 (Supplementary Fig. 1d,e), suggesting that Tsc1 deficiency affects T-cell homeostasis but not development, as previously reported. We further revealed that the Tsc1−/− mice had normal number of pro-B cells, but they had reduced numbers of pre-B, immature and mature B cells, suggesting that the deletion of Tsc1 blocked B-cell development at an early stage (Supplementary Fig. 3). Taken together, Tsc1 has different roles in the development and homeostasis of adaptive immune cells.

Tsc1-deficient mice have a minimal pool of NK cells. Next, we focused on the roles of Tsc1 in innate NK cell physiology. Notably, the NK cell numbers were reduced by over 90% in the spleens and bone marrow of the Tsc1−/− mice (Fig. 2a,b). Remarkable reductions in the numbers of NK cells were also observed in other organs, including the lymph nodes, liver and lungs (Fig. 2a,b). To address whether the diminished NK cell pools in the Tsc1−/− mice were cell-autonomous defects, and were not due to the possible defect of HSC generation, Lin−CD122+ NK1.1− NKp cells isolated from CD45.1+ WT and CD45.2+ Tsc1−/− mice were mixed at a 1:1 ratio, then adoptively transferred into immunodeficient Rag1−/−γc−/− mice. NKp cells from the CD45.1+ mice showed much greater repopulation activity than those from the Tsc1−/− mice (Fig. 2c). Therefore, the critical requirement of Tsc1 for NK cell development is cell intrinsic.

Tsc1 regulates immature NK cell development. NK cells sequentially acquire differentiation markers with their maturation. Based on these markers, NK cell development is divided into multiple stages. Tsc1−/− mice had comparable numbers of NKp cells. The proportion of early CD3−CD122+ NK1.1− CD11b− immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e). The proportions and absolute numbers of mature NK cells that are CD3+CD122−NK1.1+CD11b+ immature NK cells was much higher in the Tsc1−/− mice, but their absolute numbers were dramatically reduced (Fig. 2d,e).
mice, the ability of NK cells to mediate ‘missing-self’ rejection was evaluated, as previously described. We found that MHC-1-missing splenocytes were largely eliminated by NK cells in control mice; however, NK cell-mediated killing was barely detectable in Tsc1−/− mice (Fig. 3a). We further examined the NK cell-mediated in vivo rejection of RMA-S cells, an NK-sensitive tumour cell line, and observed that the severe defect in the Tsc1−/− mice was nearly comparable to that in immunocompromised mice (Fig. 3b). Lastly, we analysed whether Tsc1 deletion affected the ability of NK cells to prevent the metastasis of B16 melanoma. Notably, the lung metastasis of the B16 tumor cell line, and observed that the severe defect in the anti-metastasis of B16 melanoma, even in the absence of T and B cells (Fig. 3d). Thus, the absolute number of NK, if NK and mNK cells in splenic (d) and bone marrow (e) cells were quantified. (f) Flow cytometry analysis of development-related NK cell receptors on NK1.1+/− B220+/− CD3− cells in the spleen. The numbers indicate percentages of receptor-positive NK cells. All data represent at least three independent experiments and calculated data are shown as means ± s.d. *P<0.05, **P<0.01 and ***P<0.001. Unpaired Student’s t-tests (two-tailed) was used to calculate these values.

**Constitutive mTORC1 activation disrupts NK cell development.** We next determined the biochemical mechanisms by which Tsc1 controls NK cell quiescence. We first measured the effect of Tsc1 deficiency on mTORC1 activity by examining phosphorylation of the ribosomal protein S6. Freshly isolated Tsc1−/− NK cells exhibited an increase in the basal activity of this conventional mTORC1 target compared with naïve WT NK cells. However, Tsc1−/− NK cells had the same level of phosphorylation of Akt (Fig. 4a,b). Using Fucci-2 reporter mice, in which cells in the S, G2 and M phases of the cell cycle were fluorescent, we observed that 5–9% of Tsc1−/− NK cells exhibited proliferating activity compared with 3% of WT NK cells (Fig. 4c). Therefore, Tsc1 is a negative regulator of NK cell proliferation.

In agreement with this finding that Tsc1−/− NK cells proliferate rapidly, freshly isolated Tsc1−/− NK cells were consistently larger size and showed more cells containing (Fig. 4d). Furthermore, the expression levels of the nutritional markers CD71 and CD98, as well as that of the activation marker CD69, were upregulated in these cells (Fig. 4e,f). Further experiments using mixed bone marrow chimera assays showed that the constitutive activation in the Tsc1−/− mice was NK cell intrinsic (Fig. 4g,h). The phenotype was also observed in Tsc1−/− Rag1−/− mice (Supplementary Fig. 4a,b). Thus, Tsc1 regulates NK cell development, likely by restricting the proliferation of immature NK cells.
To genetically confirm the requirement of optimal mTORC1 activity for early NK cell differentiation, we generated Tsc1\(^{-/-}\) mice that were heterozygous for Raptor, a scaffold protein of mTORC1 (Tsc1\(^{-/-}\)/Raptor\(^{+/+}\)+/Vav1-Cre\(^{+}\) mice, referred to as Tsc1\(^{-/-}\)-Raptor\(^{+/+}\) mice in brief), or for Rictor, which is a major component of mTORC2 (Tsc1\(^{-/-}\)/Rictor\(^{+/+}\)+/Vav1-Cre\(^{+}\) mice, referred to as Tsc1\(^{-/-}\)-Raptor\(^{+/+}\) mice in brief). The genetic knockdown of Raptor markedly increased the percentage and absolute number of NK cells in the Tsc1\(^{-/-}\) mice. However, the inactivation of a single allele of Rictor exacerbated the defective NK cell development in Tsc1\(^{-/-}\) mice, suggesting that mTORC2 plays a role in NK cell development (Fig. 5c). Furthermore, the partial inactivation of mTORC1 activity appeared to attenuate the increased activation in Tsc1\(^{-/-}\) NK cells (Fig. 5d,e). These results clearly demonstrate that the exacerbated mTORC1 activity in Tsc1\(^{-/-}\) mice is detrimental to the early development of immature NK cells.

**Tsc1 deficiency decreases the expression of Eomes and CD122.**

Metabolic activation plays an essential role in T-cell differentiation by regulating the expression of transcription factors. Thus, we sought to determine whether the over-activation of mTORC1 altered the expression profile of transcription factors in Tsc1\(^{-/-}\) mice. Intracellular staining of T-bet and Eomes, two T-box family transcription factors involved in the NK cell development, was performed (Fig. 6a). T-bet was over-expressed in Tsc1\(^{-/-}\) NK cells compared with Tsc1-intact NK cells. By contrast, the amount of Eomes was dramatically reduced in Tsc1-null NK cells, which was more apparent in relatively immature CD27\(^{-}\)CD122\(^{-}\)NK cells, as compared with Tsc1-intact NK cells (Fig. 6b). The decreased amount of CD122 in Tsc1\(^{-/-}\) NK cells could antagonize one another's expression in NK cells. To avoid confounding effects of comparing NK cell development in two different genotypes, we also examined the expression of the two transcription factors in a developmentally matched comparison, which revealed that Tsc1\(^{-/-}\) NK cells exhibited more T-bet, but less Eomes, at all stages (Fig. 6c). Thus, Tsc1-deficient NK cells had a deficiency in the expression of transcription factors involved in NK cell development.

Eomes regulates NK cell development mainly through transcriptional regulation of CD122, such that Eomes-deficient NK cells have less CD122 (ref. 32). These previous data motivated us to examine CD122 level in Tsc1\(^{-/-}\) NK cells. As expected, CD122 expression was dramatically reduced in Tsc1\(^{-/-}\) NK cells (Fig. 6d). The decreased amount of CD122 in Tsc1\(^{-/-}\) NK cells...
Tsc1-deficient NK cells undergo apoptosis. NK cells usually require high levels of CD122 to respond to IL-15. Deficient IL-15 signalling leads to Bim-dependent NK cell apoptosis. We noted that Tsc1−/− NK cells exhibited increased caspase activity and Annexin V staining, which are two hallmarks of apoptotic cell death (Fig. 7a). The increased apoptosis might result from the over-activation of mTORC1 because the reduction of Raptor could largely diminish the number of apoptotic NK cells in Tsc1−/− mice (Fig. 7b). Interestingly, upon IL-15 stimulation, Tsc1−/− NK cells were more likely to undergo apoptosis, whereas the apoptotic death of Tsc1−/− Raptor−/− NK cells was much less pronounced (Fig. 7b). These data suggest that Tsc1 plays an important role in preventing activation-induced NK cell apoptosis.

The balance of pro- and anti-apoptotic members of the Bcl2 family controls the survival of immune cells. Among these members, Bim is considered one of important initiators of NK cell apoptosis. Although the expression of anti-apoptotic Bcl2 was not significantly affected by Tsc1 deficiency, Tsc1−/−
Figure 5 | Constitutive activation of mTORC1 is detrimental to NK cell development. (a) Intracellular phosphorylated S6 and AKT5473 in splenic NK cells from WT and Tsc1<sup>−/−</sup> mice were detected by flow cytometry. Representative overlaid histogram are shown (left panels). WT (grey dash lines) or Tsc1<sup>−/−</sup> (grey solid lines) NK cells stained with isotype antibody; WT (red lines) or Tsc1<sup>−/−</sup> (blue lines) NK cells stained with the antibodies against pS6 (up) or AKT5473 (bottom). The mean fluorescence intensity (MFI) was calculated (right panels). (b) Left, representative flow cytometric plots showing the percentages of CD3<sup>+</sup> NK cells (10<sup>5</sup>) NK cells in spleens from WT and Tsc1<sup>−/−</sup> mice administered a mock injection (DMSO) or treated with a daily injection of rapamycin for 5 days. The numbers near the indicated square boxes show the percentages of CD3<sup>+</sup> NK cells in the spleens of the indicated mice. Right panel, the absolute numbers of CD3<sup>+</sup> NK cells (10<sup>5</sup>) NK cells in the spleens of the indicated mice. The quantification of percentages and absolute numbers of CD3<sup>+</sup> NK cell was also presented. (d,e) FSC and SSC (d), CD71 and CD98 (e) on freshly isolated NK cells from the indicated mice were detected by flow cytometry, and the MFI was calculated. Each symbol represents an individual mouse. Data are showed as mean ± s.d. and are representative of two independent experiments. **P<0.01 and ***P<0.001. Unpaired Student’s t-tests (two-tailed) was used to calculate these values.

NK cells showed elevated Bim expression in the resting state (Fig. 7c). To determine whether Tsc1<sup>−/−</sup> NK cells undergo Bim-dependent apoptosis, Tsc1<sup>−/−</sup> mice were bred with Bim<sup>+/+</sup> mice to yield Tsc1<sup>−/−</sup>Bim<sup>−/−</sup>/Vav1-Cre<sup>+</sup> mice (referred to as Tsc1<sup>−/−</sup>Bim<sup>−/−</sup> mice). As a result, the additional deletion of Bim partially but significantly increased the frequency and absolute number of NK cells in Tsc1<sup>−/−</sup>Bim<sup>−/−</sup> mice compared with those in Tsc1<sup>−/−</sup> mice (Fig. 7d). Moreover, the removal of Bim largely counteracted the increased sensitivity of Tsc1<sup>−/−</sup> NK cells to IL-15-induced apoptosis (Fig. 7e). However, the Bim deletion failed to correct the abnormal activation of Tsc1<sup>−/−</sup> NK cells (Supplementary Fig. 5), suggesting that Bim-dependent NK cell death in Tsc1<sup>−/−</sup> mice is most likely a consequence of the activation. Taken together, the
Bim-dependent apoptotic pathway only partially mediates the death of \( \text{Tsc} ^{1/−} \) NK cells, indicating that other apoptosis pathways may exist\(^4\).

**Tsc1 is dispensable for NK cell terminal differentiation.** The above findings reveal a very unique role for Tsc1 in early NK cell differentiation. To further investigate whether Tsc1 is required for NK cell terminal differentiation and function, we generated a \( \text{Tsc} ^{1/β}/\text{Ncr1-Cre} \) mouse model in which Tsc1 was deleted during the late stage of NK cell development by the enzyme Cre, which is controlled by the promoter of Ncr1, which encodes Nkp46 in mice. Cre expression in terminal NK cells lead to an 80% reduction of \( \text{Tsc} ^{1/−} \) NK cells at the metabolic and transcriptional levels\(^33,34\). Although IL-15 mismatched \( \beta 2m ^{−} \) splenocytes and killed the RMA-S cells, comparable to the control mice (Fig. 8e,f).

To exclude the possibility that the dispensable activity of Tsc1 observed during the late stage of NK cell differentiation and function was due to the unsuccessful deletion of Tsc1 in the model, we further bred \( \text{Tsc} ^{1/β}/\text{Ncr1-Cre} \) mice with eYFP\(^{STOP} \) reporter mice to yield \( \text{Tsc} ^{1/β}/\text{eYFP}\(^{STOP}/\text{Ncr1-Cre} \) mice, in which the Cre-expressing NK cells were mostly eYFP-positive. Tsc1 messenger RNA was hardly detectable in the eYFP\(^{+} \) \( \text{Tsc} ^{1/β}/\text{Ncr1-Cre} \) NK cells (Supplementary Fig. 6a). Using this fate-mapping strategy, we failed to observe apparent differences in NK cell functions, such as cytokine production and degranulation, between the two genotypes when NK cells were triggered by several types of stimuli, including tumour cells, plate-coated antibodies and a cytokine cocktail (Supplementary Fig. 6). We therefore concluded that Tsc1 is not required for terminal NK cell development and effector functions.

**Discussion**

NK cells are the most important innate lymphocytes that mediate tumour surveillance. A fundamental immunological question is how the development and homeostasis of NK cells are regulated at the metabolic and transcriptional levels\(^33,34\). Although IL-15 has been confirmed to be indispensable for these processes\(^35,36\).
and multiple intracellular signalling pathways and transcription factors that promote NK cell development have been extensively studied, knowledge is limited regarding how these signalling networks are negatively regulated. In addition, NK cells are regulated to maintain their quiescence for immune homeostasis, but the manner by which their quiescence is carefully maintained also remains undefined. In this study, using two genetic mouse models, we dissected the critical role of Tsc1 in the maintenance of NK cell quiescence. The disruption of Tsc1 during the early stage of NK cell commitment (using Vav1-Cre) led to a large decrease in the number of NK cells. Intriguingly, inactivation of Tsc1 at the late stage (using Ncr1-Cre) had no obvious effect on terminal NK cell maturation or function. Therefore, our results reveal a unique role for Tsc1 in regulating NK cell development in the early stage by controlling IL-15-stimulated mTORC1 activation.

Human patients with Tsc1 deficiency have a higher tumour incidence; in fact, Tsc1 was originally regarded as a tumour repressor\cite{37,38}, and it is intrinsically required for normal cells to suppress the undue activation of mTORC1, which is believed to have increased activity in most tumors\cite{39}. The immune system plays an important role in the surveillance of malignancy. With increased understanding of the comprehensive roles of Tsc1 in immune cells, such as adaptive T and B cells, as well as in innate immune cells, including dendritic cells, it is reasonable to speculate that the increased tumour incidence in Tsc1-deficient patients is likely correlated with compromised antitumor immunity. Among the immune cells, NK cells are generally considered to be the innate lymphocytes responsible for the immunesurveillance of tumour cells. In this study, we discovered that the loss of Tsc1 caused a decrease in the number of NK cells and that this decrease was strongly associated with the lung metastasis of melanoma. Further research is required to determine whether NK cells are defective in patients with Tsc1 mutations, which may explain the high incidence of tumors in Tsc1-null patients. Therefore, Tsc1 may play an extrinsic role in the suppression of tumour growth via regulation of the cellularity of NK cells.

Emerging evidence obtained using mouse genetic models has revealed that IL-15-mediated metabolic activation is critical for NK cell development via the triggering of mTOR activation and the upregulation of nutritional receptors\cite{13,18,40}. The NK cell-specific deletion of mTOR (Ncr1-cre generated by Eric Vivier’s laboratory) markedly impaired NK cell differentiation and activation\cite{18}. In addition, our recent results have revealed that the deletion of PDK1, an upstream mTOR kinase, causes severe defects in NK cell development\cite{13}; however, the manner by which mTOR activation regulates NK cell development remains unknown. Further, it is unclear whether mTORC1 or 2 is involved in IL-15 signalling and how the distal signalling is negatively controlled. In the current study, Tsc1 expression was found to be dynamic following IL-15 stimulation. During the very early stage, IL-15 slightly inhibited Tsc1 expression, accompanied by an increase in mTORC1 activation. Thus, the transient downregulation of Tsc1 likely highlights a novel mechanism that facilitates transcriptional events promoting early NK cell development via a positive feedback loop involving PDK1-Mtor/E4BP4/Eomes/CD122 (ref. 13); however, continuous mTORC1 activation could increase Tsc1 expression during the late stage of IL-15 stimulation, thereby initiating a mechanism of feedback inhibition to suppress the prolonged mTOR activation. Therefore, we have identified a regulatory mechanism of IL-15...
crelymphocytes were prepared from poly I:C-treated mice and stimulated with plate-coated antibodies or cytokine cocktail; the bar graphs show the average significant difference.

Moreover, Rictor-containing mTORC2 may participate in early NK cell differentiation independent of mTORC2 activation. This finding has two defects in Tsc1-deficient NK cells. This finding indicates that the removal of one allele of Raptor also largely diminishes the abnormal phenotype of Tsc1-deficient mice. Thus, the Tsc1-dependent control of mTORC1 is vital for the functioning of the innate and adaptive immune systems.

Although convincing evidence suggests that increased mTORC1 activity contributes to the disruption of early NK cell quiescence, we unexpectedly found that inactivation of a main component of mTORC2, Rictor, aggravated the developmental defects in Tsc1-deficient NK cells. This finding has two implications. First, it further confirms that Tsc1 regulates early NK cell differentiation independent of mTORC2 activation. Moreover, Rictor-containing mTORC2 may participate in early NK cell development in an mTORC1-independent manner. Thus, further studies involving the genetic inactivation of mTORC1 and mTORC2 will help to elucidate the multifaceted role of mTOR in NK cell development.

A previous study has reported that Tsc1 is critical for the regulation of T-cell quiescence and function. Tsc1 deletion causes no obvious developmental defects in T cells, with the exception of NKT cells in the thymus; therefore, Tsc1 only affects the quiescence of terminally differentiated T cells. Similarly, Tsc1 is only involved in NKT cell terminal maturation through the regulation of T-box transcription factor expression. Moreover, Tsc1 regulates effector functions; for example, Tsc1-deficient T cells also exhibit hyporesponsiveness to T-cell receptor triggering. In contrast, in this study, we have revealed a distinct role of Tsc1 in NK cell biology. The stage-specific deletion of Tsc1 allowed us to observe the dispensable role of Tsc1 in terminal NK cell differentiation and effector functions. NK cells with terminal deletion of Tsc1 exhibit normal survival and function, strongly suggesting that mTORC1 signalling is not strictly required for other receptors involving NK activation. Furthermore, although IL-15 is mandatory for NK cell survival and homeostasis, terminal deletion of Tsc1 had no visible effect on peripheral NK cell homeostasis. In contrast, early deletion of Tsc1 caused severe defects in NK cell differentiation. These findings indicate that Tsc1 plays a unique role in IL-15-mediated mTORC1 activation, which is critical for NK cell development.

In summary, NK cell development in the early stage is tightly controlled by IL-15 signalling. Negative regulation of this process by Tsc1 is essential for NK cell development. Tsc1 deficiency causes the hyper-activation of mTORC1, which is detrimental to
immature NK cell development. Thus, we have identified a unique checkpoint that is crucial for NK cell development. Given the importance of the adoptive transfer of NK cells in immunotherapy, the optimal mTORC1 activation triggered by IL-15 may provide new strategies for the ex vivo expansion of NK cells.

Methods

Mice. The Tsc1<sup>flox/flox</sup> mice were a gift from Dr. Hongbin Zhang (Beijing University Medical College). Chidar, Tsc1<sup>cKO</sup> mice were backcrossed to C57BL/6 background for eight generations before being bred with the other mice. Haematopoietic or NK cell-specific Tsc1-deficient mice were generated by crossing Tsc1<sup>fl</sup> with Vav1-Cre (B6.Cg-Tg(Vav1-cro)2Akie/J, Jackson lab, stock Number, 008610) or Ncr1-Cre mice generated in our lab<sup>1</sup>. Fucci-2 reporter mice were a gift from Dr. Hau Qi (Tsinghua University) and crossed with Tsc1<sup>fl</sup>/Vav1-Cre<sup>-</sup> mice to obtain Tsc1-deficient Fucci-2 transgenic mice, where cells in S, G2 and M phases of the cell cycle are fluorescent<sup>29</sup>, Rag1<sup>−/−</sup> mice was described previously<sup>13</sup>. β2m-deficient mice, C57BL/6 mice, CD45.1, Rput<sup>fl</sup> mice, Rictor<sup>fl</sup> mice and Bim<sup>−/−</sup> mice were purchased from Jackson lab. Both female and male mice with age of 8-12 weeks were used in our experiments. All the mice are C57BL/6 background and maintained under specific pathogen-free animal facilities of Tsinghua University. All procedures involving animals were approved by the Animal Ethics Committee of Tsinghua University.

Flow cytometry. Flow cytometry was performed on a BD LSR II (four-laser Blue/Red/Violet/ultraviolet flow cytometry analyzer, BD Biosciences). Monoclonal antibodies against mouse CD3 (548-0032, 17A2, 1:200), NKp46 (11-3351, 29A1.4, 1:100), NK1.1 (17-5941, PK136, 1:200), CD117 (48-1171, 2B8, 1:200), CD11b (17-0112, M1/70, 29A1.4, 1:100), IFN-γ<sup>+</sup> (12-0271, LG.7F9, 1:200), CD27 (12-0271, LG.7F9, 1:200), CD127 (12-0273, SB2, 1:100), Ly40 (12-0276, A1, 1:200), Ly49C1 (555327, 5E6, 1:200), Ly49H1 (17-5886, 3D10, 1:200), Ly6G<sup>+</sup> (46-5781, 4D11, 1:200), CD113 (12-1351, A2F10, 1:200), SCA-1 (45-5981, D7, 1:200), NK2D2 (25-5882, CX5, 1:200), NK2G2/Crl (46-5897, 16a11, 1:200), CD11b (17-0112, M1/70, 1:200), CD207 (12-0271, LC2F9, 1:200), IFN-γ (12-7311, XMG1.2, 1:200), CD107a (12-0271, LG.7F9, 1:100) and isotype controls were purchased from eBioscience (San Diego, CA) or BD Biosciences (Mississauga, Ontario, Canada). Anti-phospho-AKT (473, 427, 1:50), and Anti-phospho-S6 (4856, 1:200) were obtained from Cell Signaling Technology (Beverly, MA). CD14-PBS157 tetramer was kindly provided by NIH tetramer facility. For analysis of surface markers, cells were stained in PBS containing 2% (wt/vol) foetal bovine serum with indicated antibodies to eBioscience or BD. The expression level was presented as net mean fluorescence intensity (AMFI), which was determined by subtracting mean fluorescence intensity of isotype control. For detection of phosphorylated signalling proteins, NK cells were fixed with Phosflow Lyse/Fix buffer, followed by permeabilization with Phosflow Perm buffer III (BD Biosciences) and stained with antibodies.

Real-time PCR. Total RNA from splenocytes, bone marrow cells or FACS-sorted NK, T and B cell subsets was extracted using Trizol Kit (Invitrogen), and reverse-transcribed using reverse transcription system (Promega, A3500). Quantitative PCR was conducted using SYBR green-based detection. The expression level of the genes of interest was determined relative to the expression of β-actin.

Detection of NK cell apoptosis. Splenocytes were stained with Annexin V (BD Biosciences), and Caspase activity was measured with fluorescein isothiocyanate conjugated z-VAD-fmk according to the manufacturer's instruction (eBiosences). In vivo detection of NK cell apoptosis was performed on the transgenic reporter mice expressing green fluorescent protein (GFP; 10<sup>5</sup>) and NK-sensitive RMA expressing DsRed (10<sup>5</sup>). 18h later the mice were killed, and cells in peritoneal cavity were collected by repeated washing with PBS containing 2% MGE EDTA. After centrifugation, cells were resuspended in 1 ml PBS. The relative percentages of RMA-S and RMA cells were measured by flow cytometry. The percentage of RMA-S cell rejection was calculated with the following formula: 100 × [1 - (percentage of residual β2m<sup>−/−</sup> population in total C57BL/6 cells of experimental group/percentage of residual β2m<sup>−/−</sup> population in total C57BL/6 cells of control group)].

In vivo spleenocytes rejection assay. Splenocytes from β2m-deficient (β2m<sup>−/−</sup>) mice were depleted of red blood cells by Ficoll-Hypaque density gradient centrifugation, and then labelled with 5 μM CFSE (Molecular Probes). At the same time, splenocytes from WT mice were labelled with 0.5 μM CFSE (10-fold < β2m<sup>−/−</sup> cells). Two types of CFSE-labelled splenocytes were mixed at 1:1 ratio. A mixture of 2 × 10<sup>6</sup> splenocytes was intravenously injected into mice pretreated with 200 μg Poly I:C for 18h, 18h later, C57BL/6-positive cells in blood, spleen and lymph nodes were determined by flow cytometry. To exclude the role of NK cells in the recipient mice, RAG1<sup>−/−</sup> mice were used. The percentage of β2m<sup>−/−</sup> spleenocytes rejection was calculated with the following formula: 100 × [1 - (percentage of residual β2m<sup>−/−</sup> population in total GFP<sup>−</sup> cells of experimental group/percentage of residual β2m<sup>−/−</sup> population in total GFP<sup>−</sup> cells of RAG1<sup>−/−</sup> mice group)].

In vivo RNA-S clearance assay. Mice treated with 200 μg Poly I:C for 18h were intraperitoneally injected with a mixture of target cells, NK-sensitive RMA-S cells expressing green fluorescent protein (GFP; 10<sup>5</sup>) and NK-non-sensitive RMA expressing DsRed (10<sup>5</sup>). 18h later the mice were killed, and cells in peritoneal cavity were collected by repeated washing with PBS containing 2% MGE EDTA. After centrifugation, cells were resuspended in 1 ml PBS. The relative percentages of RMA-S and RMA cells were measured by flow cytometry. The percentage of RMA-S cell rejection was calculated with the following formula: 100 × [1 - (percentage of residual GFP<sup>−</sup> population in total GFP<sup>−</sup> and DsRed<sup>−</sup> of experimental group/percentage of residual GFP<sup>−</sup> population in total GFP<sup>−</sup> and DsRed<sup>−</sup> cells of RAG1<sup>−/−</sup> mice group)].

B16 melanoma lung metastasis mouse model. B16F10 melanoma cells in the log phase were resuspended in 1 × Hanks Balanced Salt Solution and intravenously injected into the mice (2 × 10<sup>5</sup> cells per mouse), fourteen days later the mice were killed. The lung was weighed and the number of lung surface nodules was counted under a dissecting microscope.

Statistical analyses. Unpaired Student’s t-tests (two-tailed) were performed using the Prism software. A P value of <0.05 was considered significant. *P<0.05, **P<0.01 and ***P<0.001.

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions

M.Y. and S.C. conceived the project, designed and performed most experiments. J.D., J.H., Y.W, Z.L., G.L., W.P., X.Z., D.L. and P.X. performed experiments and analysis. W.G., Z.C., S.W. and Z.T. conceived the study. Z.D. and M.Y. co-wrote the paper.

Additional information

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