PDK1 orchestrates early NK cell development through induction of E4BP4 expression and maintenance of IL-15 responsiveness

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E4BP4, a circadian protein, is indispensable for NK cell development. It remains largely unknown which signal is required to induce E4BP4 expression and what effects it has during NK cell differentiation. Here, we reveal that PDK1, a kinase upstream of mTOR, connects IL-15 signaling to E4BP4. Early deletion of PDK1 caused a severe loss of NK cells and compromised antitumor activity in vivo. PDK1–deficient NK cells displayed much weaker IL-15–induced mTOR activation and E4BP4 induction, as well as remarkable reduction in CD122, a receptor subunit specifying NK cell responsiveness to IL-15. The phenotypes were partially reversible by ectopic expression of E4BP4 or bypassed activation of mTOR. We also determined that PDK1–mediated metabolic signaling was dispensable for NK cell terminal maturation and survival. Thus, we identify a role for PDK1 signaling as a key mediator in regulating E4BP4 expression during early NK cell development. Our findings underscore the importance of IL-15 self-responsiveness through a positive feedback loop that involves PDK1–mTOR–E4BP4–CD122 signaling.

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As a circadian clock gene, E4BP4 expression is dynamic (Doi et al., 2004; Male et al., 2012). In mice, feeding can quickly induce the up-regulation of E4BP4 expression, whereas inhibition of insulin signaling can abolish this activity (Tong et al., 2010). These data raise the possibility that E4BP4 induction in NK cells relies on metabolic signaling, which may be required for NK cell development. The mammalian target of rapamycin (mTOR) is the central checkpoint molecule in the regulation of cell metabolism. mTOR senses and integrates diverse environmental cues, including nutrients and growth factors (Powell et al., 2012; Waickman and Powell, 2012), and exists in two complexes: mTOR complex 1 (mTORC1) and mTORC2. The well-established molecular function of mTORC1 is the initiation of protein translation by phosphorylating p70 S6 kinase (S6K) and the translation-initiating, eIF4E-binding protein (4EBP1). The intimate interaction between metabolism and immunity has attracted much attention (Chi, 2012; Powell et al., 2012; Waickman and Powell, 2012). Most of the metabolic control over cell fate is focused on the activation of adaptive immune cells, such as T cells (Kim et al., 2013; Zeng et al., 2013; Wu et al., 2014). In contrast, the function of mTOR signaling in the development of lymphocytes, particularly NK cells, is rarely reported. Recently, NK cell–specific deletion of mTOR revealed its critical, nonredundant role in the regulation of two key checkpoints in NK cell biology: proliferation in the bone marrow, and activation in the periphery (Marchis et al., 2014).

The PI3K pathway is a major upstream regulator of mTOR–dependent metabolic activation and plays a critical role in cell proliferation and differentiation. Mice simultaneously lacking the PI3K subunits P110γ and δ exhibit a severe defect in early NK cell development (Tassi et al., 2007; Guo et al., 2008). Similarly, NK cell differentiation is also retarded in mice lacking the PI3K subunit p85 (Awashti et al., 2008). 3′-phosphoinositide–dependent kinase 1 (PDK1) has been considered a critical metabolic regulator connecting PI3K and downstream mTOR activation (Finlay et al., 2012). An important role for PDK1 is to phosphorylate the T308 site of AKT and synergize with mTORC2 to fully activate downstream AKT. In the immune system, PDK1 has been shown to be critical for the development of T and B cells (Hinton et al., 2004; Park et al., 2013; Venigalla et al., 2013; Baracho et al., 2014). However, the role of PDK1 in NK cell development has not been directly addressed. Loss of NK cells in mice lacking PI3K activity could imply a role for PDK1 in NK cell development. However, PI3K signaling can also result in the activation of phospholipase C γ and the Vav family independent of the PDK1–Akt–mTOR pathway (Tassi et al., 2005, 2008; Graham et al., 2006).

To address whether induction of E4BP4 in NK cells is regulated by metabolic signaling and a potential role for PDK1 in NK cell development, we generated conditional PDK1–deficient mice to block the connection between PI3K and mTOR signaling. We found that PDK1-dependent metabolic signaling functions as a key regulator for IL-15 signaling to stimulate E4BP4 expression, which in turn regulates CD122 expression and confers IL-15 responsiveness.

RESULTS

PDK1–mTOR signaling is required for IL-15–induced E4BP4 expression in vitro

E4BP4 was reported to be the most specific transcription factor required for the NK cell lineage development (Gascogne et al., 2009; Kamizono et al., 2009), but how it is regulated remains unknown. A real-time PCR assay revealed that IL-15 could preferentially and significantly up-regulate mRNAs encoding E4BP4 and Eomes (Fig. 1 A). To evaluate the E4BP4 protein level, we developed an intracellular staining assay to quantify its dynamic changes before and after IL-15 stimulation. To our surprise, E4BP4 was rarely detectable in resting NK cells. Upon IL-15 triggering, E4BP4 exhibited a more than fivefold increase in NK cells, and Eomes, which is considered to be involved downstream of E4BP4 (Male et al., 2014), exhibited slight up-regulation (Fig. 1 B). However, T-bet expression was not enhanced by IL-15 stimulation (Fig. 1 B).

To examine whether the induction of E4BP4 by IL-15 requires metabolic signaling, several pharmacological inhibitors of varying specificity, including GSK2334470, AKT1/2, and Torin1, were chosen to suppress PDK1, AKT, and mTOR, respectively. As expected, pharmacological inhibition of their activities led to a twofold decrease in IL-15–triggered up-regulation of E4BP4 at the transcriptional and translational levels, (Fig. 1, C and D). To genetically confirm this finding, we generated hematopoietic cell–specific PDK1-deficient mice, PDK1fl/fl/Vav-Cre (hereafter, referred to as PDK1−/−), and then detected E4BP4 expression in the splenic NK cells. As expected, PDK1−/− NK cells failed to significantly up-regulate E4BP4 expression upon IL-15 stimulation. These data indicate that PDK1–mediated metabolic signaling is indispensable for E4BP4 induction by IL-15.

PDK1 is intrinsically required for early NK cell development in vivo

To identify a potential role for PDK1–mediated signaling in NK cell physiology, we extensively analyzed NK cell development and function in PDK1−/− mice. Compared with PDK1–sufficient mice, PDK1−/− mice exhibited a nearly 95% reduction in the number of NK cells in the spleen and bone marrow (Fig. 2, A and B). The remarkable reduction in NK cell pool could also be found in other lymphoid organs, including the lymph nodes, liver, and lungs (Fig. 2 B). To investigate the cell–intrinsic effect of PDK1 for NK cell development, bone marrow mixtures were adoptively transferred into sublethally irradiated immunodeficient RAG1−/−γ− mice. In contrast to CD45.2 WT, PDK1−/− bone marrow cells failed to reconstitute NK cell pool efficiently (Fig. 2 C), suggesting the critical requirement for PDK1 in NK cell development is cell intrinsic.

To confirm the diminished NK cell population in PDK1−/− mice, the ability to eliminate “missing-self” MHC-I− splenocytes was measured in vivo as previously described (Dong et al., 2009, 2012). In the model, mismatched MHC-I− splenocytes were preferentially killed by control mice due to NK cell activity. In contrast, minimal NK cell activity was detectable in PDK1−/− mice (Fig. 2 D). We also observed that in vivo NK
dramatically fewer CD3−CD122+ high NK1.1+ cells (R1) in the detected lymphoid organs, like bone marrow, spleen, lung, and liver (Fig. 3A and not depicted). Additionally, the CD122 intensity on PDK1−/−/CD3−NK1.1+ cells was significantly lower and had a tendency to be gradually attenuated (Fig. 3, A and B). Surprisingly, there was a noticeable population of CD122-negative NK cells, which exhibited the distinct phenotype of intermediate expression of NK1.1, called NK1.1int. These cells accumulated mainly in the bone marrow rather than other organs (data not shown), whereas the population was rarely seen in WT mice (Fig. 3 A). To further characterize this population, we confirmed that the cells expressed high levels of CD117 and CD127, the two earliest markers for NK cells (Fig. 3 C), and were not reactive for CD1d tetramer binding (unpublished data); this excluded the possibility of the cells being NK-T cells, which also exhibit NK1.1int. The population was negative for NKp46, a marker for type I group of cytotoxicity against hematopoietic tumor RMA-S cells was minimal in PDK1−/− mice as well, nearly comparable with NK cell–lacking RAG1−/−γ− mice (Fig. 2 E). Therefore, PDK1−/− mice effectively lack peripheral NK cells and innate cytotoxic activity.

**PDK1 maintains CD122 expression and regulates NK-cell responsiveness to IL-15**

Because PDK1-deficient NK cells were resistant to IL-15–induced E4BP4 up-regulation, the impaired NK cell development probably resulted from the compromised IL-15 signaling. We initially detected IL-15–dependent lineage cells in PDK1−/− mice. The absolute numbers of NK cells (CD3−NK1.1+), NK-T cells (CD3+NK1.1int), and memory CD8+ T cells (CD8+CD122+) were remarkably diminished in PDK1−/− mice (unpublished data). To further clarify this effect, we analyzed CD122 expression on CD3−NK1.1+ cells. PDK1−/− mice exhibited dramatically fewer CD3−CD122+ high NK1.1+ cells (R1) in the detected lymphoid organs, like bone marrow, spleen, lung, and liver (Fig. 3 A and not depicted). Additionally, the CD122 intensity on PDK1−/−/CD3−NK1.1+ cells was significantly lower and had a tendency to be gradually attenuated (Fig. 3, A and B). Surprisingly, there was a noticeable population of CD122-negative NK cells, which exhibited the distinct phenotype of intermediate expression of NK1.1, called NK1.1int. These cells accumulated mainly in the bone marrow rather than other organs (data not shown), whereas the population was rarely seen in WT mice (Fig. 3 A). To further characterize this population, we confirmed that the cells expressed high levels of CD117 and CD127, the two earliest markers for NK cells (Fig. 3 C), and were not reactive for CD1d tetramer binding (unpublished data); this excluded the possibility of the cells being NK-T cells, which also exhibit NK1.1int. The population was negative for NKp46, a marker for type I group of
innate lymphoid cell (ILC1; Fig. 3 C). Collectively, these data indicate that PDK1 is essential for preserving a CD122high state during early NK cell development.

NK cells need high status of CD122 to ensure IL-15 responsiveness. To detect the in vivo IL-15 responsiveness of PDK1−/− NK cells, mice were intraperitoneally injected with recombinant IL-15–IL-15R complexes. Peripheral blood NK cells underwent a greater than twofold expansion in PDK1-sufficient mice but not in PDK1−/− mice (Fig. 3 D). Therefore, PDK1−/− mice exhibit hyporesponsiveness to IL-15 stimulation in vivo.

Reduced or failed expression of CD122 likely causes NK cell death. We found that PDK1−/− mice indeed exhibited about twofold more Annexin V+ and Caspase3+ NK cells in the spleen (Fig. 3 E). Thus, increased NK cell apoptosis likely contributes to the compromised NK cell development in PDK1−/− mice. In parallel, we detected survival-related intracellular molecules, including antiapoptotic Bcl-2 and pro-apoptotic Bim, both of them were reported to be involved in NK cell survival or death (Huntington et al., 2007). Intriguingly, NK cells from PDK1−/− mice did not exhibit more Bim and less Bcl-2 expression as we expected; in contrast, Bcl2 were really up-regulated in PDK1−/− NK cells (Fig. 3 F), suggesting that the PDK1-deficient NK cells maybe undergo cell cycle arrest like B cells to a great extent, rather than apoptosis (Venigalla et al., 2013).
Ectopic expression of E4BP4 rescues NK cell development in PDK1−/− mice
To investigate whether the compromised NK cell development and low expression level of CD122 correlated with less E4BP4 induction by IL-15 in PDK1−/− mice, actively dividing PDK1−/− bone marrow cells with enriched HSCs were infected by retroviruses encoding E4BP4 or Eomes. 6 wk after in vivo differentiation, exogenous expression of E4BP4 gave...

Figure 3. PDK1 regulates CD122 expression and IL-15 responsiveness in vivo. (A) Representative flow cytometry profile of CD122 versus NK1.1 expression on CD3− bone marrow cells. Three populations, CD122+/NK1.1+ (R1), CD122+/NK1.1− (R2), and CD122− NK1.1− (R3), are outlined (left). Absolute numbers of indicated NK cell populations from spleen and bone marrow are also shown (right). Data were pooled from two independent experiments (n = 6). **, P < 0.005; ***, P < 0.0005. (B). Representative overlaid histograms demonstrating CD122 expression on gated R1 NK cells in the spleens and BM of PDK1fl/fl (WT) and PDK1fl/fl/Vav1-Cre+ (KO) mice (left); the absolute MFI (ΔMFI) were quantified (right). Data were pooled from two independent experiments (n = 6). *, P < 0.05. (C). Profiling developmental markers on BM CD3− CD122+NK1.1+ cells from PDK1fl/fl mice and BM CD3− CD122− NK1.1− NK cells in PDK1fl/fl/Vav1-Cre+ mice. n = 5 per group. ***, P < 0.0005. (D). PDK1fl/fl or PDK1fl/fl/Vav1-Cre+ mice were injected with IL-15−IL-15R complexes every 3 d. The absolute number of peripheral blood CD3− NKp46+ NK cells was monitored on the indicated days. Fold change was calculated simply as the ratio of the NK cell number at each time point after IL-15−IL-15R treatment to the initial NK cell number in untreated mice (day 0). Data represent the mean ± SEM of 3 mice per time point and are representative of two independent experiments. *, P < 0.05. (E) Representative flow cytometry plot showing Annexin V staining and Caspase activity in naive NK cells from the indicated mice (left). Numbers adjacent to the outlined areas (left) indicate the percentage of Annexin V− or caspase-positive cells. Quantifications were performed from two independent experiments (right, n = 5). *, P < 0.05. (F). Intracellular staining of Bcl2 and Bim in naive NK cells from the indicated mice (left). Quantifications were performed from two independent experiments (right, n = 5). **, P < 0.005.
PDK1 regulates IL-15–stimulated metabolic NK cell activation via activating mTOR

PDK1 is a master molecule linking the PI3K pathway with mTOR activation via Akt. To explore whether mTOR-dependent PDK1 signaling is involved in the early NK cell development promoted by IL-15, we stimulated PDK1−/− splenocyte with recombinant IL-15–IL-15R complexes and detected the expression of nutritional receptors and mTOR signaling in CD3−CD122highNK1.1+ cells, which could eliminate the developmental discrepancy in CD122 levels between the two genotypes. We found that after overnight IL-15 stimulation, PDK1-sufficient NK cells displayed a two-to fivefold increase in CD71 and CD98, two nutritional receptors, whereas PDK1−/− NK cells nearly lost the ability to up-regulate these receptors (Fig. 5 A), and these cells consistently displayed impaired activation of AKT-mTOR1 signaling upon IL-15 exposure (Fig. 5 B). To further exclude the possibility that the impaired metabolic activation by IL-15 is caused by the variation in CD122 levels between the genotypes, several pharmacological inhibitors were chosen. Blockage of PI3K-PDK1-mTOR activation largely prevented the IL-15–triggered up-regulation of CD71 and CD98 on bone marrow and splenic NK cells (Fig. 5 C and not depicted), further demonstrating that IL-15 is able to trigger NK cell metabolic activation, which requires mTOR-dependent PI3K signaling. To directly test mTOR signaling in NK cell development, wild-type mice were injected with Torin1 to suppress mTOR activity. As expected, the proproliferative role of IL-15 was notably diminished by mTOR inhibition (Fig. 5 D). Together, these data demonstrate that PDK1 signaling is required for NK cell metabolic activation and proliferation downstream of the IL-15 receptor via activating mTOR.

PTEN deletion partially rescues E4BP4 expression and NK cell development in the absence of PDK1

Previous studies have revealed that the deletion of PTEN resulted in enhanced mTOR activation, which consequently promoted more dendritic cells generation (Sathaliyawala et al., 2010). To ascertain whether PTEN deletion could increase NK cell number and rescue the impaired NK cell development in absence of PDK1, PDK1−/− mice were further bred with PTEN+/− mice to yield PTEN+/−/PDK1−/−/Vav1-Cre+ mice. Similarly, PDK1−/− mice were also bred onto a SHIP1+/− background. We first found that deletion of PTEN, but not SHIP1, could notably enhance metabolic activation of NK cell via IL-15 stimulation, even in the absence of PDK1 (Fig. 6 A). Most importantly, PTEN inactivation significantly restored IL-15–triggered E4BP4 expression by PDK1−/− NK cells (Fig. 6 B). To test whether the rescued mTOR activation and increased E4BP4 expression could recover NK cell generation in PTEN+/−/PDK1−/−/Vav1-Cre+ mice, NK cell development was carefully examined (Fig. 3 A). In brief, PTEN deletion could significantly increase the absolute number of NK cells in the spleen and bone marrow (Fig. 6, C and D). Most importantly, the population of CD3−CD122−NK1.1+ cells previously observed in PDK1−/− mice was also dramatically reduced (Fig. 6, C and D). In contrast, SHIP1 deletion had little effect on PDK1−/− NK cell development, likely due to...
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its inability to up-regulate mTOR-dependent metabolic signaling (Fig. 6 E). We further evaluated the developmental process of the rescued NK cells in the PTEN<sup>fl/fl</sup>/PDK1<sup>fl/fl</sup>/Vav1-Cre<sup>+</sup> mice and found that bypassed activation of mTOR could restore the transitional process through the NK progenitor to CD27<sup>+</sup>CD11b<sup>+</sup> NK cell stage in the absence of PDK1. Nevertheless, this activation failed to complete the terminal maturation of NK cells (Fig. 6 F). Collectively, PDK1 plays a critical role in early NK cell differentiation mainly through activating mTOR signaling, which is critical for E4BP4 induction downstream IL-15 signaling.

PDK1 signaling is not required for terminal NK cell maturation and survival

To accurately understand at which stage PDK1 signaling is required for NK cell development. We extensively analyzed NK cell receptor profile, and revealed that a population of NK1.1<sup>+</sup>NKp46<sup>−</sup> cells accumulated in the bone marrow of PDK1-deficient mice, indicating the critical role of PDK1 for relatively early NK cell development (Fig. 7 A). Then, we further investigated whether deletion of PDK1 at a late stage of NK cell differentiation could affect NK cell terminal maturation or survival. Ncr1-Cre mice have previously been used for NK cell evaluation by two other groups (Walzer et al., 2007; Eckelhart et al., 2011). We regenerated the mice with Cre expression under the control of the mouse Ncr1 promoter and established a stable line with Cre expression. To characterize this new line, Ncr1-Cre mice were intercrossed with Rosa26<sup>DTA STOP</sup> mice. The splenic NK cell number was 75% reduced in Rosa26<sup>DTA STOP</sup>/Ncr1-Cre<sup>+</sup> mice (Fig. 7, B and C), and the remaining NK cells were mostly immature CD3<sup>−</sup>NK1.1<sup>+</sup>CD27<sup>−</sup>CD11b<sup>−</sup> (Fig. 7, B and C). Therefore, the newly generated Ncr1-Cre mice will be a useful tool for gene deletion at a late stage of NK cell differentiation.

Next, our Ncr1-Cre mice were bred with PDK1<sup>fl/fl</sup> mice. Terminal deletion of PDK1 did not obviously impair the NK cell percentage or number either in the spleen or the bone marrow (Fig. 7, D and E), and the remaining NK cells were mostly immature CD3<sup>−</sup>NKp46<sup>−</sup>NK1.1<sup>−</sup>CD27<sup>−</sup>CD11b<sup>−</sup> (Fig. 7, B and C). Therefore, the newly generated Ncr1-Cre mice will be a useful tool for gene deletion at a late stage of NK cell differentiation.
DISCUSSION
In this study, we used two different genetic approaches to disconnect PI3Ks with mTOR signaling at the early (Vav1-Cre) or late (Ncr1-Cre) stage of NK cell development. We revealed that the PDK1-mediated metabolic signaling works as a fundamental switch downstream of IL-15R signaling on early NK cells to...
induce the expression of E4BP4 that maintain IL-15 responsiveness via preserving the high level of CD122. Our study identifies a positive feedback loop via PDK1–mTOR–E4BP4–Eomes–CD122, a mechanism involving NK cell development.

NK cells develop from hematopoietic stem cells through multiple stages. The central issues are how NK cells become committed to be responsive to IL-15, how NK cells maintain IL-15 responsiveness during their differentiation. Previous study
has reported that Eomes could bind CD122 promoter region, and Eomes deficiency caused significantly low CD122 expression on IL-15–dependent cell lineages, like NK cells and memory CD8+ T cells (Intlekofer et al., 2005). Recent studies highlighted the critical role of E4BP4 as upstream of Eomes, so that E4BP4 deficiency caused severe defects in NK cell development (Kamizono et al., 2009; Male et al., 2014). We reported here a novel role for IL-15 in regulating self-responsiveness via the induction of CD122 in a PDK1-dependent manner. PDK1–deficient NK cells exhibited reduced CD122 expression and were hyporesponsive to IL-15 stimulation in vivo. Ectopic expression of E4BP4 or Eomes could largely rescue CD122 expression and NK cell generation. Therefore, PDK1 signaling is critical for NK cell development via induction of E4BP4 and Eomes. However, we also observed distinct phenotypes in mice lacking either E4BP4 or PDK1. E4BP4 deficiency displayed more severe phenotype and did not result in a population of NK cells without CD122. This discrepancy between the two genotypes may be explained by recent findings that E4BP4 may also be involved in the earliest NK cell commitment at the preNK stage (Male et al., 2014). Thus, E4BP4 may have dual activity, establishing NK cell commitment by inducing CD122 expression and maintaining IL-15 responsiveness by preserving the CD122$^{\text{high}}$ status during early NK cell development. The PDK1–mTOR–E4BP4 axis is only required for early differentiation after NK cell commitment through maintaining IL-15 responsiveness, whereas E4BP4 has an additional role in establishing NK cell commitment in a PDK1–mTOR–independent manner. Additionally, Firth et al. (2013) showed that late-stage deletion of E4BP4 did not affect NK cell differentiation. Here, we also observed normal NK cell development in the absence of PDK1 at the terminal stages, indicative of the dispensable role of PDK1–mediated E4BP4 induction for NK cell terminal maturation and survival.

In addition to the essential role of E4BP4 in conventional NK cell development, more recent studies have revealed that innate lymphoid cells (ILCs) also need E4BP4 for their generation (Geiger et al., 2014; Seillet et al., 2014), and nonclassical tissue-resident NK cells in the liver, thymus, and salivary glands are developmentally E4BP4–independent (Cortez et al., 2014; Crotta et al., 2014; Sojka et al., 2014). In PDK1–deficient mice, there is a noticeable population with distinct phenotype, CD3$^{\text{–}}$CD122$^{+}$CD117$^{+}$CD127$^{-}$NK1.1$^{\text{+}}$, which only accumulates in the bone marrow. We assume that the population belongs to NK cell lineage, but wonder how these cells maintain survival in the absence of IL-15 signaling. Although we ruled out the possibility of these cells being NKT cell and ILC1, it remains to be determined whether this unusual population is ILC progenitor or other ILCs, and whether PDK1–mediated metabolic signaling is required for the development of E4BP4–dependent ILCs.

The prevailing working mechanism of IL-15 is to activate the JAK–STAT family downstream of IL-15 β and γ, accounting for the fact that humans or mice that lack STAT5 have impaired NK cell development (Imada et al., 1998; Eckelhart et al., 2011). IL-15 also has the ability to biochemically activate PI3K pathways (Nandagopal et al., 2014). In this study, pharmacological or genetic inactivation of PDK1 activity could not completely prevent E4BP4 induction in NK cells by IL-15 triggering, suggesting that other signaling, particularly JAK–STAT pathway, is probably also involved in the induction of E4BP4 by IL-15, though PI3K–mediated metabolic signaling is the major regulator of E4BP4 expression.

Mice simultaneously lacking PI3K subunit P110 γ and δ had severe defect in early NK cell development (Tassi et al., 2007; Guo et al., 2008). We observed that PDK1 deficiency caused an almost 95% reduction in NK cells, comparable to p110 γ and δ doubly–deficient mice (Tassi et al., 2005), strongly suggesting that PI3K regulates NK cell development largely through the recruitment of PDK1, which may further activate AKT phosphorylation. Pharmacological inhibition of total AKT activity using AKTi, which inhibits all three isoforms, resulted in decreased metabolic activation of NK cells. Importantly, AKT inhibition recapitulated the phenotype that less E4BP4 induction by IL-15 was found in the absence of PDK1. Thus, the PI3K–PDK1–AKT axis is critical to NK cell development via regulating E4BP4 expressing downstream IL-15 signaling.

AKT activates mTOR by phosphorylating tuberous sclerosis complex 1, a negative regulator of mTOR signaling. As hematopoietic–specific deletion of mTOR is embryonic lethal to mice in our hands, we were hindered from extensively analyzing its role in early NK cell development directly. Fortunately, Marçais et al. (2014) revealed the critical role of mTOR kinase as key metabolic checkpoint for NK cell proliferation and activation during the manuscript preparation. In this study, we also revealed that pharmacological inhibition of mTOR via the chemical Torin1, which is an inhibitor specific for mTOR enzymatic activity as part of both mTORC1 and mTORC2, could suppress NK cell differentiation and abolish the proproliferative role of IL-15 in vivo. We confirmed these results when rapamycin, a widely used mTORC1 inhibitor, was used, suggesting the central role of mTORC1 in the PDK1–mediated regulation of early NK cell development, but we could not exclude the possibility that mTORC2 is also involved, as long-term rapamycin treatment likely suppresses mTORC2 activity via a feedback mechanism. Genetic disruption of the major components of mTORC1 and mTORC2, including RAPTOR and RICTOR, respectively, will be useful for further studies.

Tumor cells always exhibit altered, usually accelerated, energy consumption. Targeting tumor metabolism represents an alternative way to suppress tumor growth. PDK1 is considered as a potential target for tumor therapy (Raimondi and Falasca, 2011). Our current study has implications for the long-term therapeutic use of PDK1 inhibitors or mTOR inhibitors, such as rapamycin, for tumor patients, which may exhibit a risk of affecting NK cell development in human patients.

**MATERIALS AND METHODS**

**Mice.** The Ncr1-Cre transgene was designed with the mouse ncr1 (gene encoding NKp46) promoter region containing 5-UTR, 3,000 bp, an optimized
variant of Cre recombinase and an SV40 polyA signal. The transgene was microinjected into the pronuclei of fertilized oocytes from C57BL/6 mice. One founder stably expressing Cre was established and used to generate mutant mice.

Hematopoietic or NK cell–specific PDK1-deficient mice were generated by crossing PDK1fl/fl (from D. Alessi, University of Dundee, Dundee, Scotland, UK) with Var1-Cre (B6.Cg-Tg(Var1-A2Kio)) (The Jackson Laboratory) or newly generated Nlr1-Cre mice in our laboratory. RAG1−/− mice were obtained by intercrossing B6.129S7-Rag1tm1Mom/J; The Jackson Laboratory). PCR was used to identify the genotype. The mice were then backcrossed to pure background at least 10 generations. B2m-deficient mice, C57BL/6 mice, and CD45.1 mice were purchased from The Jackson Laboratory. All mice were bred and maintained in specific pathogen-free animal facilities of Tsinghua University. All procedures involving animals were approved by the Animal Ethics Committee of Tsinghua University.

Reagents. Antibodies recognizing mouse CD3 (mAb 145-2C11), NKp46 (mAb 29A14), NK1.1 (PK136), CD117 (2B8), CD127 (A1237), Ly49A (A1), Ly49C/1 (5E6), CD135 (A2F10), SCA-1 (D7), NKG2D (A10), NKG2A/C/E (2D5), CD11b (mAb M1/70), CD27 (LG.7F9), and isotype controls were purchased from eBioscience or BD. CD1d-PBS157 tetramer was provided by the National Institutes of Health tetramer facility. To inhibit the activities of PI3K–mTOR pathway, NK cells were treated with various inhibitors: 1 µM Torin1 (Tocris), 1 µM GSK2334470 (Sigma-Alrich), 1 µM AKT1i/2 (EMD Millipore), or 10 µM Ly294002 (EMD Millipore).

Real-time PCR. Splenic NK cells were sorted by fluorescence-activated cell sorting, and stimulated with IL-15–IL-15R complex overnight. Total RNA was extracted using TRIzol kit (Invitrogen), and reverse-transcribed using reverse transcription system (Promega). qPCR was performed using SYBR Green-based detection. The expression level of the genes of interest was determined relative to the expression of β-actin. The results are presented as relative to unstimulated, set as 1.

Flow cytometry. For analysis of surface markers CD71, CD98, and CD122, cells were stained in PBS containing 2% (v/v) FBS with antibodies from eBioscience or BD. Their expression level was presented as net mean fluorescence intensity (ΔMFI), which was determined by subtracting MFI of isotype control, or as fold relative to unstimulated, set as 1. Intracellular staining was used for NK cell transcription factor and phosphorylated proteins. NK cell transcription factors E4BP4, Eomes, and T-bet were stained with anti–mouse E4BP4 antibody (S2M-E19; eBioscience), anti-Eomes (Dan11mag; eBioscience), and anti–T-bet (eBio4B10; eBioscience) before and after overnight stimulation with IL-15–IL-15R complex according to the manufacturer’s instruction (eBioscience), similar to Foxp3 staining, respectively. For detection of phosphorylated signaling proteins, NK cells were fixed with Phosflow Lyse/Fix buffer, followed by permeabilization with Phosflow Perm buffer III (BD) and staining with antibodies to S6 phosphorylated at Ser235 and Ser236 (D57.2.2E; Cell Signaling Technology), Akt phosphorylated at Ser473 (M89-61; BD), and Thr308 (1:223.371; BD). Flow cytometry data were acquired on LSRII or LSR Fortessa (BD) and analyzed using FlowJo software (Tree Star). Net mean fluorescence intensity (ΔMFI) was calculated. Expression levels were presented as fold relative to unstimulated, set as 1.

Detection of NK cell apoptosis. Splenic NK cells were staining with Annexin V (BD), and Caspase activity was measured with FITC-conjugated z-VAD-fmk according to the manufacturer’s instruction (eBioscience).

Adoptive cell transfer. Donor mice were treated with 5-fluorouracil (5-FU) for 4 d before bone marrow cells were harvested. A mixture of 2 × 10⁶ CD3−CD19−NK1.1−-depleted bone marrow cells from either WT CD45.1 B6 mice with same number of WT or PDK1fl/fl/Var1-Cre mice expressing CD45.2 were intravenously transferred into sublethally irradiated RAG1−/− mice. For in vivo rescue, recipient RAG1−/− mice were sublethally irradiated and then transplanted with 2 × 10⁶ retrovirally transduced bone marrow cells from 5-FU-treated PDK1-deficient mice. Reconstitution of recipients was assessed by flow cytometry of bone marrow and spleen at 6 wk after transplantation.

In vivo NK cell functions. In vivo splenocyte rejection assay has been previously described (Dong et al., 2009). For RMA-S clearance assay, mice treated with 200 µg Poly I:C for 18 h were intraperitoneally injected with a mixture of target cells, NK-sensitive RMA-S cells expressing GFP (10⁴), and NK-sensitive RMA expressing DsRed. 18 h after the tumor injection, mice were killed, and cells in peritoneal cavity were collected by repeated washing with PBS containing 2 µM EDTA, and after centrifugation, cells were finally suspended in 1 ml PBS. The relative percentages of RMA-S and RMA cells were monitored with flow cytometry. The percentage of RMA-S cell rejection was calculated as followed formula: 100 × (1 − [percentage of residual GFP+ population in total GFP+ and DsRed+ of experimental group/ percentage of residual GFP+ population in total GFP+ and DsRed+ of control group])

Detection of in vivo IL-15 responsiveness. Mice were intravenously injected with 500 ng IL-15–IL-15R complexes every 3 d, and NK cell numbers were dynamically monitored by flow cytometric analysis of peripheral blood CD3−NKp46+ cells at indicated time-points. The data were shown as the increase fold relative to untreated mice.

Statistical analyses. Unpaired Student’s t tests (two-tailed) were performed using Prism software.

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M. Yang, D. Li, and Z. Dong performed and analyzed experiments. Z. Chang, Z. Yang, and Z. Tian provided critical reagents and advice. M. Yang and Z. Dong designed experiments, analyzed data, and wrote the manuscript.

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