A Unique Role for ITK in Survival of Invariant NKT Cells Associated with the p53-Dependent Pathway in Mice

Qian Qi, Weishan Huang, Yuting Bai, Gabriel Balmus, Robert S. Weiss and Avery August

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Invariant NKT (iNKT) cells play important roles in the immune response. ITK and TXK/RLK are Tec family kinases that are expressed in iNKT cells; the expression level of ITK is ∼7-fold higher than that of TXK. Itk<sup>−/−</sup> mice have reduced iNKT cell frequency and numbers, with defects in development and cytokine secretion that are exacerbated in Itk/Txk double-knockout mice. In contrast, there is no iNKT cell defect in Txk<sup>−/−</sup> mice. To determine whether ITK and TXK play distinct roles in iNKT cell development and function, we examined mice that overexpress TXK in T cells at levels similar to Itk. Overexpression of TXK rescues the maturation and cytokine secretion of Itk<sup>−/−</sup> iNKT cells, as well as altered expression of transcription factors T-bet, eomesodermin, and PLZF. In contrast, the increased apoptosis observed in Itk<sup>−/−</sup> splenic iNKT cells is not affected by TXK overexpression, likely due to the lack of effect on the elevated expression of p53 regulated proapoptotic pathways Fas, Bax, and Bad in those cells. Supporting this idea, p53 overexpression, likely due to the lack of effect on the elevated expression of p53 regulated proapoptotic pathways Fas, Bax, and Bad in those cells. Supporting this idea, p53<sup>−/−</sup> and Bax<sup>−/−</sup> mice have increased splenic iNKT cells. Our results suggest that TXK plays an overlapping role with ITK in iNKT cell development and function but that ITK also has a unique function in the survival of iNKT cells, likely via a p53-dependent pathway.

**A Unique Role for ITK in Survival of Invariant NKT Cells Associated with the p53-Dependent Pathway in Mice**

Qian Qi,†,‡,§ Weishan Huang, *,†,‡,§ Yuting Bai, *,†,‡,§ Gabriel Balmus, ‡ Robert S. Weiss, ‡ and Avery August*,†,‡

In natural killer T cells are a distinct subset of T cells that recognize glycolipid Ag presented by the CD1d glycoprotein. NKT cells are composed of type 1 NKT cells and type 2 NKT cells. Type 1 NKT cells, also called invariant NKT (iNKT) cells, express an invariant Vα-chain (Vα14Jα18 in mice and Vα24Jα18 in human), together with certain types of Vβ-chains, and can be detected by α-galactosyl ceramide (α-GalCer)-loaded CD1d tetramer (1). Upon stimulation, iNKT cells are able to rapidly secrete large amounts of multiple cytokines, such as IL-4, IFN-γ, and IL-17, and play important roles in the pathogenesis of allergic asthma, bacterial infection, and cancer (1).

iNKT cells initiate their development from CD4<sup>+</sup>CD8<sup>+</sup> (double-positive) T cells and are selected by glycolipid Ags in the context of CD1d molecules expressed on double-positive T cells. After selection, iNKT cells further develop through four stages to become mature. The developmental stages of iNKT cells are defined based on the expression of their surface markers CD24, CD44, and NK1.1. The most immature iNKT cell (stage 0) is phenotypically characterized as CD24<sup>−</sup>CD44<sup>−</sup>NK1.1<sup>−</sup>, followed in a stepwise manner by downregulation of CD24 (CD24<sup>−</sup>CD44<sup>−</sup>NK1.1<sup>−</sup>; stage 1) and upregulation of CD44 (CD24<sup>−</sup>CD44<sup>−</sup>NK1.1<sup>−</sup>; stage 2) and NK1.1 (CD24<sup>−</sup>CD44<sup>−</sup>NK1.1<sup>−</sup>; stage 3) to become the final mature iNKT cell. In addition, some of the iNKT cells at developmental stage 2 also migrate to the spleen, where they upregulate NK1.1 as a final maturation step (1). The signaling events that are required for the selection and maturation of iNKT cells have been studied extensively. Both TCR and SLAM–SAP–Fyn signaling pathways are required for the selection of iNKT cells (2–5). Transcription factors PLZF, Egr2, NF-κB family members, signaling proteins Dock2 and PDK1, and microRNA processor Dicer were shown to be important for the maturation of iNKT cells from stage 1 to stage 2 (6–12). Transcription factor T-bet, vitamin D receptor, cytokine IL-15, cell surface receptor CD28 and ICOS, SLP-76, and PTEN are important for the final maturation of iNKT cells from stage 2 to stage 3 (1, 13–22).

ITK and TXK/RLK (referred to as TXK hereafter) are two Tec family tyrosine kinase members expressed in T cells. ITK is composed of five domains, which, from the N terminus to the C terminus, are PH, TH, SH3, SH2, and kinase domain. ITK is phosphorylated in an ATP-dependent manner by downregulation of CD24 (CD24<sup>−</sup>CD44<sup>−</sup>NK1.1<sup>−</sup>; stage 1) and upregulation of CD44 (CD24<sup>−</sup>CD44<sup>−</sup>NK1.1<sup>−</sup>; stage 2) and NK1.1 (CD24<sup>−</sup>CD44<sup>−</sup>NK1.1<sup>−</sup>; stage 3) to become the final mature iNKT cell. In addition, some of the iNKT cells at developmental stage 2 also migrate to the spleen, where they upregulate NK1.1 as a final maturation step (1). The signaling events that are required for the selection and maturation of iNKT cells have been studied extensively. Both TCR and SLAM–SAP–Fyn signaling pathways are required for the selection of iNKT cells (2–5). Transcription factors PLZF, Egr2, NF-κB family members, signaling proteins Dock2 and PDK1, and microRNA processor Dicer were shown to be important for the maturation of iNKT cells from stage 1 to stage 2 (6–12). Transcription factor T-bet, vitamin D receptor, cytokine IL-15, cell surface receptor CD28 and ICOS, SLP-76, and PTEN are important for the final maturation of iNKT cells from stage 2 to stage 3 (1, 13–22).

ITK and TXK/RLK (referred to as TXK hereafter) are two Tec family tyrosine kinase members expressed in T cells. ITK is composed of five domains, which, from the N terminus to the C terminus, are PH, TH, SH3, SH2, and kinase domain. ITK is normally found in the cytoplasm of resting T cells, and, upon activation, PI3K is activated, generating PI3P in the cell membrane. The generation of PI3P recruits ITK from the cytoplasm to the cell membrane via the PH domain of Itk. The structure of TXK is very similar to ITK, with the exception of the N terminus. Instead of a PH domain, TXK has a cysteine-string motif that allows for constitutive localization at the plasma membrane. Both ITK and TXK are expressed in iNKT cells, with the expression level of ITK being ∼7-fold higher than TXK (23). Previous studies showed that, in the absence of Itk, the number of iNKT cells is decreased, development is blocked at developmental stage 2, and cytokine production is defective (23, 24). All of these defects are exacerbated in Itk<sup>−/−</sup>Txk<sup>−/−</sup> double-knockout (DKO) mice; however, Txk<sup>−/−</sup> mice have normal iNKT cell maturation and number (23). Given the similarities, as well as the differences, in the structure of ITK and TXK, we wanted to determine whether TXK is truly functionally redundant to ITK in iNKT cells. However, because of the low expression levels of TXK in T cells, analysis of Txk<sup>−/−</sup> mice

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*Abbreviations used in this article: DKO, double knockout; α-GalCer, α-galactosyl ceramide; iNKT, invariant NKT; WT, wild-type.

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is not as informative. Therefore, we used transgenic mice that overexpress TXX at levels similar to endogenous ITK in wild-type (WT) mice (Tg(CD2-Txk)Itk<sup>+/−</sup>−) and show that TXX has overlapping roles with ITK in iNKT cell development, transcription factor expression, and cytokine production but that ITK has a distinct role in regulating the survival of iNKT cells.

**Materials and Methods**

**Mouse**

Tg(CD2-Txk)Itk<sup>+/−</sup>− mice were a kind gift from Dr. Paul Love (via Dr. Pamela Schwartzberg, National Human Genome Research Institute/National Institutes of Health) and were generated as described previously (25). WT, Itk<sup>−/−</sup>, Tg<sup>−/−</sup>, and Tg(CD2-Txk)Itk<sup>+/−</sup>− mice were on a C57BL/6 background and kept in specific pathogen-free conditions. Bax-deficient mice on a C57BL/6 background were from The Jackson Laboratory. Mice lacking p53, along with WT controls, were maintained on a 129S6 background (26). All of the mice (with the exception of p53<sup>−/−</sup> mice) were 6–9 wk of age, whereas p53<sup>−/−</sup> mice were used at 4–5 wk old, prior to tumor development normally observed in these mice. Cells from 4–5-wk-old Bad<sup>−/−</sup> mice on a C57BL/6 background were from Dr. Nika Daniel (Dana Farber Cancer Institute/Harvard Medical School) and were compared with those from age-matched WT mice. Experiments were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University and Cornell University.

**Flow cytometry**

Single-cell suspensions from tissues collected from the indicated mice were incubated with Fc block (BD Biosciences, San Jose, CA) for 10 min and then were stained with fluorescent Abs for 30 min on ice. PE-BS57/CD1d tetramer was kindly provided by the National Institutes of Health tetramer core facility. Anti–FITC-TCRβ, V500-CD44, allophycocyanin–Cy7–TCRβ, PECy5-CD44, and PECy7-CD4 were from BD Biosciences; anti-PECy7-NKL1, FITC-CD44, allophycocyanin–NK1.1, FITC-CD122, and allophycocyanin–Alexa Fluor 750–TCRβ were from ebioScience (San Diego, CA); and anti-PE/Texas Red CD8 and anti-PE/Texas Red CD4 were from Caltag (Burlingame, CA).

**In vivo BrdU-incorporation assay**

WT, Itk<sup>−/−</sup>, and Tg(CD2-Txk)Itk<sup>+/−</sup>− mice were injected i.p. with 1 μg BrdU in 100 μl PBS, and drinking water containing 0.8 mg/ml BrdU was provided for 6 d. BrdU-containing drinking water was changed every 2 d. Cells from thymus, spleen, and liver were then isolated and stained with surface Abs, followed by BrdU staining using an APC-BrdU kit (BD Biosciences).

**Induction of Fas-mediated apoptosis in vitro**

Splenocytes were collected from WT, Itk<sup>−/−</sup>, and Tg(CD2-Txk)Itk<sup>+/−</sup>− mice and directly plated in 24-well plates at a density of 2 × 10<sup>4</sup>/ml in each well. Cells were treated or not with 5 μl/ml anti-CD95 (Fas) Ab Jo2 (BD Biosciences) for the indicated time to induce Fas-mediated apoptosis. Cells were stained for surface markers, washed in PBS, and further stained with PE-BS57/CD1d for the indicated time to induce Fas-mediated apoptosis. Cells were incubated with Fc block (BD Biosciences, San Jose, CA) for 10 min and then were stained with fluorescent Abs for 30 min on ice. PE-BS57/CD1d tetramer was kindly provided by the National Institutes of Health tetramer core facility. Anti–FITC-TCRβ, V500-CD44, allophycocyanin–Cy7–TCRβ, PECy5-CD44, and PECy7-CD4 were from BD Biosciences; anti-PECy7-NKL1, FITC-CD44, allophycocyanin–NK1.1, FITC-CD122, and allophycocyanin–Alexa Fluor 750–TCRβ were from ebioScience (San Diego, CA); and anti-PE/Texas Red CD8 and anti-PE/Texas Red CD4 were from Caltag (Burlingame, CA).

**Cell sorting and real-time PCR analysis**

Thymic iNKT cells (TCRβ<sup>+</sup>/BS57/CD1d tetramer<sup>+</sup>) were sorted from WT, Itk<sup>−/−</sup>, and Tg(CD2-Txk)Itk<sup>+/−</sup>− mice, and naive conventional CD4<sup>+</sup> T cells (TCRβ<sup>+</sup>/CD4<sup>+</sup> CD44<sup>+</sup>) were sorted from spleens of WT and Itk<sup>−/−</sup> mice, using a Cytopeia Influx Cell Sorter (Cytopeia, Seattle, WA). RNA was isolated using an RNeasy Mini kit (QIAGEN, Valencia, CA), followed by generation of cDNA with You Prime First-Strand beads kit (GE Healthcare, Piscatway, NJ). Quantitative real-time PCR was carried out using Taqman primer/probe sets for Bax, Bad, Bcl-2, Bcl-6, Bcl2L1, T-bet, comedosomin, Eg2r, Fas, p53, and PLZF with GAPDH as a housekeeping gene (Applied Biosystems, Foster City, CA). Data were analyzed using the comparative threshold cycle 2<sup>−ΔΔCT</sup> method, normalized to the expression of GAPDH, and the values were expressed as fold increase compared with WT iNKT cells, which was set as 1. To compare the expression level of Itk and Txk, eQPCR was performed on cDNA from sorted WT or Txk<sup>+/−</sup>/Itk<sup>−/−</sup>− iNKT cells. Signals were adjusted using standards generated from plasmids carrying the cDNA for murine Itk or Txk and normalized to the expression of GAPDH. The values were expressed as fold increase compared with the Itk expression in WT iNKT cells sorted from thymus or spleen, which was set as 1.

**α-GalCer stimulation and intracellular cytokine staining**

WT, Itk<sup>−/−</sup>, and Tg(CD2-Txk)Itk<sup>+/−</sup>− mice were injected i.p. with 100 μl PBS containing either 2 μg α-GalCer or 1 μl DMSO. After 2 h, splenocytes were collected and cultured in vitro for 4 h in the presence of 10 μg/ml brefeldin A. Cells were stained for surface markers, followed by intracellular staining of IL-4 and IFN-γ using a BD fixation/permeabilization kit.

**Data analysis**

Statistical analyses were determined using GraphPad Prism (GraphPad Software, La Jolla, CA), using the Student t test, with p ≤ 0.05 considered statistically significant.

**Results**

**Overexpression of TXK does not restore the number of iNKT cells in Itk<sup>−/−</sup> mice**

Previous studies demonstrated that the absence of ITK results in defects in iNKT cell development, which are exacerbated in Itk<sup>−/−</sup> mice but not in Itk<sup>−/−</sup>/Txk<sup>−/−</sup> double knockout (DKO) mice, suggesting that TXK also has a role in iNKT cell development and function (23, 27). However, the number of iNKT cells is unchanged in the absence of TXK alone (23). In addition, we determined that the development of iNKT cells is also normal in the absence of TXK (Supplemental Fig. 1A). Because TXK is expressed at ~7-fold lower levels compared with ITK in iNKT cells (23) (Supplemental Fig. 1B), this normal iNKT cell number and development in Itk<sup>−/−</sup> mice may be due to the redundant functions of ITK and TXK in iNKT cells. Given the exacerbation of iNKT cell defects in Itk<sup>−/−</sup>/Txk<sup>−/−</sup> DKO mice, we examined whether increased expression of TXK is able to rescue defects in iNKT cells in the absence of Itk. To do this, we used Tg(CD2-Txk)Itk<sup>+/−</sup>− mice, which overexpress TXK specifically in T cells at levels that are similar to endogenous ITK in T cells (23, 25, 28) (Supplemental Fig. 1B).

Flow cytometric analysis revealed that, in the absence of Itk, the percentage and number of iNKT cells in the thymus and periphery were significantly decreased (Fig. 1A–C). Overexpression of TXK restored the percentage of iNKT cells in the thymus of Itk<sup>−/−</sup> mice to WT levels, although the numbers of these cells were not restored because of decreased total thymocyte number in Tg(CD2-Txk)Itk<sup>+/−</sup>− mice (Supplemental Fig. 2, note that the Itk transgene fails to restore thymic cellularity in Itk<sup>−/−</sup> mice). In contrast, the frequency and number of iNKT cells in the spleen of Itk<sup>−/−</sup> mice were not restored by overexpressing TXK (Fig. 1A–C). The percentage and number of hepatic iNKT cells in Tg(CD2-Txk)Itk<sup>+/−</sup>− mice were increased compared with Itk<sup>−/−</sup> mice but remained lower than those in WT mice. This result demonstrates that overexpression of TXK can restore the percentage of iNKT cells in the thymus of Itk<sup>−/−</sup> mice but not the numbers or percentages in the periphery of these mice.

**Overexpression of TXK rescues the maturation of iNKT cells in the absence of Itk**

To determine the role of TXK in the development of iNKT cells, we analyzed the expression of CD44 and NK1.1 on thymic iNKT cells of WT, Itk<sup>−/−</sup>, and Tg(CD2-Txk)Itk<sup>+/−</sup>− mice. The majority of WT iNKT cells were CD44<sup>+</sup>NK1.1<sup>+</sup>, suggesting that most of them are mature and in developmental stage 3 (Fig. 1D). In contrast, a significantly higher percentage of Itk<sup>−/−</sup>− iNKT cells were CD44<sup>+</sup>NK1.1<sup>+</sup>, indicating that iNKT cells are blocked in developmental stage 2 in the absence of Itk. However, in the thymus of Tg(CD2-Txk)Itk<sup>+/−</sup>− mice, the percentage of CD44<sup>+</sup>NK1.1<sup>+</sup> iNKT cells was significantly increased compared with those in Itk<sup>−/−</sup> mice, and it was similar to WT iNKT cells. This indicates that overexpression...
of TXK can rescue the development of iNKT cells in the thymus in the absence of ITK (Fig. 1D). In addition, because CD44\(^+\) NK1.1\(^+\) (developmental stage 2) iNKT cells also migrate to spleen to undergo final maturation, we assessed the development of iNKT cells in the spleen. Similarly to the case in the thymus, overexpression of TXK restored the percentage of CD44\(^+\)NK1.1\(^+\) iNKT cells in \(\text{Itk}^{-/-}\) mice to similar levels as in WT mice (Fig. 1E). By contrast and as noted before, the number of mature iNKT cells was partially rescued, although not to the level of WT mice. These data suggest that pathways regulated by TXK can function similarly to ITK in regulating the full maturation of iNKT cells.

Overexpression of TXK rescues \(\alpha\)-GalCer–induced cytokine secretion by iNKT cells in the absence of Itk

iNKT cells are able to secrete large amounts of IL-4 and IFN-\(\gamma\) in response to \(\alpha\)-GalCer stimulation, a response that is defective in the absence of ITK (23, 27). To determine whether TXK can function in the absence of ITK in regulating iNKT cell cytokine secretion, we injected \(\alpha\)-GalCer i.p. into WT, \(\text{Itk}^{-/-}\), and \(\text{Tg}(\text{CD2-Txk})\text{Itk}^{-/-}\) mice, followed by analysis of IL-4 and IFN-\(\gamma\) production by iNKT cells as determined ex vivo using intracellular cytokine staining. Compared with WT iNKT cells, \(\text{Itk}^{-/-}\) iNKT cells produced much less IL-4 and IFN-\(\gamma\) (Fig. 2). In contrast, overexpression of TXK restored cytokine production in \(\text{Itk}^{-/-}\) iNKT cells to WT levels, indicating that TXK can regulate the signaling pathway downstream of ITK for cytokine production in iNKT cells (Fig. 2).

Overexpression of TXK normalizes expression of CD122 and transcription factors in \(\text{Itk}^{-/-}\) iNKT cells

IL-15 signals and IL-15R CD122 were shown to be important for the final maturation of iNKT cells, and the expression of CD122 was suggested to correlate with the maturation of iNKT cells in \(\text{Itk}^{-/-}\) mice. We previously showed that a kinase-defective mutant of ITK can partly restore the expression of CD122 on iNKT cells in the absence of Itk, suggesting that the signaling pathways that lead to CD122 expression in iNKT cells may be, in part, kinase independent (27). To determine whether TXK can also restore the expression of CD122 in iNKT cells in the absence of Itk, we determined CD122 expression levels on iNKT cells in the thymus

FIGURE 1. Overexpression of TXK rescues frequency and maturation, but not number, of iNKT cells in the absence of Itk. (A) Flow cytometric analysis of iNKT cells from thymus, spleen, and liver of WT, \(\text{Itk}^{-/-}\), and \(\text{Tg}(\text{CD2-Txk})\text{Itk}^{-/-}\) mice. Determination of the percentage (B) and number (C) of iNKT cells from thymus (\(n > 9\)), spleen (\(n > 9\)), and liver (\(n = 4\)). Thymocytes (D) and splenocytes (E) from WT, \(\text{Itk}^{-/-}\), and \(\text{Tg}(\text{CD2-Txk})\text{Itk}^{-/-}\) mice were gated on iNKT cells (tetramer\(^+\)/TCR\(\beta\)^+\()\) and analyzed for the expression of CD44 and NK1.1 (top panel). The percentage and number of CD44\(^-\)NK1.1\(^-\), CD44\(^+\)NK1.1\(^-\), and CD44\(^+\)NK1.1\(^+\) iNKT cells were determined (bottom panels) (\(n > 8\)). *\(p < 0.05\), **\(p < 0.01\).

FIGURE 2. Overexpression of TXK rescues \(\alpha\)-GalCer–induced cytokine secretion of iNKT cells in the absence of ITK. WT, \(\text{Itk}^{-/-}\), and \(\text{Tg}(\text{CD2-Txk})\text{Itk}^{-/-}\) mice were injected i.p. with either PBS or 2 \(\mu\)g \(\alpha\)-GalCer. After 2 h, splenocytes were collected and cultured in vitro for 4 h in the presence of 10 \(\mu\)g/ml brefeldin A. Cells were analyzed by flow cytometry, gated on iNKT cells (tetramer\(^+\)/TCR\(\beta\)^+\()\), and analyzed for IL-4 and IFN-\(\gamma\) expression. Data are representative of two independent experiments.
and spleen of the three mouse strains. Although the majority of WT thymic and splenic iNKT cells express CD122, the percentage of iNKT cells that express CD122 in Itk−/− mice was dramatically decreased, as previously reported (23, 27). In contrast, the percentage of iNKT cells that express CD122 in Tg(CD2-Txk)Itk−/− mice was similar to those in WT mice (Fig. 3A).

Several transcription factors have been reported to be important for the development and function of iNKT cells. PLZF and Egr2 are important for the development of NKT cells from stage 1 to stage 2 (6–8), whereas T-bet is critical for the final maturation of iNKT cells (14, 15). In addition, the expression level of eomesodermin, another member of the T-box family of transcription factors, is important for regulating CD122 expression in innate CD8 T cells, although it is not normally expressed in iNKT cells (14, 29). In the absence of Itk, the expression of these transcription factors is deregulated (23, 27). To determine the effect of TXK on these transcription factors, their expression levels in thymic and splenic iNKT cells of WT, Itk−/−, and Tg(CD2-Txk)Itk−/− mice were analyzed by quantitative RT-PCR. In the absence of Itk, levels of mRNA for T-bet in thymic iNKT cells were decreased, whereas the levels of mRNA for PLZF, Egr2, and eomesodermin were significantly increased, indicating that signals from ITK are required for proper expression of these transcription factors in thymic iNKT cells (23, 27) (Fig. 3B). Overexpression of TXK restored this altered expression of transcription factors in Itk−/− thymic iNKT cells to WT levels (Fig. 3B). In the splenic iNKT cells, TXK was able to restore the expression pattern of eomesodermin but not PLZF (Fig. 3C). These data suggest that similarly to ITK, TXK can regulate the pattern of expression of these transcription factors in iNKT cells.

Overexpression of TXK does not restore survival of Itk−/− iNKT cells

To determine the reason for the inability of TXK to restore iNKT cells numbers in Tg(CD2-Txk)Itk−/− mice, we examined whether these cells could proliferate similarly to those in WT mice. To do this, we analyzed incorporation of BrdU in iNKT cells in the thymus, spleen, and liver of these mice. We found that iNKT cells in WT and Tg(CD2-Txk)Itk−/− mice incorporated similar levels of BrdU in these three tissues, whereas iNKT cells in the thymus of Itk−/− mice had increased BrdU uptake, which reflected the block in iNKT cell development at the NK1.1 stage in these mice, where iNKT cells have higher cycling rates (Fig. 4) (23, 27). This finding suggests that ITK and TXK do not regulate the proliferation of iNKT cells and that the differences in iNKT cells numbers are not due to differences in the proliferation of these cells. However, this result indicates that TXK may alleviate the block in differentiation of iNKT cells seen in the absence of Itk, thus normalizing proliferation of thymic iNKT cells.

Differences in iNKT cells numbers may be due to increased cell death. Therefore, we determined the level of apoptosis in these cells by staining with Annexin V. Although the percentage of Annexin V+ iNKT cells was similar in the three strains in the thymus, the percentage of those cells in Itk−/− spleens that were Annexin V+ was much higher than that in WT spleens, and overexpression of TXK did not alter this increased percentage. This result suggests that apoptosis of iNKT cells is increased in the absence of Itk and that overexpression of TXK does not restore the survival of these cells (Fig. 5A). We further determined whether the expression of molecules that could regulate apoptosis is altered in Itk−/− and Tg(CD2-Txk)Itk−/− iNKT cells and found that expression of FasL was similar in WT, Itk−/−, and Tg(CD2-Txk)Itk−/− splenic iNKT cells. In contrast, Fas expression was much higher in splenic Itk−/− iNKT cells compared with WT iNKT cells, and overexpression of TXK did not normalize this pattern (Fig. 5B). We next determined whether the increased expression of Fas on Itk−/− and Tg(CD2-Txk)Itk−/− splenic iNKT cells reflected increased
susceptibility to apoptotic signals in vitro. Splenocytes from WT, Itk−/−, and Tg(CD2-Txk)Itk−/− mice were treated with agonistic Fas mAb in vitro and analyzed for levels of apoptosis. We found that Itk−/− and Tg(CD2-Txk)Itk−/− iNKT cells exhibited increased apoptosis compared with WT iNKT cells in response to Fas triggering, suggesting that reduced numbers of iNKT cells in the periphery of the former strains may be due to increased susceptibility to death signals (Fig. 5C). Similar analysis of the expression levels of receptors for survival cytokines IL-2 and IL-7 revealed no differences between the different strains (Supplemental Fig. 3). This inability of the Txk transgene to restore survival was not due to reduced expression of the transgene in the peripheral iNKT cells, because the expression levels were similar in both thymic and splenic iNKT cells (Supplemental Fig. 1B).

**Txk overexpression does not normalize the increased expression of proapoptotic genes in Itk−/− splenic iNKT cells**

To determine whether the elevated apoptotic response observed in Itk−/− and Tg(CD2-Txk)Itk−/− splenic iNKT cells represented a proapoptotic state, we analyzed these cells for expression of proapoptotic genes Bad, Bax, and Fas; antiapoptotic genes Bcl-2 and BclXL; and p53 and its negative regulator Bcl-6. Comparison of splenic iNKT cells from WT, Itk−/−, and Tg(CD2-Txk)Itk−/− mice showed that the latter mice exhibit higher levels of mRNA for Fas and its downstream proapoptotic mediators Bax and Bad, indicating that TXK fails to restore normal levels of Fas and Fas-mediated proapoptotic proteins caused by the absence of ITK (Fig. 6A). However, in thymic iNKT cells, increased TXK expression reduced the expression of these genes that are elevated in the absence of ITK, correlating with the lack of enhanced apoptosis of iNKT cells observed in the thymus (Fig. 6B). In contrast, although mRNA for antiapoptotic gene Bcl-2 was reduced in the absence of ITK in both thymic and splenic iNKT cells, increased expression of TXK is able to restore the expression of this gene to WT levels, whereas no difference was observed in BclXL expression (Fig. 6A). The expression of Bcl-6 was reduced in the absence of ITK in splenic iNKT cells but not in thymic iNKT cells, and it was normalized in Tg(CD2-Txk)Itk−/− thymic iNKT cells but not in Tg(CD2-Txk)Itk−/− splenic iNKT cells (Fig. 6). By contrast, p53 expression was induced in the absence of ITK in thymic and splenic iNKT cells; however, although its expression was normalized in Tg(CD2-Txk)Itk−/− thymic iNKT cells, this was not the case in splenic Tg(CD2-Txk)Itk−/− iNKT cells (Fig. 6). In addition, Bcl-6 expression was elevated in the absence of ITK in thymic iNKT cells, potentially counteracting the effects of elevated p53, but it was not elevated in Itk−/− and Tg(CD2-Txk)Itk−/− splenic iNKT cells. Because Bax, Bad, and Fas are regulated by p53, and Bcl-6 can regulate the activity of p53, these data suggest that ITK, but not TXK, specifically regulates the survival of peripheral iNKT cells by regulating the expression and function of the Fas pathway, perhaps via a p53-dependent pathway.

**Regulation of percentage and number of splenic iNKT cells by p53 and p53-regulated apoptotic regulators Bax and Bad**

The data shown in Fig. 6 suggest that the p53 pathway may regulate the survival of iNKT cells in the spleen. To determine whether this is the case, we analyzed mice lacking p53 and the p53-regulated apoptotic regulators Bax and Bad for levels of iNKT cells. This analysis revealed that although the absence of p53 did not affect the percentage or numbers of thymic iNKT cells, the percentage and numbers of these cells in the spleen were increased in the absence of p53 (Fig. 7A). Similarly, mice deficient in the p53-regulated proapoptotic molecule Bax had...
increased percentage and number of splenic iNKT cells (Fig. 7B), although Bad-deficient mice did not exhibit analogous changes in the percentage or numbers of iNKT cells (Fig. 7C). This suggests that the p53 pathway can regulate the survival of splenic iNKT cells and that the absence of ITK may affect the p53 pathway, thus regulating the survival of iNKT cells. The results also indicate that TXK cannot rescue this function of Itk.

**Discussion**

ITK and TXK are both expressed in T cells, and ITK was demonstrated to have a critical role in the development of naive T cells (30–36). In contrast, a role for TXK has been less clear, because Txk single knockouts do not exhibit any obvious phenotype, although this kinase does play a role in T cell development, because...
Itk/Txk DKO exhibit a more severe phenotype (32–34). More recent studies examining the role of these kinases in iNKT cell development revealed that, similarly to conventional T cells, ITK plays an important role in iNKT cell development, with TXK having a less well-defined role (23, 24, 27, 37). This is likely due to the fact that the expression of TXK in iNKT cells is ~7–10 fold less than Itk. In this study, we used Tg(CD2-Txk)Itk+/− mice, which have an expression level of TXK that is similar to endogenous ITK in iNKT cells, and show that, at this expression level, TXK rescues defects in the maturation and cytokine production of Itk−/− iNKT cells. This may be due to normalized expression of transcription factors T-bet, Egr2, PLZF, and eomesoderm. In contrast, increased expression of TXK does not restore the reduced numbers nor affect the elevated apoptosis of Itk−/− iNKT cells, which may be due to the failure to normalize elevated expression of the death receptor Fas and associated proapoptotic molecules. We also show that this may be due to deregulation of a p53 pathway leading to increased apoptosis, as well as that lack of p53 or Bax results in increased splenic iNKT cells. These data suggest that ITK and TXK have overlapping roles in the development and function of iNKT cells; however, ITK has a distinct role in the suppression of apoptosis of iNKT cells via a p53-dependent mechanism.

TCR stimulation leads to the recruitment of ITK to the cell membrane and subsequent activation of its kinase activity, which is due, in part, to the interaction between the PH domain of ITK and PI3K lipids at the cell membrane, a PI3K-dependent event (38). In contrast, TXK lacks a PH domain found in the other Tec kinase family members; instead, it has an N-terminal palmitoylation site (39). Among the Tec kinases, this would allow TXK to be uniquely independent of PI3K signaling and suggest unique functions in activation and downstream signaling for this kinase (38, 39). However, as noted above, Txk single knockouts do not exhibit an obvious phenotype in T cell development, activation, or function (40). Indeed, we previously showed that, although the absence of ITK affects naive T cell differentiation into Th2 cells, increased expression of TXK to levels equivalent to those for ITK can rescue this Th2 response (28). In addition, increased expression of TXK can improve positive-selection defects observed in the absence of ITK (41). This suggests that TXK can couple to similar intracellular signaling pathways that ITK uses for this function, including the activation of PLCγ1 and increases in intracellular calcium responses (28, 41). Therefore, it is surprising that, when examined in iTNK cells, increased expression of TXK can rescue the function of ITK in iTNK cell development, maturation, and function, but it cannot restore the survival of these cells. This indicates that ITK plays a unique role in regulating iTNK cell survival.

It is clear that TXK makes some contribution to the development of iNKT cells, because mice lacking both ITK and TXK exhibit more severe reductions in the numbers of iNKT cells compared with Itk−/− mice (23). This is largely due to a reduction in maturation from stage 2 to stage 3, which is accompanied by reduced expression of CD122 and T-bet, increased expression of Egr2, PLZF, and eomesodermin, and enhanced proliferation of cells at this stage of development. It is noteworthy that T-bet deficiency causes a block in iTNK cell development largely at stage 2, similar to that seen in the absence of Itk; therefore, this block that is observed in the Itk-deficient iTNK cells may be, in part, a consequence of T-bet deficiency and the subsequent reduction in CD122 expression and reduced responses to IL-15 (14, 19, 42). However, we recently reported that there is an ITK kinase domain-independent pathway that can partially restore T-bet expression and induce further maturation along the stage 3 pathway; but full maturation is not restored (27), supporting an important role for T-bet in this process (14). Eomesodermin is not normally expressed in iTNK cells (14), but we observed increased expression in the absence of ITK, and it is possible that aberrant expression of this factor affects the maturation of these cells. Although eomesodermin is related to T-bet and can have overlapping functions (e.g., in CD8+ T cells) (14, 29), it does have unique functions, which may be the case in iTNK cells. Furthermore, although PLZF is important for iTNK cell development (with a block at stage 1), as well as to gain effector function, overexpression of PLZF results in a block in maturation at stage 2 (7, 43). Normalization of expression of these factors, a function of both the ITK kinase-independent pathway and TXK, may be critical for further maturation of iTNK cells. Note that the effect of the ITK kinase-independent pathway on iTNK cell maturation is dependent on iTNK expression, suggesting that TXK collaborates with ITK to regulate iTNK cell maturation. Our current findings show that increasing TXK’s expression can fully rescue maturation and function and supports this idea. Given the ability of ITK to partially rescue iTNK cell development in a kinase-independent fashion, we speculate that TXK may also be able to do this, because the two kinases interact with similar proteins via their SH2 and SH3 domains (36). However, we expect that the kinase activity of both is critical for full maturation of these cells.

Despite the absence of a PH domain, TXK can engage pathways regulated by ITK for conventional T cell positive selection, Th2 differentiation, and now iTNK cell development, maturation, and function (25, 28, this work). Tg(CD2-Txk)Itk+/− mice have iTNK cells with normal maturation and function with regard to cytokine secretion, indicating that ITK and TXK share the same or overlapping pathways for the activation of these downstream effects. It is also possible that the reduced cytokine secretion in Itk-deficient iTNK cells may be related to the immature state of these cells, and that by rescuing maturation, overexpression of TXK also restores the ability of these cells to produce these cytokines. However, Itk−/− iTNK cells express similar levels of preformed mRNA for IL-4 and IFN-γ as expected in more mature iTNK cells. Moreover, Itk−/− iTNK cells are able to secrete both cytokines after PMA/ionomycin stimulation, which bypasses ITK and TCR, suggesting a signaling defect in cytokine secretion in addition to any maturational defects (23, 24, 27, 37). Nevertheless, our findings also reveal that there may be ITK-specific signals that result in the activation of pathways that prevent increased apoptosis of iTNK cells, leading to normal numbers of iTNK cells. Our findings suggest that this may be related to p53 pathway activation and expression of Fas and proapoptotic pathways in iTNK cells, which is elevated in the absence of Itk and not normalized when TXK expression is increased. The mechanism by which ITK suppresses the p53 pathway is unclear; however, Ras-regulated pathways, such as the PI3K, MEK, and JNK pathways, were shown to suppress p53 activation (44, 45). With a PH domain, ITK interacts with lipids generated by PI3K, as well as PI3K itself, which TXK may not be able to do because it lacks a PH domain (38, 46). In addition, although TXK can rescue PLCγ1 tyrosine phosphorylation and calcium responses in the absence of ITK (25), ITK may be required for optimal activation of Ras, MEK, and/or JNK pathways necessary for the suppression of p53 activity in iTNK cells.

Fas is expressed on all iTNK cells, and these cells can be regulated by Fas-mediated death (47). iTNK cells accumulate in older C57BL/6-lpr/lpr mice that lack Fas function, suggesting that this pathway plays a role in the homeostasis of peripheral numbers of these cells (47). Our findings indicate that Fas expression is elevated in the absence of ITK and that Itk−/− and Tg(CD2-Txk)
\textit{Itk}^{−/−} iNKT cells are more sensitive to Fas-induced apoptosis. It is likely that an increase in Fas expression can contribute to the reduction in iNKT cells in \textit{Itk}^{−/−} and \textit{Tg}(CD2-Txk)\textit{Itk}^{−/−} mice by enhancing apoptosis. However, the reverse may not apply (i.e., a reduction in Fas may not lead to an increase at this time point, because C57BL/6-lpr/lpr mice do not have higher numbers of iNKT cells at steady state at 6–8 wk, although it clearly occurs later) (47). It is possible that homeostasis of iNKT cells is such that these cells slowly build up in the absence of Fas, but increased Fas can accelerate their demise.

Fas expression was shown to be regulated by SP1 and inducible by p50/p65 NF-κB transcription factors, GA-binding protein, and AP-1 (48, 49). Bax and Bad expression are also elevated in the absence of Itk. Genotoxic damage was also shown to induce the expression of Fas, Bax, and Bad via a p53-dependent mechanism, and Bcl-6 is a negative regulator of p53, such that reduced Bcl-6 levels unleash p53 transcriptional activity (50–53). The absence of ITK compromises the activation of both NF-κB and AP-1 (54), suggesting that elevated Fas expression via these transcription factors is unlikely. Furthermore, in conventional T cells, TXK can rescue upstream pathways that lead to activation of these transcription factors (25, 28, 55–57). It is likely that the absence of ITK results in the activation of other pathways, such as the p53 pathway, that would lead to increases in Fas expression, along with other proapoptotic factors Bax and Bad, making iNKT cells more susceptible to Fas-mediated death and apoptosis. This may partially explain the reduced numbers of iNKT cells in the \textit{Itk}^{−/−} mouse, as well as \textit{Tg}(CD2-Txk)\textit{Itk}^{−/−} mice, because TXK is unable to normalize the increases in these proapoptotic factors. Our finding that p53 expression is elevated in \textit{Itk}^{−/−} splenic iNKT cells and is not restored by the increased expression of TXK further supports this view. Additional support for a role for p53 in this process includes our finding that p53^{−/−} and Bax^{−/−} mice have increased percentages and numbers of splenic iNKT cells, which would be expected if this pathway regulates the survival of these cells in vivo. In contrast, analysis of the expression of these genes in conventional naïve WT and \textit{Itk}^{−/−} CD4^{+} T cells reveals that, although there are changes in expression, the pattern is different from that seen in iNKT cells, suggesting that naïve CD4^{+} T cells behave differently from inNKT cells (Supplemental Fig. 4).

A number of genes have differential function in iNKT cells versus conventional T cells, as evidenced by the analysis of components of the TCR pathway or other transcription factors that differentially affect the development of these cells (1–16). Thus, pathways activating the p53 pathway may also be differentially regulated in iNKT cells compared with conventional T cells. These differences may be due to the different function of these cells: conventional T cells generally undergo additional differentiation into effector and memory cells, whereas iNKT cells are already effectors and do not undergo further differentiation. It would be of interest to determine whether the p53 pathway in terminally differentiated innate T cells (such as γδ T cells or other nonconventional T cells), or effector and memory T cells, behaves similarly to iNKT cells. Differences in the expression and function of p53 in thymic and splenic iNKT cells may be due to differential signals for maturation of these cells in the thymus versus the spleen, such that TXK is able to reduce p53 expression in the thymus but not in the spleen. In addition, the development process for thymic iNKT cells may make them less likely to use the p53 pathway, whereas splenic iNKT cells may be sensitive to this pathway, as we find.

Tec kinases are being considered as targets for the treatment of a number of diseases, and selective inhibitors targeting ITK and the related kinase BTK have been developed (58–62). Because TXK is related to ITK and is also expressed in T cells, it is of interest to determine whether this kinase has overlapping function. Our results suggest that TXK and ITK have overlapping functions in iNKT cell maturation and function; however, targeting ITK may uniquely affect the survival of iNKT cells, which TXK cannot rescue. These findings provide novel insight into the relationship between these two related Tec kinases in iNKT cell development and function.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure Legends

Supplemental Figure 1. No defect in iNKT cell development in the absence of TXK. A) Thymocytes (top) and splenocytes (bottom) from WT and Txk−/− mice were gated on iNKT cells (tetramer+/TcRβ+) and analyzed for the expression of CD44 and NK1.1. Percentages of CD44−NK1.1−, CD44+NK1.1− and CD44+NK1.1+ iNKT cells were determined. Expression of TXK transgene. B) mRNA from sorted thymic and splenic iNKT cells from WT or Tg(CD2-Txk)Itk−/− mice were analyzed for expression of endogenous Itk (WT) or TXK as described in the materials and methods section.

Supplemental Figure 2. TXK over expression does not restore thymic cellularity in the absence of ITK. A) Thymocytes from WT, Itk−/− or Tg(CD2-Txk)Itk−/− mice were analyzed for CD4 and CD8. B) Analysis of percentage and numbers of double positive (DP) CD4 or CD8 single positive thymocytes in the indicated mice. *p<0.05.

Supplemental Figure 3. No difference in expression of IL-7Rα (CD127) or IL-2Rγ (CD132) expression on iNKT cells from WT, Itk−/− and Tg(CD2-Txk)Itk−/− mice. Thymocytes and splenocytes from WT, Itk−/− and Tg(CD2-Txk)Itk−/− mice were gated on iNKT cells and analyzed for the expression of IL-7Rα (CD127) or IL-2Rγ (CD132). Total thymocytes or splenocytes were used as control (grey).

Supplemental Figure 4. Over expression of TXK regulates expression of pro- and anti-apoptotic genes in Itk−/− conventional naïve CD4+ T cells. Relative gene expression levels of Fas, Bax, Bad, Bcl-2, BclXL, Bcl-6 and p53 in WT and Itk−/− naïve CD4+
conventional T cells thymus and spleen were determined by real-time quantitative PCR (*p<0.05 vs. WT).
A) 

Thymus

Spleen

CD44

NK1.1

WT

Txk\(^{-/-}\)

B) 

Thymus

Spleen

Relative Gene Expression

WT (ltk)  WT (Txk)  Tlxk\(_{tg}\)ltk\(^{-/-}\) (Txk) 

Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3

Thymus

Spleen

- WT
- Itk\(^{-/-}\)
- T\(xk_{Tg}\)/Itk\(^{-/-}\)

IL-7R\(\alpha\)

IL-2R\(\gamma\)
Supplemental Figure 4