TBK-binding protein 1 regulates IL-15-induced autophagy and NKT cell survival

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The cytokine IL-15 mediates development and survival of immune cells, including natural killer T (NKT) cells, but the underlying mechanism of IL-15 function is incompletely understood. Here we show that IL-15 induces autophagy in NKT cells with a mechanism that involves a crucial signaling component, TBK-binding protein 1 (Tbkbp1). Tbkbp1 facilitates activation of the autophagy-initiating kinase Ulk1 through antagonizing the inhibitory action of mTORC1. This antagonization involves the recruitment of an mTORC1-opposing phosphatase to Ulk1. Tbkbp1 deficiency attenuates IL-15-stimulated NKT cell autophagy, and is associated with mitochondrial dysfunction, aberrant ROS production, defective Bcl2 expression and reduced NKT cell survival. Consequently, Tbkbp1-deficient mice have profound deficiency in NKT cells, especially IFN-γ-producing NKT1. We further show that Tbkbp1 regulates IL-15-stimulated autophagy and survival of NK cells. These findings suggest a mechanism of autophagy induction by IL-15, and establish Tbkbp1 as a regulator of NKT cell development and survival.
Autophagy is a multi-step cellular process that delivers unused proteins and damaged organelles to the lysosome for breakdown, thereby promoting cell survival under extreme conditions such as nutrient deprivation. The initiation of autophagy involves formation of a protein complex, composed of UNC51-like kinase (ULK1 or ULK2), the scaffold protein FIP200 (also called RB1CC1), autophagy-related (ATG) 13 and ATG101. Upon activation, ULK1/2 phosphorylates downstream targets, including BECLIN1 and VPS34, involved in phagophore formation. Subsequent events involve lipidation of microtubule-associated protein 1 light chain 3 (LC3) to convert it from a cytosolic form (LC3-I) to a lipidated form (LC3-II) that is recruited to autophagosomal membranes, where it mediates cargo recruitment and autophagosome completion. Eventually, autophagosomes fuse with lysosomes to form autolysosomes, in which cargos are degraded by lysosomal hydrolases. A key step in autophagy induction is activation of ULK1, which is reciprocally regulated by mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and AMP-activated kinase alpha (AMPKα). Under nutrient-replete conditions, mTORC1 inhibits autophagy through phosphorylating ULK1 at serine 757, which prevents ULK1 binding and activation by AMPKα; nutrient deprivation inactivates mTORC1, allowing the activated AMPKα to phosphorylate ULK1 at serine 555 and other activation sites for autophagy initiation. Recent studies demonstrate that autophagy also plays a crucial role in physiological processes, including immune cell development and homeostasis. However, it is unclear how autophagy is induced along with the physiological processes of immune cell development and homeostasis and how autophagy regulates immune cell survival.

Natural killer T (NKT) cells are a subset of innate-like T cells responding to lipid antigens and regulating diverse aspects of immune and autoimmune responses. The development of NKT cells occurs in the thymus, where they originate from CD4+CD8+ double-positive (DP), and possibly also CD4−CD8− double-negative (DN) thymocytes, with a rearranged semi-invariant T-cell receptor (TCR). In contrast to the development of conventional T cells, which rely on self-peptide antigens presented on classical MHC molecules for positive selection, the development of NKT cells requires self-lipid antigens presented by CD1d expressed on DP thymocytes. Following positive selection, immature NKT cells go through sequential stages of maturation that can be defined based on surface expression of CD44 and NK1.1 markers, including stage 1 (CD44+NK1.1−), stage 2 (CD44+NK1.1−), and stage 3 (CD44+NK1.1+). Recent studies suggest that mature NKT cells can be classified into three sublineages, NKT1, NKT2, and NKT17, characterized by expression of the transcription factors T-bet, GATA3, and RORγt, respectively, and production of the cytokines IFNγ, IL-4, and IL-17, respectively. In fact, the previously defined stage 2 cells include not only immature NKT1 cells but also mature NKT2 and NKT17 cells that display CD44−NK1.1− surface markers. The expression of IL-17 receptor beta (IL-17RB) on NKT2 and NKT17 cells, but not on NKT1 sublineage cells, provides a means of lineage distinction.

The requirement of autophagy in NKT cell survival and maturation has been demonstrated using mouse models carrying deficiencies in major components of the autophagy pathway. Deletion of ATG5 or ATG7 results in severe loss of NKT cells, with predominant effect on the mature NKT cells producing interferon gamma (IFNγ). However, how autophagy is induced and regulated under the physiological conditions of NKT cell development and homeostasis has been undefined. Common gamma chain (γc) family of cytokines, particularly IL-15, are crucial for the survival and maturation of iNKT cells. IL-15 deficiency predominantly impairs the homeostasis and survival of IFNγ-producing stage 3 NKT (NKT1) cells, which is consistent with the high level expression of the beta chain of IL-2 and IL-15 receptors (IL-2Rα/IL-15Rα) on these cells. On the other hand, the survival of RORγt+ NKT17 cells is independent of IL-15 but relies on IL-7. However, the molecular mechanism underlying the survival function of IL-15 is incompletely understood.

In the present study, we show that IL-15 stimulates an autophagy pathway that is crucial for the survival of NKT cells. In contrast to nutrient deprivation, which inactivates mTORC1 and activates AMPKα, IL-15 activates both AMPKα and mTORC1 and requires a signaling factor, Tbk-binding protein 1 (Tbkbp1), for ULK1 activation. We provide genetic evidence that Tbkbp1 deficiency attenuates IL-15-stimulated NKT cell autophagy, causing mitochondrial dysfunction and aberrant ROS production, as well as impaired survival gene expression and apoptosis of the Tbkbp1-deficient NKT cells. Consequently, the Tbkbp1-deficient mice have a profoundly reduced number of NKT cells, predominantly the IFNγ-producing NKT1 cells. We further show that Tbkbp1 is also required for IL-15-induced autophagy and survival of NK cells. These findings provide insight into the mechanisms underlying autophagy induction and function in the physiological process of immune cell development and establish Tbkbp1 as a regulator of NK and NKT cell survival.

**Results**

Tbkbp1 deficiency reduces IFNγ-producing NKT1 cells. Tbkbp1, also called SINTBAB, was identified as a protein physically interacting with the protein kinase TBK1, although its physiological function has not been defined. A recent study has identified Tbkbp1 as a gene highly expressed in mature NKT cells. Consistently, we found that thymic and splenic NKT cells, as well as NK cells, expressed much higher levels of Tbkbp1 than DP and DN thymocytes and conventional CD4+ and CD8+ T cells. Similar results were obtained with human NKT cells (Supplementary Fig. 1a-c). To examine the function of Tbkbp1, we generated Tbkbp1 germ-line knockout (Tbkbp1-KO) and wild-type (WT) control mice (Supplementary Fig. 1d-g). The Tbkbp1-KO mice had normal frequencies of thymocyte and peripheral T cell populations, except for a moderate increase in CD8+ single-positive (SP) thymocytes and CD8+ splenic T cells (Supplementary Fig. 1h-j). However, compared to the WT control mice, the Tbkbp1-KO mice had a significant reduction in the frequency and absolute numbers of thymic, splenic, and liver NK cells, detected based on their binding to a glycolipid antigen (PB57)-loaded CD1d-tetramer but not to an unloaded CD1d-tetramer (Fig. 1c and Supplementary Fig. 2a). Further analysis based on the CD44 and NK1.1 surface markers revealed that the Tbkbp1 deficiency caused a predominant loss of the stage 3 NKT cells (CD44+NK1.1+) and a relative increase in the stage 1 (CD44+NK1.1−) and stage 2 (CD44+NK1.1−) NKT cells (Fig. 1d, e).

Recent studies suggest that mature NKT cells include three distinct sublineages, NKT1, NKT2, and NKT17, with the latter two sublineages being included in the previously defined stage 2 NKT cells. So, the question was raised as to whether Tbkbp1 regulated the development or survival of the different sublineages of NKT cells. We thus analyzed the NKT sublineages by flow cytometry based on their lineage transcription factors as well as another NKT-associated transcription factor, PLZF, as previously described. The Tbkbp1 deficiency resulted in a severe loss of the PLZFflowT-bet+ NKT1 subset, with a concomitant increase in the frequency of PLZFhiT-bet− NKT2 cells, in the thymus and spleen (Fig. 1f, g). The Tbkbp1-KO mice also had a moderate increase in the frequency of PLZFflowRORγt+ NKT17 cells (Fig. 1f, g). These
results suggest that Tbkbp1 is selectively required for the development or survival of NKT1 cells.

To further determine the role of Tbkbp1 in regulating NKT subsets, we analyzed the NKT cell sublineages based on expression of IL-17Rb, a marker expressed on NKT2 and NKT17 cells but not on NKT1 cells. We also included CD4 as a marker to further distinguish NKT2 (CD4⁺) from NKT17 (CD4⁻) cells (NKT1 cells include both CD4⁺ and CD4⁻ populations). Consistent with the loss of NKT1 cells (IL-17Rb⁻), the Tbkbp1-KO mice had a drastic increase in the frequency of IL-17Rb⁺ NKT cells, most strikingly the CD4⁺IL-17Rb⁺ NKT2 cells (Fig. 1h). This result further confirmed the selective loss of NKT1 and relative accumulation of NKT2 cells in Tbkbp1-KO mice. We next examined the possible role of Tbkbp1 in NKT17 sublineage commitment by gating on immature stage 1 (CD44⁺NK1.1⁻) NKT cells (Supplementary Fig. 2b) known to be the branching point of NKT1 cells from NKT2 and NKT17 cells. Interestingly, the WT and Tbkbp1-KO mice had comparable frequencies of IL-17Rb⁺ cells during the CD44⁺NK1.1⁻ immature stage but display a striking difference in the later stage (CD44⁺NK1.1⁺), suggesting that Tbkbp1 was dispensable for NKT17 sublineage commitment but required for NKT1 maturation or survival (Supplementary Fig. 2c). Taken together, these results suggest that the Tbkbp1 deficiency causes a predominant loss of NKT1 cells and relative accumulation of NKT2 and NKT17 cells.

A major functional characteristic of NKT2 cells is abundant expression of IL-4 as opposed to the predominant production of IFNγ by NKT1 cells. Consistent with their severe loss of NKT1 and relative increase in NKT2 subpopulation, the
Tbkbp1-deficient NKT cells were largely devoid of IFNγ-producing NKT cells with a relative increase in IL-4-producing NKT cells (Fig. 1b). ELISA also revealed that Tbkbp1-KO NKT cells produced much less IFNγ and profoundly more IL-4 than WT NKT cells upon in vivo activation by anti-CD3 plus anti-CD28 or in vitro activation by α-GalCer (Fig. 1j, k). Thus, Tbkbp1 deficiency causes a severe loss of IFNγ-producing NKT cells and a relative accumulation of IL-4-producing NKT cells.

**Tbkbp1 is a cell-intrinsic regulator of NKT cell development.** To examine the cellular mechanism of Tbkbp1 function, we generated T-cell-conditional Tbkbp1 KO (Tbkbp1-1TKO) mice (Supplementary Fig. 1d–g). As seen with the whole-body Tbkbp1-KO mice, the Tbkbp1-1TKO mice had reduced frequency and number of NKT cells in both the thymus and peripheral organs (Fig. 2a, b). Moreover, the loss of NKT cells in the Tbkbp1-1TKO mice was predominantly in the CD44+CD1dhi (previously known as stage 3) population, which was coupled with a relative increase in the CD44−CD1d− (stage 1) and CD44−CD1d+ (stage 2) cells producing IL-4 (Fig. 2c–e). These results suggested a T cell-specific function of Tbkbp1 in regulating NKT cell development.

Since thymocytes serve as both NKT precursors and antigen-presenting cells that support NKT cell development, the Tbkbp1-1TKO mice could not determine whether Tbkbp1 had a cell-intrinsic or cell-extrinsic function in NKT cell regulation. To solve this problem, we carried out mixed bone marrow (BM) adoptive transfer studies by transferring Rag1-KO mice with a mixture of WT (CD45.1+) and Tbkbp1-KO mice (CD45.2+) BM cells. The chimeric mice had a drastically reduced frequency of stage 3 NKT cells derived from the CD45.1+ or CD45.2+ cells and presented as representative FACS plots and summary graphs (n = 5 chimeric mice). Flow cytometric analysis of cell surface expression of CD1d in DP thymocytes from WT or Tbkbp1-1KO mice, presented as a representative FACS plot and summary graph (n = 5 mice per genotype). ELISA of IL-2 produced by NKT hybridoma cells cocultured for 24 h with total thymocytes from WT or Tbkbp1-1KO mice in the absence or presence of α-GalCer. Data are representative of at least three independent experiments, and bar graphs are presented as mean ± s.d. values. *P < 0.05; **P < 0.01; ***P < 0.001. Student’s t-test (b, d–i).

**Tbkbp1 deficiency increases memory-like CD8+ T cells.** The NKT1+ (predominantly NKT2) cells mediate steady-state production of IL-4, which promotes development of memory-like CD8+ T cells. Consistent with their relative increase in IL-4-producing NKT cells, the Tbkbp1-1KO mice had a drastic increase in the frequency of thymic and splenic memory-like CD8+ T cells, characterized by the CD44hiCD122+ and CD44hiCXCR3+ surface...
The tumors (size reaching to 225 mm$^2$). Derived from 6-week-old WT and representative plots (left) and summary graphs (right). Data are representative of at least three independent experiments, and bar graphs are presented as mean ± s.d. values. *CD8$^+$ Tbkbp1-KO IL-4 production in naïve mice is known to occur predominantly in NK1.1 memory-like CD8$^+$ T cells. In support of this, deletion of IL-4 in Tbkbp1-KO mice, which blocked hyperproduction of memory-like CD8$^+$ T cells (Supplementary Fig. 3e), abrogated their ability to mediate stronger tumor rejection and CD8$^+$ effector T cell responses (Supplementary Fig. 4a–c). Furthermore, deletion of CD8$^+$ T cells using an anti-CD8 neutralizing antibody (Supplementary Fig. 4d) markedly attenuated the antitumor immunity of the Tbkbp1-KO mice and erased the difference between the Tbkbp1-KO and WT control mice (Supplementary Fig. 4g). These results suggest that Tbkbp1 regulates IL-4-dependent generation of memory-like CD8$^+$ T cells with antitumor function.
**Tbkbp1 is required for IL-15-stimulated NKT cell survival.** The cytokine IL-15 plays a crucial role in NKT survival and maturation. In particular, the IL-17Rb-IFNγ-producing NKT1 cells express high levels of IL-2/IL-15Rb and rely on IL-15 for survival, although the survival of IL-17Rb+ NKT2 and NKT17 cells is independent of IL-15. Because of the selective role of Tbkbp1 in NKT1 regulation, we examined the role of Tbkbp1 in regulating IL-15-induced NKT cell survival and proliferation. IL-15 induced time-dependent expansion of WT NKT cells, which was almost completely blocked in the Tbkbp1-KO NKT cells (Fig. 4a, b). Parallel apoptosis analysis revealed that in vitro cultured NKT cells were undergoing massive apoptosis (Fig. 4c, d). Importantly, the apoptosis of WT NKT cells, but not Tbkbp1-KO NKT cells, was efficiently protected by IL-15 (Fig. 4c, d). The Tbkbp1-KO NKT cells also had a moderate defect in IL-15-induced proliferation (Fig. 4e). IL-15-mediated inhibition of NKT cell apoptosis involves induction of survival factors, including Bcl-2. Flow cytometry and qRT-PCR analyses of freshly isolated NKT cells revealed a reduction in Bcl-2 expression in the Tbkbp1-KO NKT cells, suggesting defective responses to in vivo homeostatic triggers (Fig. 4f, g). Moreover, IL-15 upregulated the expression level of Bcl-2 protein and mRNA in WT NKT cells but barely induced Bcl-2 expression in Tbkbp1-KO NKT cells (Fig. 4f, g). These results suggest that Tbkbp1 is a critical mediator of IL-15 induced Bcl2 expression and NKT cell survival.

Since IL-15 and IL-2 stimulate signaling via the same receptor subunits, IL-2/IL-15b and common gamma chain (γc), we wondered whether Tbkbp1 also played a role in regulating IL-2-stimulated signaling in NKT cells. Like IL-15, IL-2 prevented apoptosis of WT NKT cells (Supplementary Fig. 5a, b). Importantly, the IL-2-stimulated NKT survival was impaired in Tbkbp1-KO NKT cells (Supplementary Fig. 5a, b). On the other hand, Tbkbp1 was largely dispensable for the induction of NKT cell survival mediated by another γc family cytokine IL-7 (Supplementary Fig. 5a, b), even when tested with a broad range of doses (Supplementary Fig. 5c, d). Consistently, Tbkbp1 deficiency attenuated Bcl-2 expression in NKT cells stimulated by IL-2 and IL-15, but not by IL-7 (Supplementary Fig. 5e).

**Tbkbp1 regulates mitochondria ROS.** We next examined the molecular mechanism by which Tbkbp1 mediates IL-15-stimulated Bcl2 expression and NKT cell survival. Surprisingly, the Tbkbp1 deficiency did not appreciably influence IL-15-stimulated phosphorylation of the transcription factor STAT5 and the survival kinase AKT (Fig. 5a), two major signaling events...
stimulated by IL-15. These results suggested that Tbkbp1 might regulate a downstream molecular event involved in Bcl-2 induction and NKT cell survival. In this regard, reactive oxygen species (ROS) are known to suppress Bcl-2 gene expression and promote cell death. Analysis of freshly isolated NKT cells revealed that while the majority of WT NKT cells had a low content of ROS, almost the entire population of Tbkbp1-deficient NKT cells had a high content of ROS, suggesting aberrant ROS production under in vivo homeostatic conditions (Fig. 5b). The increased ROS production in the Tbkbp1-KO NKT cells was also detected based on staining using MitoSOX Red (Fig. 5b), a dye known to detect mitochondrial superoxide anion.

Mitochondria is a major source of ROS, and aberrant ROS production is associated with mitochondrial dysfunction. The increased ROS production in Tbkbp1-deficient NKT cells prompted us to examine the effect of Tbkbp1 deficiency on mitochondrial function based on staining with MitoTracker Green and MitoTracker Red CMXRos, known to detect mitochondrial content and membrane potential, respectively. Compared to the WT NKT cells, the Tbkbp1-deficient NKT cells displayed profoundly increased mitochondrial mass and membrane potential, indicative of mitochondrial dysfunction (Fig. 5b).

To assess the possible connection between aberrant ROS production and impaired survival of Tbkbp1-deficient NKT cells, we performed in vitro studies by employing nicotinamide (NAM), a precursor of the coenzyme NAD+ known to regulate mitochondrial potential and inhibit ROS production. Following in vitro cultivation in the presence of IL-15, KO NKT cells still had substantially higher levels of ROS than WT NKT cells (Fig. 5c, d). As expected, the ROS level in both WT and KO NKT cells could be efficiently lowered down upon incubation with NAM (Fig. 5c). Importantly, NAM treatment largely, although not completely, rescued the defect of the Tbkbp1-deficient NKT cells in IL-15-stimulated expression of Bcl-2 and Bcl-XL (Fig. 5e, f). The NAM treatment also partially restored IL-15-induced survival of the Tbkbp1-deficient NKT cells (Fig. 5g). These results suggest that Tbkbp1 deficiency in NKT cells causes mitochondrial dysfunction and aberrant ROS production, which contributes to the defect in IL-15-induced survival gene expression and apoptosis inhibition.

**Tbkbp1 mediates IL-15-stimulated autophagy in NKT cells.** Autophagy is a fundamental mechanism that removes damaged mitochondria to prevent abnormal ROS production and maintain cell survival. Accumulating studies have demonstrated the requirement of autophagy in NKT cell maturation, although the underlying mechanism is obscure. Because of the aberrant ROS production in Tbkbp1-deficient NKT cells, we examined the role of Tbkbp1 in regulating autophagy. Consistent with a recent study performed with conventional T cells, we found that IL-15 could stimulate autophagy in NKT cells, as revealed by generation of the modified form of LC3, LC3II, and reduction in the level of the autophagy substrate, p62 (Fig. 6a and Supplementary Fig. 6a).

![Graph](https://example.com/graph.png)
attenuated in Tbkbp1-KO NKT cells (Fig. 6b). To assure that these phenotypes were not due to developmental effect, we knocked down Tbkbp1 in an NKT hybridoma, NKT1.2, using two different Tbkbp1 shRNAs. As seen with the primary NKT cells, IL-15 stimulated LC3II generation and p62 loss in NKT1.2 cells, which were blocked upon Tbkbp1 knockdown (Fig. 6c and Supplementary Fig. 6b). The IL-15-stimulated p62 loss was inhibited by a lysosomal inhibitor, bafilomycin A, consistent with its degradation by the autophagy pathway (Fig. 6c and Supplementary Fig. 6b). Moreover, in the presence of bafilomycin A, the Tbkbp1-knockdown cells still had a lower level of LC3II than control cells, confirming a defect of the Tbkbp1-knockdown cells in LC3II generation (Fig. 6c and Supplementary Fig. 6b). Consistently, the IL-15-stimulated Ulk1 S555 phosphorylation was also attenuated in the Tbkbp1-knockdown NKT1.2 cells (Fig. 6d). We further confirmed the Tbkbp1-dependent autophagy induction by IL-15 based on formation of LC3 puncta (Fig. 6e) and staining with a commercial mitophagy antibody (Fig. 6f). Confocal assays revealed mitochondria-lysosome colocalization in WT, but not Tbkbp1-KO, NKT cells (Supplementary Fig. 6e), further emphasizing the role of Tbkbp1 in regulating autophagy. IL-2 also stimulated NKT cell autophagy in a Tbkbp1-dependent manner, but IL-7 only induced weak NKT autophagy in a Tbkbp1-independent manner (Supplementary Fig. 6f).
Tbkbp1 facilitates AMPKa-mediated Ulk1 activation by antagonizing mTORC1. **a**, **b** IB analysis of the indicated phosphorylated (P-) and total proteins in whole-cell lysates of IL-15-stimulated WT and Tbkbp1-KO thymic NKT cells (a) or NKT hybridoma cells transduced with a non-silencing control shRNA or two different Tbkbp1-specific shRNAs (b). **c** IB analysis of the indicated phosphorylated (P-) and total proteins in whole-cell lysates of IL-15-stimulated NKT hybridoma cells. **d**, **e** IB analysis of phosphorylated Ulk1 on Ser757 or Ser555 using WT or Tbkbp1-KO NKT cells incubated for 60 min with the mTOR inhibitor Torin or solvent control DMSO (d) or IL-15-stimulated NKT hybridoma cells transduced with a nonsilencing control shRNA or two different Tbkbp1-specific shRNAs (e). **f** IB analysis of phosphorylated Ulk1 on Ser757 or Ser555 in IL-15-stimulated NKT hybridoma cells transduced with an empty vector or HA-Tbkbp1 expression vector. **g** Co-IP analyses of AMPKα-Ulk1 interactions (upper panels) and direct IB analyses of the indicated proteins (lower panels) in NKT hybridoma cells transduced with a control or Tbkbp1-specific shRNA (#1) incubated with IL-15 as indicated. IgG was used as a negative control for IP. Data are representative of three or more independent experiments.

Fig. 6f, g). Together, these findings establish Tbkbp1 as a crucial mediator of autophagy stimulated by the cytokines IL-15 and IL-2.

To determine the functional significance of autophagy in NKT cell survival, we examined the effect of an autophagy inhibitor, 3MA, on IL-15-induced Bcl-2 expression and survival in NKT cells. As expected, IL-15 strongly induced the expression of Bcl-2 in WT, but not Tbkbp1-deficient, NKT cells (Fig. 6f). Importantly, the autophagy inhibitor 3MA completely blocked IL-15-stimulated Bcl-2 expression in WT NKT cells, thereby erasing the differences between the WT and the Tbkbp1-deficient NKT cells (Fig. 6f). In the presence of 3MA, IL-15 also failed to prevent apoptosis in WT NKT cells, causing a high level of apoptosis in both WT and Tbkbp1-KO NKT cells even in the presence of IL-15 (Fig. 6g). The IL-15-induced Bcl-2 expression in WT NKT cells was also inhibited by several other autophagy inhibitors (Supplementary Fig. 6h) known to interfere with different steps in the autophagy pathway. Autophagy inhibition by bafilomycin A only moderately increased the mitochondrial mass in Tbkbp1-deficient NKT cells, which was consistent with the attenuated autophagy in these mutant cells. Thus, Tbkbp1-mediated autophagy regulation appears to contribute to IL-15-induced mitochondria function, Bcl-2 expression, and survival of NKT cells.

Tbkbp1 regulates the autophagy and survival of NK cells. Like NKT cells, NK cells express high levels of the IL-2/IL-15R and rely on IL-15 for survival and maturation. We thus examined whether Tbkbp1 also played a similar role in NK cells. Compared to WT control mice, the Tbkbp1-KO mice had a significant reduction in the frequencies and absolute numbers of splenic NK cells, although the BM NK cells were only moderately affected (Supplementary Fig. 7a). NK cell maturation is defined into four stages based on their surface expression of CD11b and CD27, including stage 1 (CD11b\textsuperscript{low}CD27\textsuperscript{low}), stage 2 (CD11b\textsuperscript{low}CD27\textsuperscript{high}), stage 3 (CD11b\textsuperscript{high}CD27\textsuperscript{high}), and stage 4 (CD11b\textsuperscript{high}CD27\textsuperscript{low}). Tbkbp1 deficiency reduced the frequency of stage 4 NK cells and currently increased frequencies of early stages, suggesting a role for Tbkbp1 in regulating NK cell maturation or...
survival (Supplementary Fig. 7b). Indeed, the Tbkbp1 deficiency impaired IL-15-stimulated NK cell survival (Supplementary Fig. 7c) as well as IL-15-induced autophagy, as revealed by attenuated LC3II generation and p62 degradation (Supplementary Fig. 7d). On the other hand, Tbkbp1 deficiency had no obvious effect on IL-15-induced survival or autophagy of CD8 memory T cells (Supplementary Fig. 7e, f). This result was consistent with the much weaker expression of Tbkbp1 in conventional T cells than in NK and NKT cells (Fig. 1a, b). Collectively, these results demonstrate an important role for Tbkbp1 in regulating IL-15-stimulated autophagy and survival of NK and NKT cells.

Tbkbp1 associates with the Ulk1-FIP200 complex. In search of the molecular mechanism by which Tbkbp1 regulates IL-15-stimulated autophagy, we found that one of the Tbkbp1-binding proteins identified by affinity capture-mass spectrometry was FIP200 (BioGrid), an essential component of the Ulk1 autophagy initiation complex.41,42 We confirmed the specific Tbkbp1/FIP200 interaction by co-immunoprecipitation (co-IP) assays (Fig. 6h). Consistent with the FIP200-Ulk1 association, Tbkbp1 also interacted with Ulk1 in transfected cells and was co-immunoprecipitated with both FIP200 and Ulk1 under endogenous conditions (Fig. 6i, j). Tbkbp1 contains several domains, including a TBK1-binding domain (TBD) (Fig. 6k) that is also present in several other TBK1-binding proteins.2 Interestingly, the TBD, located between amino acids 280 and 330 of Tbkbp1, was required for Tbkbp1/Ulk1 interaction (Fig. 6l, m). Mutation of two conserved residues (Q315 and L319) in the Tbkbp1 TBD severely crippled the Tbkbp1-Ulk1 association, whereas mutation of another residue (Y298) did not affect the binding (Fig. 6m).

Consistently, Tbkbp1 Y298A but not the Ulk1 interaction-defective Tbkbp1 mutants, Q315A and L319A, was able to rescue the defect of another residue (Y298) did not affect the binding. Fig. 6m). These findings suggest that Tbkbp1 may regulate autophagy induction by physically interacting with Ulk1 autophagy initiation complex.

Tbkbp1 facilitates Ulk1 activation by antagonizing mTORC1. Ulk1 activation is a crucial step in autophagy induction, which is reciprocally regulated by two kinases: mTORC1 and AMPKα.2,3,5 AMPKα activates Ulk1 by phosphorylating Ulk1 at S555 and additional activation sites, whereas mTORC1 inhibits Ulk1 activation by phosphorylating Ulk1 at S757 and, thereby, inhibiting Ulk1-AMPKα interaction and AMPKα-mediated Ulk1 phosphorylation. Autophagy induction by nutrient deprivation involves mTORC1 inactivation and AMPKα activation, switching Ulk1 from mTORC1-suppressed state to AMPKα-activated state. Consistent with its ability to induce autophagy, IL-15 stimulated the activation of AMPKα in primary NKT cells and NKT hybridoma cells (Fig. 7a, b).
deprivation, IL-15 did not inhibit but rather activated the AMPKa-opposing kinase mTORC1, as revealed by the phosphorylation of its substrate S6K and the downstream ribosomal protein S6 (Fig. 7a, b). Moreover, Tbkbp1 knockout or knockdown had no effect on IL-15-stimulated activation of AMPKa and mTORC1 (Fig. 7a, b). A more detailed time-course analysis revealed that IL-15 stimulated rapid activation of mTORC1 but delayed activation of AMPKa, which was associated with a shift of Ulk1 phosphorylation from S757 to S555 (Fig. 7c). These findings raised the question of how the Ulk1 phosphorylation events were modulated.

Because of the physical interaction between Tbkbp1 and FIP200/Ulk1, we surmised that Tbkbp1 might act on Ulk1 to regulate its phosphorylation by AMPKa or mTORC1. Indeed, Tbkbp1 knockout or knockdown profoundly enhanced Ulk1 S757 phosphorylation under both basal and IL-15-stimulated conditions, which was associated with reduced Ulk1 phosphorylation at S555 (Fig. 7d, e). Consistent with a prior study4. The Ulk1 S757 phosphorylation was mediated by mTORC1, since it was blocked by mTOR inhibitor Torin (Fig. 7d). Importantly, Torin-mediated inhibition of S757 phosphorylation promoted Ulk1 S555 phosphorylation and overrode the suppression of Ulk1 S555 phosphorylation in Tbkbp1-deficient NKT cells (Fig. 7d). In line with these findings, overexpression of Tbkbp1 inhibited Ulk1 S757 phosphorylation and concomitantly promoted Ulk1 S555 phosphorylation (Fig. 7f). It is known that the mTORC1-mediated Ulk1 S757 phosphorylation inhibits Ulk1-AMPKa interaction4. Consistently, Tbkbp1 knockdown enhanced Ulk1 S757 phosphorylation (Fig. 7e) and concomitantly inhibited Ulk1-AMPKa interaction (Fig. 7g). These results suggest that Tbkbp1 facilitates IL-15-stimulated autophagy by antagonizing mTORC1-mediated Ulk1 S757 phosphorylation and, thereby, promoting Ulk1-AMPKa association and AMPKa-mediated Ulk1 S555 phosphorylation.

Tbkbp1 recruits an mTORC1-opposing phosphatase to Ulk1.
Since Tbkbp1 deficiency enhanced mTORC1-mediated Ulk1 S757 phosphorylation without promoting mTORC1 activation (Fig. 7b–d), we examined whether Tbkbp1 regulates the association of mTORC1 with Ulk1. To our surprise, Tbkbp1 knockdown did not significantly enhance the binding of mTORC1 to Ulk1 (Fig. 8a), suggesting the involvement of a different mechanism. In this regard, mTORC1-mediated protein phosphorylation is subject to regulation by phosphatases, especially protein phosphatase 6 (PP6)13. Notably, proximity label-mass spectrometry analysis identified physical association between Tbkbp1 and a regulatory subunit of PP6, ANKRD28 (BioGrid database). We confirmed the strong Tbkbp1-ANKRD28 interaction by coIP assays (Fig. 8b). Moreover, although ANKRD28 did not interact with Ulk1 directly, these two proteins formed a complex in the presence of Tbkbp1, suggesting that Tbkbp1 functioned as an adapter to recruit ANKRD28 to Ulk1 (Fig. 8c). Moreover, endogenous ANKRD28 was recruited to the Ulk1-FIP200 complex, along with Tbkbp1, in IL-15-stimulated NKT hybridoma cells, and this inducible recruitment was abolished in the Tbkbp1 knockout cells (Fig. 8d). These results suggested the intriguing possibility that Tbkbp1-mediated Ulk1 regulation involves recruitment of PP6. In further support of this idea, silencing ANKRD28 with 2 different shRNAs caused a profound increase in Ulk1 S757 phosphorylation and a concomitant decrease in Ulk1 S555 phosphorylation (Fig. 8e). This result was more striking for ANKRD28 knockdown with shRNA2 than that with shRNA1, which was consistent with the higher knockdown efficiency of shRNA2 (Fig. 8e). Similarly, PP6C knockdown also promoted Ulk1 S757 phosphorylation and reduced Ulk1 S555 phosphorylation (Fig. 8f). Together, these results suggest that Tbkbp1-mediated regulation of Ulk1 activation may involve recruitment of PP6 to Ulk1.

Discussion
The results presented in this paper established Tbkbp1 as a crucial regulator of NKT cell survival and development. Tbkbp1 deficiency caused a selective loss of IFNγ-producing NKT1 cells, resulting in a relative increase in the frequency of IL-4-producing NKT2 cells. The enhanced steady-state IL-4 production in turn contributed to increased generation of memory-like CD8+ T cells in the Tbkbp1-KO mice. Tbkbp1 had a cell-intrinsic role in NKT cell regulation and acted by mediating the survival signal of IL-15. Our data revealed that IL-15 induces NKT cell autophagy via a Tbkbp1-dependent mechanism.

Autophagy activation has been extensively studied as a response to stress conditions, such as nutrient deprivation, but how autophagy is induced during physiological processes is still less well understood. Our present study provided an example of physiological autophagy induction in the immune system. Unlike nutrient deprivation, which triggers autophagy by inactivating mTORC114, the IL-15-mediated autophagy induction occurred along with mTORC1 activation. Tbkbp1 antagonized the autophagy-inhibitory function of mTORC1 by preventing mTORC1-mediated Ulk1 phosphorylation at an inhibitory residue (S757), thereby facilitating Ulk1 activation by AMPKa. This function of Tbkbp1 was specific and involved physical interaction with the Ulk1-FIP200 complex and recruitment of an mTORC1-opposing phosphatase, PP6. Thus, Tbkbp1 enables autophagy induction by IL-15 without inactivation of mTORC1.

We showed that Tbkbp1 deficiency impaired IL-15-induced expression of Bcl2 and Bcl-XL and survival of NKT cells without compromising the major signaling events, including activation of STAT5 and AKT. We obtained evidence that autophagy induction is an integral part of the survival function of IL-15, since pharmacological inhibition of autophagy impaired IL-15-induced Bcl2 expression and apoptosis inhibition. Of note, mice deficient in autophagy have selective loss of stage 3 NKT (or NKT1) cells8, a phenotype that is similar to that of the Tbkbp1- or Il15-deficient mice. Autophagy also plays a role in the regulation of NK cells and conventional T cells6–10. We found that Tbkbp1 was required for IL-15-mediated autophagy and survival of NK cells but not of memory CD8+ T cells. One possible reason for such functional selectivity of Tbkbp1 is its abundant expression in NK and NKT cells and relatively weak expression in conventional T cells.

Tbkbp1 deficiency had little or no effect on the development of NK1.1+ (stages 1 and 2) NKT cells but caused a severe loss of the NK1.1+CD44+ (stage 3) NKT cells. This phenotype is reminiscent of the mice with deficiencies in IL-15 signaling, which have selective loss of stage 3 NKT cells18,20. While these results may suggest a role for Tbkbp1 in regulating NKT terminal maturation, recent studies suggest that the previously defined stage 2 NKT cells actually contain the mature NKT2 and NKT17 sublineages, whereas the stage 3 cells form the mature NKT1 cells. Our data, based on analysis of the lineage marker IL-17Rb and transcription factors, suggest an essential role for Tbkbp1 in regulating the homeostasis and survival of NKT1, but not NKT2 or NKT17, cells. These results are consistent with the previous finding that NKT1 cells, but not NKT2 or NKT17 cells, abundantly express IL-15Rb and rely on IL-15 for survival and homeostasis19,20. A recent study further suggests that the survival of NKT17 cells is mediated by IL-7 but not IL-1521. Consistently, we found Tbkbp1 to be required for NKT cell survival induced by IL-15 but not by IL-7.
The Tbkbp1-KO mice generally resemble Il15-KO mice in the phenotype of NKT cell development, but there seem to be some minor differences between these two mutant strains. Although both Tbkbp1-KO and Il15-KO mice have a drastically reduced absolute number of stage 3 NKT cells, the former also have a more severe reduction in the percentage of this NKT population. While this could be due to different experimental conditions, it is also likely that Tbkbp1 may play a crucial, but not the only, role in mediating IL-15-induced NKT cell survival. One possibility is that Tbkbp1 also modulates the signaling function of additional cytokines in the cytokine family. In support of this possibility, we found that Tbkbp1 was also required for IL-2-induced NKT cell autophagy and survival. Future studies will examine the role of Tbkbp1 in regulating the signaling function of additional cytokines in NKT cells.

Flow cytometry and intracellular cytokine staining. Suspensions of thymocytes, splenocytes, and liver cells were prepared and stained with PE-conjugated fluorochrome-conjugated antibodies and flow cytometry analysis as described [14] using LSRII (BD). NKT cells were detected using specific blue-labeled TCRβ and APC- or PE-labeled CD1d-tetramer loaded with PBS57. The unloaded CD1d-tetramer was used as a negative control. For intracellular cytokine staining (ICS), NKT and T cells were stimulated with 4 h with PMA (10 nM) and ionomycin (1 μM) and monensin (2 μM). NKT cells were harvested by FACS and intracellular cytokine staining was performed following staining of cells with Fixable Viability Kit (C10491) and LysoTracker Deep Red (M36008), and then subjected to ICS and flow cytometry analysis. Gating strategies are shown in Supplementary Fig. 8 and 9. The data were analyzed using FlowJo software.

Autophagy detection by confocal microscopy. Cells were either not treated or stimulated with IL-15 for 24 h and fixed with 4% (w/v) paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were washed three times with PBS and then permeabilized with digitonin (20 μg/ml in PBS) for 5 min at room temperature and blocked with 10% goat serum in PBS. For autophagy detection, the cells were incubated with anti-LC3 antibody (1:1000), secondary antibody (anti-rabbit Alexa 488, 1:2000) and then subjected to ICS and flow cytometry analysis.

NKT and NK cell isolation and stimulation. Thymocytes were prepared from young adult mice (6-8 week old) and incubated with PE-labeled anti-CD1d-tetramer antibody for isolation of NKT cells using anti-PE magnetic beads. The NKT cells were further purified by flow cytometric cell sorting based on TCRβ expression and Ulk1 phosphorylation. The cells were stimulated with IL-15 and subjected to IB analysis of autophagy induction based on LC3 modification and Ulk1 phosphorylation.

Cell proliferation and apoptosis assays. Enriched NKT Cells were stained for 5 min with carboxyfluorescein succinimidyl ester (CFSE) in PBS with a final concentration of 5 μM and then washed with ice cold RPMI-1640 medium supplemented with 10% FBS. CFSE-labeled NKT cells were cultured with anti-I-A^d, anti-CD49d, anti-LFA-1 and anti-CD49d coated plate at a density of 10^5/ml in T-25 flasks at 37°C. NKT cells were further purified by flow cytometric cell sorting based on TCRβ staining. Purified NKT cells were stimulated with IL-15 and subjected to IB analysis of autophagy induction based on LC3 modification and Ulk1 phosphorylation.

Plasmids. pCLXSN(GFP)-HA-Tbkbp1 was generated by inserting the mouse Tbkbp1 cDNA, along with a N-terminal HA tag, into the pCLXSN(GFP) retroviral vector [12], and the same approach was used to construct pCLXSN(GFP)-based expression vectors encoding truncated forms of Tbkbp1, 1-280 and 1-330. The Tbkbp1 cDNA, along with an N-terminal HA tag, was inserted into the pCLXSN(GFP) retroviral vector. The same approach was used to construct pCLXSN(GFP)-based expression vectors encoding truncated forms of Tbkbp1, 1-280 and 1-330. The Tbkbp1 cDNA, along with an N-terminal HA tag, was inserted into the pCLXSN(GFP) retroviral vector. The same approach was used to construct pCLXSN(GFP)-based expression vectors encoding truncated forms of Tbkbp1, 1-280 and 1-330. The Tbkbp1 cDNA, along with an N-terminal HA tag, was inserted into the pCLXSN(GFP) retroviral vector.
were incubated for 15 min with FITC-annexin V and propidium iodide (PI) and subjected to flow cytometry to quantify the apoptotic cell population.

**Memory CD8** T cell proliferation and in vitro, Memory CD8^+ T cells were generated as described. In brief, naïve CD8^+ T cells were activated using plate bound α-CD3 (5 μg/ml), soluble α-CD28 (0.5 μg/ml), and IL-2 (100 U/ml) for 3 days. T cells were then cultured in mIL-15-supplemented medium (100 U/ml) for more days with daily changes of fresh mIL-15 medium. Memory CD8^+ T cells were washed and starved for overnight and then were restimulated with mIL-15 for apoptosis and IB assays.

**Human T cell and NKT cell culture.** Human peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% human serum (Gemini Bio, 100-512), 10 mM HEPES buffer solution, 2 mM GlutaMAX, 1 mM sodium pyruvate, 5.5 mM 2-ME, 100 unit/ml penicillin, and 100 μg/ml streptomycin. For preparing conventional T cells, PBMCs were cultured with 1000 U IL-2 and 2 × 10^5 cells/ml in a 24-well plate by changing media every 2 days. On day 7, CD4^+ and CD8^+ T cells were analyzed by flow cytometry and sorted for RNA isolation and IB assays. For NKT cell culture, PBMCs were cultured with 100 U IL-2 and 100 ng/ml α-GalCer at a density of 2 × 10^5 cells/ml in a 24-well culture plate by changing media every 2 days. On day 14, NKT cells were analyzed by flow cytometry and sorted for RNA isolation and immunoblot assays.

**Cell culture and viral transduction.** Vali41 NKT cell hybridoma 1.2 (NK1.2) was provided by M Kronenberg (La Jolla Institute for Allergy and Immunology, La Jolla, California) and described previously. For gene silencing, lentiviral particles were prepared by transfecting HEK293 cells (ATCC) with pgPiZ lentiviral vectors encoding specific shRNAs or control shRNAs along with packaging plasmids. The packages were used to infect NKT1.2 cells, followed by selection of the infected cells by flow cytometric cell sorting based on GFP expression (pgPiZ vector carries the GFP gene). The infected cells were stimulated with IL-15 for signaling and autophagy analyses. For overexpression studies, the cells were infected with retroviral vectors for the indicated cDNAs.

**IB and colP assays.** Whole-cell lysates were prepared and subjected to IB and colP assays as described previously. The density of the protein bands in photographic films was determined by densitometry using the ImageJ software, and the level of LC3II and p62 proteins was presented as ratios to that of loading control (tubulin). SD is calculated from the mean of three independent experiments. Statistical analysis was performed by two-way ANOVA with Bonferroni multiple comparison test. Uncropped gel images are shown in Supplementary Fig. 10–14.

**BM adoptive transfer.** BM cells isolated from Tbkbp1-KO or WT mice (CD45.2^+^) were mixed with BM cells from B6.SJL (CD45.1^+^) mice. The challenged mice were monitored for tumor growth, and the studied mice with murine B16 melanoma cells were administered i.p. with rat anti-mouse CD8 antibody or isotype control rat IgG2a (2A3) at the dose of 150 μg per mouse. The challenged mice were analyzed by flow cytometric analysis of draining lymph node cells.

**Statistical analysis.** Statistical analysis was performed using Prism software (GraphPad Software 6.0). The Kolmogorov–Smirnov test was used to tests for normal distribution of the data. If the samples were normally distributed, unpaired two-tailed Student’s t-test was used to determine the statistical difference between two groups. For comparison of more than two groups, one-way ANOVA followed by Bonferroni multiple comparisons post-test were performed. If the samples were not normally distributed, the Mann-Whitney test was performed for two groups, Two-way ANOVA with Bonferroni multiple comparison test was used for B16-OVA tumor growth. Kaplan-Meier analyses was used and the log-rank Mantel-Cox test was employed to infer significance between the cumulative death curves of two groups. All data are presented as mean ± SD. A p value <0.05 was considered significant, and the level of significance expressed as follows: *P < 0.05; **P < 0.01; ***P < 0.001. The number of animals used (n), and the specific statistical tests used are indicated for each experiment in the figure legends.

**Data availability.** The datasets generated during the current study are available from the corresponding author on reasonable request.

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Author contributions
Lele Z. designed the study, performed experiments, analyzed data, and wrote part of the manuscript; X.X., Lingyun Z., Z.J., X.Z., J.S., Z.S., B.Z., and X.C. contributed to the performance of the experiments; and S.-C.S. supervised the work and wrote the manuscript.

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