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A unique role for ITK in survival of α NKT cells associated with the p53 dependent pathway in mice[§]

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Abstract

Invariant natural killer T (α NKT) cells play important roles in the immune response. ITK and TXK/RLK are Tec family kinases that are expressed in α NKT cells, with the expression level of ITK about 7-fold higher than that of TXK. *Itk*^{-/-} mice have reduced α NKT cell frequency and numbers, with defects in development and cytokine secretion, which are exacerbated in the *Itk/Txk* DKO mice. By contrast, there is no α NKT cell defect in *Txk*^{-/-} mice. To determine whether ITK and TXK play distinct roles in α NKT cell development and function, we examined mice that over express TXK in T cells to similar levels as *Itk*. Over expression of TXK rescues the maturation and cytokine secretion of *Itk*^{-/-} α NKT cells, as well as altered expression of transcription factors T-bet, eomesodermin and PLZF. By contrast, the increased apoptosis observed in *Itk*^{-/-} splenic α NKT cells is not affected by TXK over expression, likely due to lack of effect on the elevated expression of p53 regulated pro-apoptotic pathways Fas, Bax and Bad in those cells. Supporting this idea, *p53*^{-/-} and *Bax*^{-/-} mice have increased splenic α NKT cells. Our results suggest that TXK plays an overlapping role with ITK in α NKT cell development and function, but that ITK also has a unique function in the survival of α NKT cells, likely via a p53 dependent pathway.

Keywords

Non-conventional T cells; Tec kinases; Fas; T cell development

Introduction

NKT cells are a distinct subset of T cells that recognize glycolipid antigen presented by the CD1d glycoprotein. NKT cells are composed of Type 1 NKT cells and Type 2 NKT cells. Type 1 NKT cells, also named as invariant NKT cells (α NKT), express an invariant V α chain (V α 14J α 18 in mice and V α 24J α 18 in human) together with certain types of V β chains and can be detected by α -GalCer loaded CD1d tetramer (1). Upon stimulation, α NKT cells are able to rapidly secrete large amount of multiple cytokines such as IL-4, IFN- γ and

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IL-17, and play important roles in the pathogenesis of allergic asthma, bacterial infection and cancer (1).

α NKT cells initiate their development from CD4⁺ CD8⁺ (DP) T cells and are selected by glycolipid antigen(s) in the context of CD1d molecules expressed on DP T cells. After selection, α NKT cells further develop through four stages to become mature. The developmental stages of α NKT cells are defined based on the expression of their surface markers CD24, CD44 and NK1.1. The most immature phase of α NKT cells (stage 0) is phenotypically characterized as CD24⁺CD44⁻NK1.1⁻, followed in a stepwise manner by CD24 down-regulation (CD24⁻CD44⁻NK1.1⁻: stage 1), upregulation of CD44 (CD44⁺NK1.1⁻: stage 2) and NK1.1 (CD44⁺NK1.1⁺: stage 3), to become the final mature α NKT cell. In addition, some of the α NKT cells at developmental stage 2 also migrate to the spleen, where they up regulate NK1.1 as a final maturation step (1). The signaling events that are required for the selection and maturation of α NKT cells have been studied extensively. Both TCR and SLAM-SAP-Fyn signaling pathways are required for the selection of α NKT cells (2–5). Transcription factors PLZF, Egr2, NF- κ B family members, signaling protein Dock2 and PDK1 and microRNA processor Dicer have all been shown to be important for the maturation of α NKT 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 α NKT 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 N-terminus to C-terminus are PH, TH, SH3, SH2 and kinase domain. ITK is normally found in the cytoplasm of resting T cells, and upon activation, PI-3 kinase is activated, generating PIP3 in the cell membrane. The generation of PIP3 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 α NKT cells, with the expression level of ITK about 7-fold higher than TXK (23). Previous studies have shown that in the absence of Itk, the number of α NKT 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/Txk* DKO mice, however, *Txk*^{-/-} mice have normal α NKT cell maturation and number (23). Given the similarities as well as differences in the structure of ITK and TXK, we wanted to determine whether TXK is truly functionally redundant to ITK in α NKT cells. However, due to the low expression levels of TXK in T cells, analysis of *Txk*^{-/-} mice is not as informative. We therefore utilized transgenic mice which over express TXK to similar levels of endogenous ITK in WT mice (*Tg(CD2-Txk)Itk*^{-/-}) and show here that TXK has overlapping roles as ITK in α NKT cell development, transcription factor expression and cytokine production, but that ITK has a distinct role in regulating survival of α NKT cells.

Material and Methods

Mice

Tg(CD2-Txk)Itk^{-/-} mice were a kind gift from Dr. Paul Love (via Dr. Pamela Schwartzberg (NHGRI/NIH)) and were generated as described previously (25). Wild type (WT), *Itk*^{-/-}, *Txk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice were on C57BL/6 background and kept in pathogen-free conditions. Bax deficient mice on a C57BL/6 background were from Jackson labs. Mice lacking p53, along with WT controls, were maintained on a 129S6 background (26). All the mice (except p53^{-/-} mice) used were 6–9 wks. of age, while p53^{-/-} mice were 4–5 weeks prior to tumor development observed in these mice. Cells from 4–5 week old *Bad*^{-/-} mice

on a C57BL/6 background were from Dr. Nika Daniel (Dana Farber Cancer/Institute Harvard Medical School), and compared to age matched WT mice. Experiments were approved by the IACUC 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) for 10 minutes, and then were stained with fluorescent antibodies for 30 min on ice. PE-PBS57/CD1d tetramer was kindly provided by the National Institute of Health tetramer core facility. anti-FITC-TCR β , V500-CD44, APC-Cy7-TCR, PECy5-CD44 and PECy7-CD4 were from BD Biosciences (San Jose, CA); anti-PECy7-NK1.1, FITC-CD44, APC-NK1.1, FITC-CD122, APC-alexa750-TCR β were from eBioscience (San Diego, CA); anti-PE/Texas Red CD8 and anti-PE/Texas Red CD4 were from Caltag (Burlingame, CA).

In vivo BrdU incorporation assay

WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice were injected intraperitoneally with 1 μ g BrdU in 100 μ l PBS, followed by drinking water containing 0.8 mg/ml BrdU for 6 days. BrdU-containing drinking water was changed every two days. Cells from thymus, spleen and liver were then isolated and stained with surface antibodies, followed by BrdU staining using APC-BrdU kit from BD biosciences.

Induction of Fas-mediated apoptosis *in vitro*

Splenocytes were collected from WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice and directly plated in 24 well plates at 2 X10⁶/ml in each well. Cells were treated with or without 5 μ g/ml anti-CD95 (Fas) antibody 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 1:50 diluted PI/RNase staining buffer (BD Biosciences) and anti-Cy5-Annexin V (BD Biosciences) in Annexin V binding buffer (BD Biosciences) for 15 minutes. Samples were analyzed within 1-hour post Annexin V staining.

Cell sorting and real-time PCR analysis

Thymic α NKT cells (TCR β ⁺PBS-57/CD1d tetramer⁺) were sorted from WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice, and naive conventional CD4⁺ T cells (TCR β ⁺CD4⁺CD44^{low}) were sorted from spleens of WT and *Itk*^{-/-} mice, using a Cytopeia Influx Cell Sorter (Cytopeia, Seattle, WA). RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA), followed by generation of cDNA with You Prime First-Strand beads kit (GE Healthcare, Piscataway, NJ). Quantitative real-time PCR was carried out using Taqman primer/probe sets for Bax, Bad, Bcl-2, Bcl-6, Bcl_XL, T-bet, eomesodermin, Egr2, Fas, p53 and PLZF with GAPDH as a housekeeping gene (Applied Biosystems, Foster City, CA). Data were analyzed using the comparative threshold cycle 2^{- $\Delta\Delta$ CT} method, normalized to the expression of GAPDH and the values were expressed as fold increase compared to WT α NKT cells, which was set as 1. To compare the expression level of *Itk* and *Txk*, qRT-PCR was performed on cDNA from sorted WT or *Txk*_{Tg}/*Itk*^{-/-} α NKT 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 to the *Itk* expression in WT α NKT cells sorted from thymus or spleen, which was set as 1.

α -Galactosyl Ceramide (GalCer) stimulation and intracellular cytokine staining

WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice were injected intraperitoneally with 100 μ l PBS containing either 2 μ g α -Galactosyl Ceramide (α -GalCer) or 1 μ l DMSO. After 2 hrs.,

splenocytes were collected and cultured in-vitro for 4 hrs. in the presence of 10 $\mu\text{g/ml}$ Brefeldin A. Cells were stained for surface markers, followed by intracellular staining of IL-4 and IFN- γ using BD fixation/permeabilization kit.

Data Analysis

Statistical analyses were determined using GraphPad Prism (GraphPad Software Inc. La Jolla, CA), using Student's *t*-test, with a probability value of $p < 0.05$ considered statistically significant.

Results

Over expression of TXK does not restore the number of αNKT cells in *Itk*^{-/-} mice

Previous studies have demonstrated that the absence of ITK results in defects in αNKT cell development, which are exacerbated in *Itk/Txk* DKO mice, suggesting that TXK also has role in αNKT cell development and function (23, 27). However, the number of αNKT cells is unchanged in the absence of TXK alone (23). In addition, we have determined that development of αNKT cells is also normal in the absence of TXK (Supplemental Figure 1A). Since TXK is expressed at about 7 fold lower levels than ITK in αNKT cells ((23) and Supplemental Figure 1B), this normal αNKT cell number and development in *Txk*^{-/-} mice may be due to the redundant functions of ITK and TXK in αNKT cells. Given the exacerbation of αNKT cell defects in the *Itk/Txk* DKO mice, we examined whether increased expression of TXK is able to rescue defects in αNKT cells in the absence of *Itk*. To do this, we used *Tg(CD2-Txk)Itk*^{-/-} mice, which over express TXK specifically in T cells to levels that are similar to endogenous ITK in T cells ((23, 25, 28) and Supplemental Figure 1B).

Flow cytometric analysis revealed that in the absence of *Itk*, the percentage and number of αNKT cells in the thymus and periphery were significantly decreased (Fig. 1A, B & C). Over expression of TXK restored the percentage of αNKT cells in the thymus of *Itk*^{-/-} mice to WT levels, although the numbers of these cells were not restored due to decreased total thymocyte number in the *Tg(CD2-Txk)Itk*^{-/-} mice (Supplemental Figure 2, note that the *Txk* transgene fails to restore thymic cellularity in *Itk*^{-/-} mice). In contrast, the frequency and number of αNKT cells in the spleen of *Itk*^{-/-} mice were not restored by over expressing TXK (Fig. 1A, B, C). The percentage and number of hepatic αNKT cells in *Tg(CD2-Txk)Itk*^{-/-} mice were increased compared to *Itk*^{-/-} mice, but remained lower than those in WT mice. This result demonstrate that over expression of TXK can restore the percentage of αNKT cells in the thymus of *Itk*^{-/-} mice, but not the numbers, or percentages in the periphery of these mice.

Over expression of TXK rescues the maturation of αNKT cells in the absence of *Itk*

To determine the role of TXK in the development of αNKT cells, we analyzed the expression of CD44 and NK1.1 on thymic αNKT cells of WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice. The majority of WT αNKT cells were CD44⁺NK1.1⁺, suggesting that most of them are mature and in developmental stage 3 (Fig. 1D). In contrast, a significantly higher percentage of *Itk*^{-/-} αNKT cells were CD44⁺NK1.1⁻, indicating that αNKT cells are blocked in development stage 2 in the absence of *Itk*. However, in the thymus of *Tg(CD2-Txk)Itk*^{-/-} mice, the percentage of CD44⁺NK1.1⁺ αNKT cells was significantly increased compared to those in *Itk*^{-/-} mice, and was similar to WT αNKT cells. This indicates that over expression of TXK can rescue the development of αNKT cells in the thymus in the absence of ITK (Fig. 1D). In addition, since CD44⁺NK1.1⁻ (developmental stage 2) αNKT cells also migrate to spleen to undergo final maturation, we also assessed the development of αNKT cells in the spleen. Similar to the case in the thymus, over expression of TXK also restored the

percentage of CD44⁺NK1.1⁺ λ NKT cells in *Itk*^{-/-} mice to similar levels as WT mice (Fig. 1E). By contrast and as noted before, the number of mature λ NKT 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 full maturation of λ NKT cells.

Over expression of TXK rescues α -Galactosyl Ceramide induced cytokine secretion by λ NKT cells in the absence of *Itk*

λ NKT cells are able to secrete large amounts of IL-4 and IFN- γ in response to α -GalCer stimulation, a response which is defective in the absence of ITK (23, 27). To determine whether TXK can function in the absence of ITK in regulating cytokine secretion in λ NKT cells, we injected α -GalCer intraperitoneally into WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice, followed by analysis of IL-4 and IFN- γ production by λ NKT cells as determined ex-vivo using intracellular cytokine staining. Compared to WT λ NKT cells, *Itk*^{-/-} λ NKT cells produced much less IL-4 and IFN- γ (Fig. 2). By contrast, over expression of TXK restored cytokine production in the *Itk*^{-/-} λ NKT cells to WT levels, indicating that TXK can regulate the signaling pathway downstream of ITK for cytokine production in λ NKT cells (Fig. 2).

Over expression of TXK normalizes expression of CD122 and transcription factors in *Itk*^{-/-} λ NKT cells

IL-15 signals and IL-15 receptor CD122 have been shown to be important for the final maturation of λ NKT cells, and the expression of CD122 has been suggested to correlate with the maturation of λ NKT cells in *Itk*^{-/-} mice. We have previously shown that a kinase defective mutant of ITK can partly restore the expression of CD122 on λ NKT cells in the absence of *Itk*, suggesting that the signaling pathways that lead to CD122 expression in λ NKT cells may be in part, kinase independent (27). To determine if TXK can also restore the expression of CD122 in λ NKT cells in the absence of *Itk*, we determined CD122 expression levels on λ NKT cells in the thymus and spleen of the three mouse strains. While the majority of WT thymic and splenic λ NKT cells express CD122, the percentage of λ NKT cells that express CD122 in *Itk*^{-/-} mice was dramatically decreased as previously reported (23, 27). In contrast, the percentage of λ NKT 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 λ NKT cells. PLZF and Egr2 are important for the development of NKT cells from stage 1 to stage 2 (6–8), while T-bet is critical for the final maturation of λ NKT cells (14, 15). In addition, the expression level of eomesodermin, another member of T-box family of transcription factors, is important for regulating CD122 expression in innate CD8 T cells, although it is not normally expressed in λ NKT cells (14, 29). In the absence of *Itk*, the expression of these transcription factors are deregulated (23, 27). In order to determine the effect of TXK on these transcription factors, their expression levels in thymic and splenic λ NKT cells of WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice were analyzed by QRT-PCR. In the absence of *Itk*, levels of mRNA for T-bet in thymic λ NKT cells was decreased, while the levels of mRNA for PLZF, Egr2 and eomesodermin were significantly increased, indicating that signals from ITK is required for proper expression of these transcription factors in thymic λ NKT cells (23, 27) and Fig. 3B). Over expression of TXK restored this altered expression of transcription factors in *Itk*^{-/-} thymic λ NKT cells to WT levels (Fig. 3B). In the splenic λ NKT cells TXK was able to restore the expression pattern of eomesodermin, but not PLZF (Fig. 3C). These data suggest that similar to ITK, TXK can regulate the pattern of expression of these transcription factors in λ NKT cells.

Over expression of TXK does not restore survival of *Itk*^{-/-} α NKT cells

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

Differences in α NKT cells numbers may be due to increased cell death. We therefore determined the level of apoptosis in these cells by staining with Annexin V. Although the percentage of Annexin V⁺ α NKT 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 over expression of TXK did not alter this increased percentage. This result suggests that apoptosis of α NKT cells is increased in the absence of *Itk*, and that over expression 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*^{-/-} α NKT cells and found that expression of FasL was similar in WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} splenic α NKT cells. In contrast, Fas expression was much higher in the splenic *Itk*^{-/-} α NKT cells compared to WT α NKT cells, and over expression of TXK did not normalize this pattern (Fig. 5B). We next determined whether the increased expression of Fas on the *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} splenic α NKT 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*^{-/-} α NKT cells exhibited increased apoptosis compared to WT α NKT cells in response to Fas triggering, suggesting that reduced numbers of α NKT 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 Figure 3). This inability of the *Txk* transgene to restore survival was not due to reduced expression of the transgene in the peripheral α NKT cells as the expression levels were similar in both thymic and splenic α NKT cells (Supplemental Figure 1B).

Txk overexpression does not normalize the increased expression of pro-apoptotic genes in *Itk*^{-/-} splenic α NKT cells

To determine whether the elevated apoptotic response observed in *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} splenic α NKT cells mice represented a pro-apoptotic state, we analyzed these cells for expression of pro-apoptotic genes Bad, Bax, as well as Fas, anti-apoptotic genes Bcl-2 and Bcl_{XL}, and p53 and its negative regulator Bcl-6. Comparison of splenic α NKT cells from WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice indicated that the latter mice exhibit higher levels of mRNA for Fas and its downstream pro-apoptotic 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 α NKT 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 α NKT cells observed in the thymus (Fig. 6B). By contrast, while mRNA for anti-apoptotic gene Bcl-2 was reduced in the

absence of ITK in splenic α NKT cells, increased expression of TXK is able to restore the expression of this gene to WT levels, while no difference was observed in Bcl_{XL} expression (Fig. 6A). The expression of Bcl-6 was reduced in the absence of ITK in splenic α NKT cells, but not in thymic α NKT cells, and was normalized in *Tg(CD2-Txk)Itk^{-/-}* thymic α NKT cells but not in *Tg(CD2-Txk)Itk^{-/-}* splenic α NKT cells (Fig. 6A, B). By contrast, p53 expression was induced in the absence of ITK in thymic and splenic α NKT cells, but while its expression was normalized in *Tg(CD2-Txk)Itk^{-/-}* thymic α NKT cells, this was not the case in splenic *Tg(CD2-Txk)Itk^{-/-}* α NKT cells (Fig. 6A, B). In addition, Bcl-6 expression was elevated in the absence of ITK in thymic α NKT cells, potentially counteracting the effects of elevated p53, but was not in *Itk^{-/-}* and *Tg(CD2-Txk)Itk^{-/-}* splenic α NKT cells. Since Bax, Bad, Fas are all 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 α NKT cells by regulating the expression of and function of the Fas pathway, perhaps via a p53 dependent pathway.

Regulation of percentage and number of splenic α NKT cells by p53 and p53 regulated apoptotic regulators Bax and Bad

The data shown in figure 6 suggest that the p53 pathway may regulate the survival of α NKT cells in the spleen. To determine if this is the case, we analyzed mice lacking p53 and the p53 regulated apoptotic regulators Bax and Bad, for levels of α NKT cells. This analysis revealed that while the absence of p53 did not affect the percentage or numbers of thymic α NKT 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 pro-apoptotic molecule Bax had increased percentage and number of splenic α NKT cells (Fig. 7B), although Bad deficient mice did not exhibit analogous changes in percentage or numbers of α NKT cells (Fig. 7C). This suggests that the p53 pathway can regulate the survival of splenic α NKT cells, and that the absence of ITK may affect the p53 pathway, thus regulating survival of α NKT 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 has been demonstrated to have a critical role in the development of naïve T cells (30–36). By contrast, a role for TXK has been less clear as *Txk* single knockouts do not exhibit any obvious phenotype, although this kinase does play a role in T cell development as *Itk/Txk* double knockouts exhibit a more severe phenotype (32–34). More recent studies examining the role of these kinases in α NKT cell development has revealed that similar to conventional T cells, ITK plays an important role in α NKT 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 α NKT cells is approximately 7–10 fold less than *Itk*. Here we have used *Tg(CD2-Txk)Itk^{-/-}* mice, which have expression of TXK similar to endogenous ITK in α NKT cells and show that at this expression level, TXK rescues defects in the maturation and cytokine production of *Itk^{-/-}* α NKT cells. This may be due to normalized expression of transcription factor T-bet, Egr2, PLZF and eomesodermin. By contrast, increased expression of TXK does not restore the reduced numbers nor affect the elevated apoptosis of *Itk^{-/-}* α NKT cells, which may be due to the failure to normalize elevated expression of the death receptor Fas and associated pro-apoptotic molecules. We also show that this may be due to deregulation of a p53 pathway leading to increased apoptosis, and show that lack of p53 or Bax results in increased splenic α NKT cells. These data suggest that ITK and TXK have overlapping roles in the development and function of α NKT cells, however, ITK has a distinct role in the suppression of apoptosis of α NKT 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, due in part to the interaction between the PH domain of ITK and PIP3 lipids at the cell membrane, a Phosphatidylinositol-3 kinase dependent event (38). By contrast, TXK lacks a PH domain found in the other Tec kinase family members, and has instead a N-terminal palmitoylation site (39). Among the Tec kinases, this would allow TXK to be uniquely independent of PI3 kinase 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 have previously shown that while the absence of ITK affects naïve T cell differentiation to Th2 cells, increased expression of TXK to levels equivalent to ITK can rescue this Th2 response (28). In addition, increased expression of TXK can also 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 PLC γ 1 and increases in intracellular calcium responses (28, 41). It is therefore surprising that when examined in α NKT cells, increased expression of TXK can rescue the function of ITK in α NKT cell development, maturation and function, but cannot restore survival of these cells. This indicates that ITK plays a unique role in regulating α NKT cell survival.

It is clear that TXK makes some contribution to the development of α NKT cells since mice lacking both ITK and TXK exhibit more severe reductions in α NKT cells numbers compared to *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 α NKT cells development largely at stage 2, similar to that seen in the absence of *Itk*, and so this block that is observed in the *Itk* deficient α NKT cells may be in part a consequence of T-bet deficiency and subsequent reduction in CD122 expression and reduced responses to IL-15 (14, 19, 42). However we have 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, however, full maturation is not restored (27), supporting an important role for T-bet in this process (14). Eomesodermin is not normally expressed in α NKT 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. While 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 α NKT cells. Furthermore, while PLZF is important for α NKT 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, as well as TXK, may be critical for further maturation of α NKT cells. Note that the effect of the ITK kinase independent pathway on α NKT cell maturation is dependent on TXK expression, suggesting that TXK collaborates with ITK to regulate α NKT cell maturation. Our current findings show that elevating TXK's expression can fully rescue maturation and function supports this idea. Given the ability of ITK to partially rescue α NKT cell development in a kinase independent fashion, we speculate that TXK may also be able to do this, since 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 α NKT cell development, maturation and function ((25, 28) and this work). The *Tg(CD2-Txk)Itk*^{-/-} mice have α NKT cells with normal maturation and function with regards 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 λ NKT cells may be related to immature state of these cells, and that by rescuing maturation, over expression of TXK also restores the ability of these cells to produce these cytokines. However, *Itk*^{-/-} λ NKT cells express similar levels of preformed mRNA for IL-4 and IFN γ as expected in more mature λ NKT cells. Moreover, *Itk*^{-/-} λ NKT cells are able to secrete both cytokines after PMA/Ionomycin stimulation, which bypasses ITK and the 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 λ NKT cells, leading to normal numbers of λ NKT cells. Our findings suggest that this may be related to p53 pathway activation and expression of Fas and pro-apoptotic pathways in λ NKT 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 PI3 kinase, MEK and JNK pathways have all been shown to suppress p53 activation (44, 45). With a PH domain, ITK interacts both with lipids generated by PI3 kinase, as well as PI3 kinase itself, which TXK may not be able to do since it lacks a PH domain (38, 46). In addition, although TXK can rescue PLC γ 1 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 suppression of p53 activity in λ NKT cells.

Fas is expressed on all λ NKT cells, and these cells can be regulated by Fas mediated death (47), and λ NKT cells accumulate in older C57BL/6-*Ipr/Ipr* 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)Itk*^{-/-} λ NKT cells are more sensitive to Fas induced apoptosis. It is likely that an increase in Fas expression can contribute to the reduction in λ NKT cells in *Itk*^{-/-} and *Tg(CD2-Txk)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, since C57BL/6-*Ipr/Ipr* mice do not have higher numbers of λ NKT cells at steady state at 6–8 weeks, although it clearly occurs later (47). Perhaps homeostasis of λ NKT cells is such that these cells slowly build up in the absence of Fas, but increased Fas can accelerate their demise.

Fas expression has been shown to be regulated by SP1 and inducible by p50/p65 NF- κ B transcription factors, GA-binding protein and AP-1 (48, 49). Bax and Bad expression are also elevated in the absence of *Itk*. Genotoxic damage has also been 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- κ B 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 pro-apoptotic factors Bax and Bad, making λ NKT cells more susceptible to Fas mediated death and apoptosis. This may partially explain the reduced numbers of λ NKT cells in the *Itk*^{-/-} mouse, as well as the *Tg(CD2-Txk)Itk*^{-/-} mice, since TXK is unable to normalize the increases in these pro-apoptotic factors. Our finding that p53 expression is elevated in *Itk*^{-/-} splenic λ NKT 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 percentage and numbers of splenic λ NKT cells, as would be expected if this pathway regulates the survival of these cells in vivo. By contrast, analysis of the expression of these genes in conventional naive WT and *Itk*^{-/-} CD4⁺ T cells reveals that while there are changes in expression, the

pattern is different from that seen in *i*NKT cells, suggesting that naïve CD4⁺ T cells behave differently from *i*NKT cells (Supplemental Figure 4). A number of genes have differential function in *i*NKT cells vs. conventional T cells, as evidenced by the analysis of components of the TcR pathway or other transcription factors that differentially affect development of these cells (1–16). Thus, pathways activating the p53 pathway may also be differentially regulated in *i*NKT cells compared to conventional T cells. These differences may be due to the different function of these cells, where conventional T cells generally undergo further differentiation to effector and memory cells, while *i*NKT 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 $\gamma\delta$ T cells or other nonconventional T cells), or effector and memory T cells, behaves similar to *i*NKT cells. Differences in the expression and function of p53 in thymic and splenic *i*NKT cells may be due to differential signals for maturation of these cells in the thymus vs. 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 *i*NKT cells may make them less likely to utilize the p53 pathway, while splenic *i*NKT cells may be sensitive to this pathway, as we find.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

α-GalCer	α -Galactosyl Ceramide
DKO	Double Knock Out
GATA3	GATA binding protein 3
<i>i</i>NKT cells	Invariant Natural Killer T cells
Itk	Interleukin-2 Inducible T cell kinase
P/I	PMA and Ionomycin
TcR	T cell Receptor

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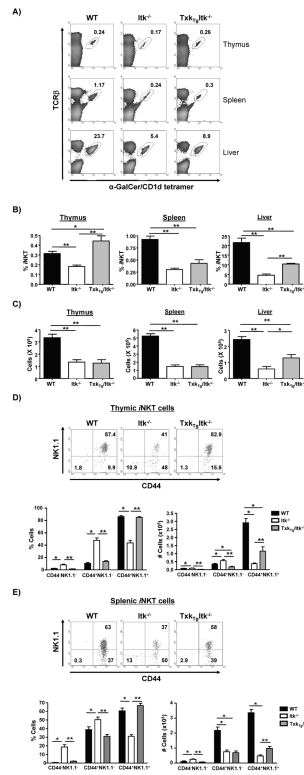


Figure 1. Over expression of TXK rescues frequency and maturation but not number of *i*NKT cells in the absence of *Itk*

A) Flow cytometric analysis of *i*NKT cells from thymus, spleen and liver of WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice. Determination of the percentage (**B**) and number (**C**) of *i*NKT cells from thymus, spleen and liver. For thymus and spleen samples, n>9, for liver samples, n=4. p* $<$ 0.05, p** $<$ 0.01. (**D**) Thymocytes and (**E**) splenocytes from WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice were gated on *i*NKT cells (tetramer⁺/TCRβ⁺) and analyzed for the expression of CD44 and NK1.1 (top graph). The percentage and number of CD44⁻NK1.1⁻, CD44⁺NK1.1⁻ and CD44⁺NK1.1⁺ *i*NKT cells were determined (bottom graph). n>8, *p<0.05, **p<0.01.

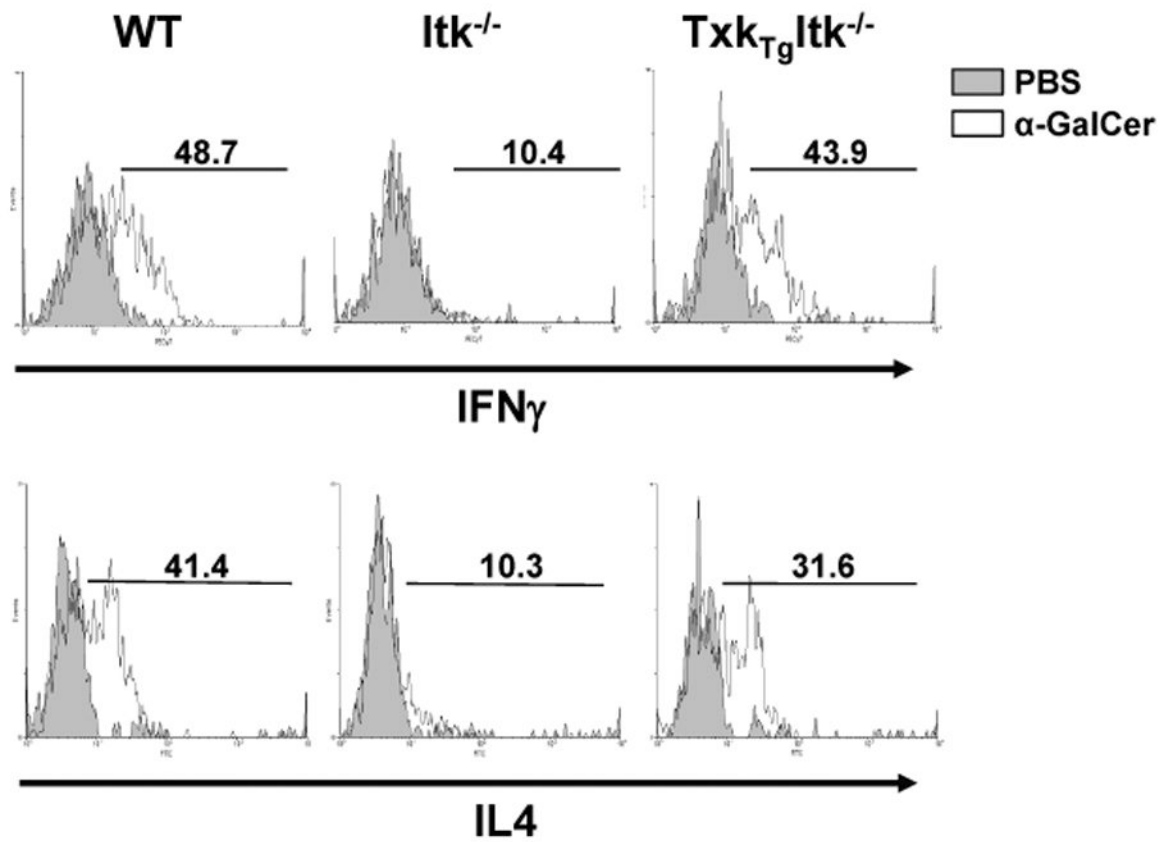


Figure 2. Over expression of TXK rescues α -GalCer induced cytokine secretion of *i*NKT cells in the absence of ITK

WT, *Itk*^{-/-} and *Tg*(*CD2-Txk*)*Itk*^{-/-} mice were injected intraperitoneally with either PBS or 2 μ g α -GalCer. After 2 hours, splenocytes were collected and cultured in-vitro for 4 h in the presence of 10 μ g/ml Brefeldin A. Cells were analyzed by flow cytometry, gated on *i*NKT cells (tetramer⁺/TcR β ⁺) and analyzed for IL-4 and IFN- γ expression. PBS control (shaded), α -GalCer (solid line). Data are representative of two independent experiments.

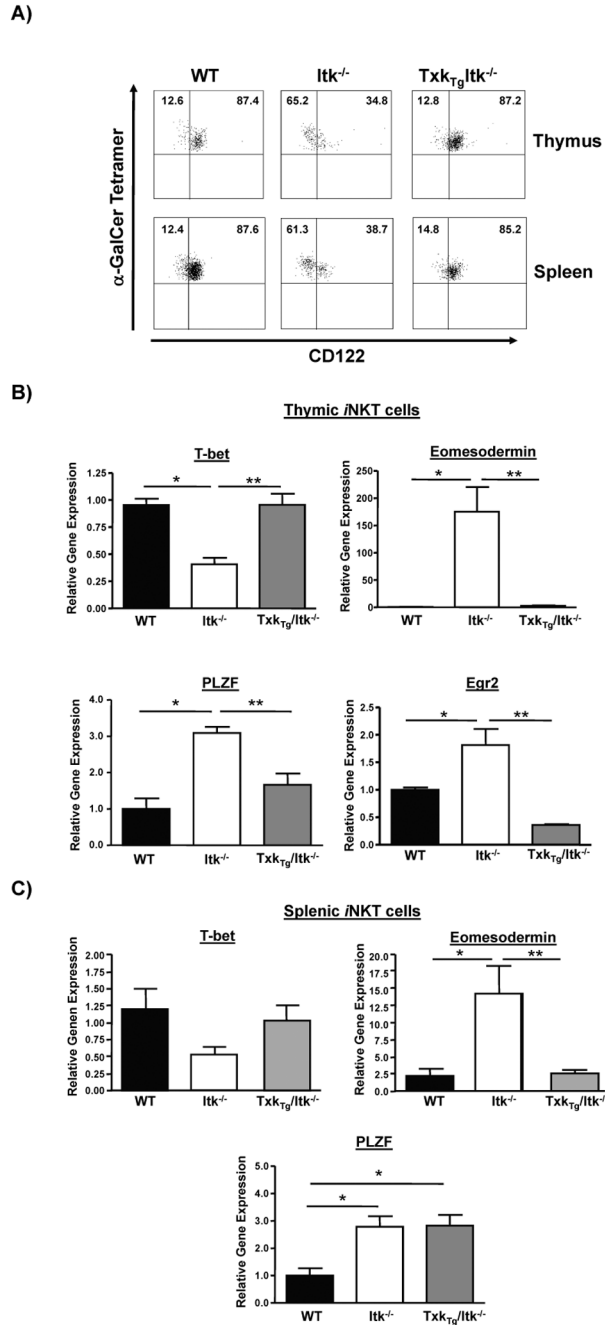


Figure 3. Over expression of TXK restores expression of CD122 and transcription factors in *Itk*^{-/-} iNKT cells

A) Thymocytes and splenocytes from WT, *Itk*^{-/-} and *Tg(*CD2-*Txk**)Itk*^{-/-} mice were gated on iNKT cells (tetramer⁺/TcR β ⁺) and stained for CD122. Data are representative of five independent experiments. **B)** Relative gene expression levels of T-bet, eomesodermin, PLZF and Egr2 in thymic iNKT cells from WT, *Itk*^{-/-} and *Tg(*CD2-*Txk**)Itk*^{-/-} mice were determined by real-time quantitative PCR (n>3, *p<0.05 vs. WT iNKT cells; **p<0.05 vs. *Itk*^{-/-} iNKT cells). **C)** Relative gene expression levels of T-bet, eomesodermin and PLZF in splenic iNKT cells from WT, *Itk*^{-/-} and *Tg(*CD2-*Txk**)Itk*^{-/-} mice were determined by real-time quantitative PCR (n>3, *p<0.05 vs. WT iNKT cells; **p<0.05 vs. *Itk*^{-/-} iNKT cells).

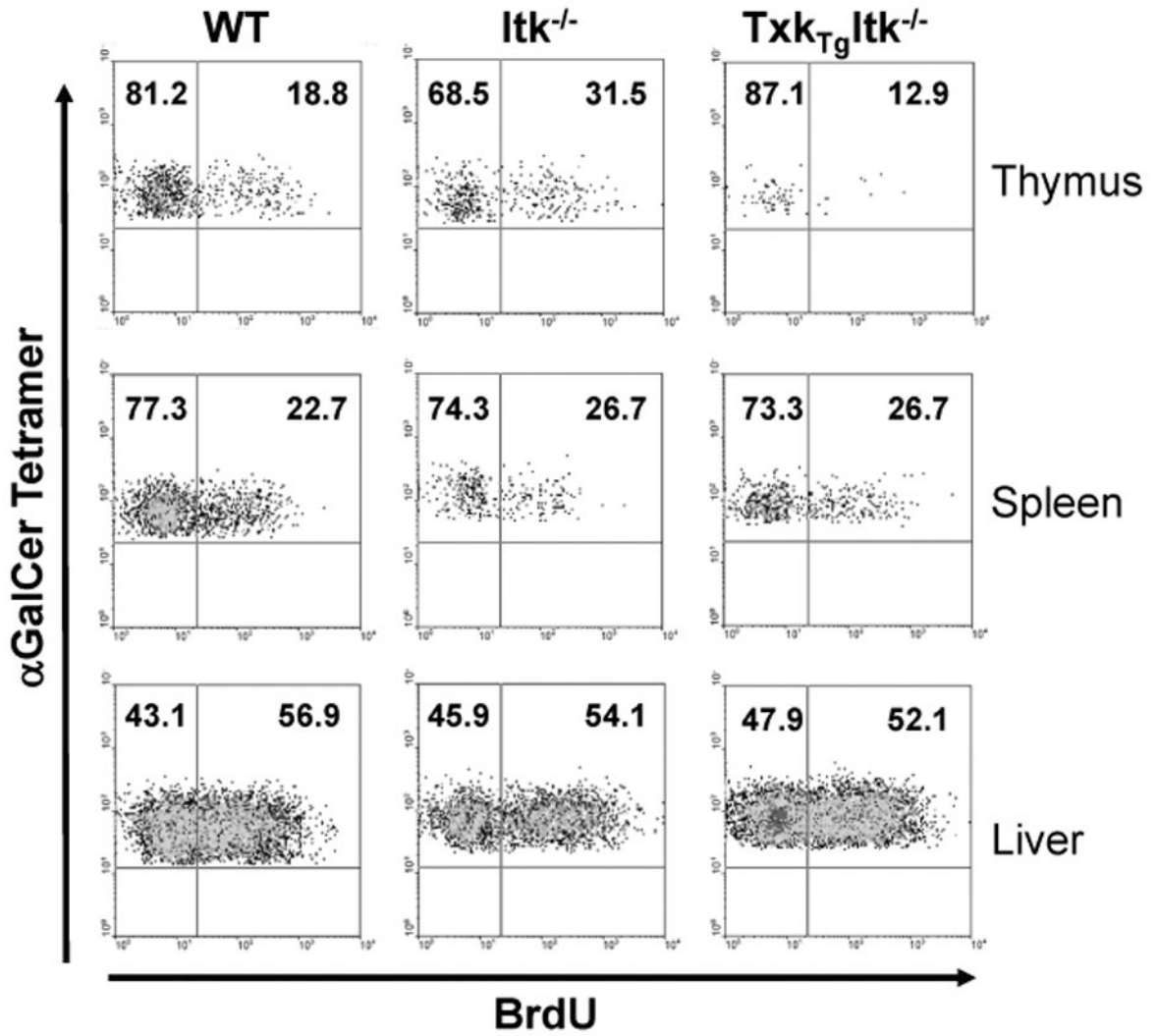


Figure 4. Over expression of TXK normalizes proliferation of thymic *i*NKT cells in the absence of ITK

WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} mice were injected intraperitoneally with 1 μ g BrdU and placed on drinking water containing 0.8 mg/ml BrdU for the next 6 days. Cells from the thymus, spleen and liver were gated on *i*NKT cells (tetramer⁺/TcR β ⁺) and analyzed for BrdU uptake. Data are representative of two independent experiments.

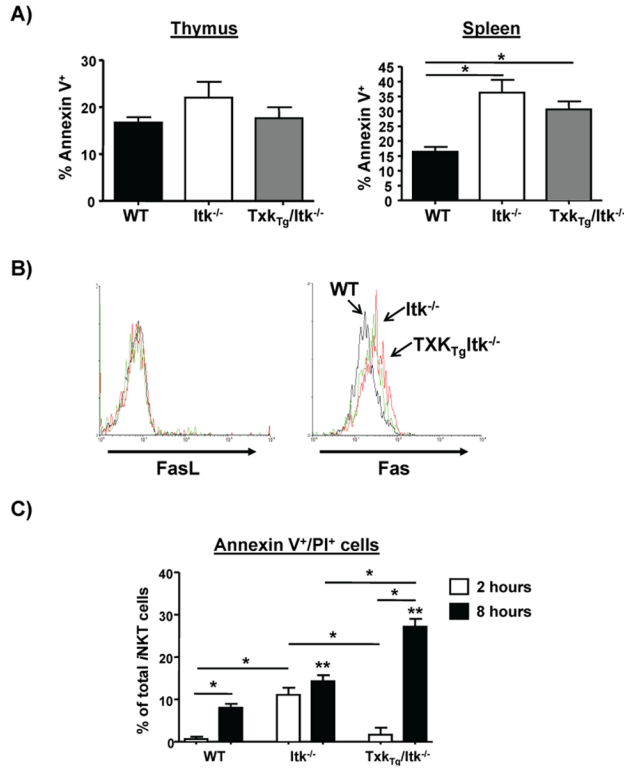


Figure 5. Over expression of TXK fails to restore cell survival of splenic *Itk*^{-/-} iNKT cells
A) Thymocytes and splenocytes from WT, *Itk*^{-/-} and *Tg(*CD2-*Txk**)Itk*^{-/-} mice were gated on iNKT cells (tetramer⁺/TcRβ⁺) and stained for the presence of Annexin V. The percentage of Annexin V⁺ iNKT cells was determined. n>4, p* $<$ 0.05. **B)** Splenocytes from WT (black line), *Itk*^{-/-} (red line) and *Tg(*CD2-*Txk**)Itk*^{-/-} mice (green line) were gated on iNKT cells (tetramer⁺/TcRβ⁺) and analyzed for the expression of Fas (left) and FasL (right). MFIs of Fas: WT=2719+/-146.9, *Itk*^{-/-}=3740+/-195.9, and *Tg(*CD2-*Txk**)Itk*^{-/-}=3386+/-224.5 (+/-SEM), p<0.05 vs. WT. **C)** Splenocytes from WT, *Itk*^{-/-} and *Tg(*CD2-*Txk**)Itk*^{-/-} mice were treated with Fas agonistic antibody or control for 2 and 8 hours, then iNKT cell apoptosis analyzed for the expression of Annexin V by gating on tetramer⁺/TcRβ⁺ cells.

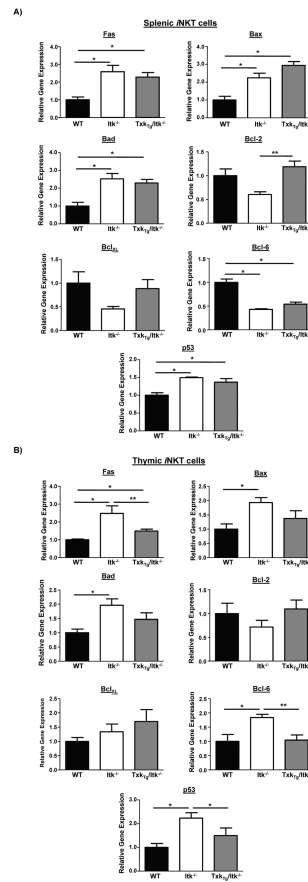


Figure 6. Over expression of TXK regulates expression of pro- and anti-apoptotic genes in *Itk*^{-/-} iNKT cells

Relative gene expression levels of Fas, Bax, Bad, Bcl-2, Bcl_{XL}, Bcl-6 and p53 in WT, *Itk*^{-/-} and *Tg(CD2-Txk)Itk*^{-/-} iNKT cells from **A**) spleen or **B**) thymus were determined by real-time quantitative PCR (*p<0.05 vs. WT iNKT cells; **p<0.05 vs. *Itk*^{-/-} iNKT cells).

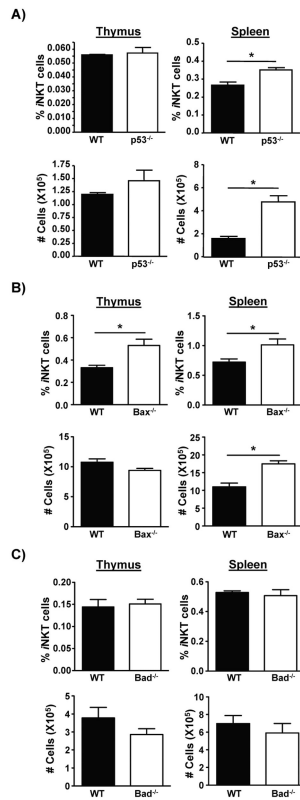


Figure 7. p53 and p53 regulated gene Bax regulate the percentage and numbers of splenic iNKT cells

A) Determination of the percentage and number of iNKT cells from thymus and spleen from WT or p53^{-/-} mice. p* < 0.05. **B)** Determination of the percentage and number of iNKT cells from thymus and spleen from WT or Bax^{-/-} mice. p* < 0.05. **C)** Determination of the percentage and number of iNKT cells from thymus and spleen from WT or Bad^{-/-} mice. p* < 0.05.