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A Key Role for Itk in Both IFNγ and IL-4 Production by NKT Cells¹

Byron B. Au-Yeung and Deborah J. Fowell²

NKT cells rapidly secrete cytokines upon TCR stimulation and thus may modulate the acquired immune response. Recent studies suggest that signaling for development and effector function in NKT cells may differ from conventional T cells. The tyrosine kinase Itk is activated downstream of the TCR, and its absence in CD4⁺ T cells results in impaired Th2, but not Th1 responses. In this study, we investigated NKT cell function in the absence of Itk as impaired type 2 responses in vivo could be manifest through IL-4 defects in a number of cell types. We show that Itk-deficient NKT cells up-regulate IL-4 mRNA in the thymus and express constitutive IL-4 and IFN-γ transcripts in peripheral organs. Thus, Itk is not required for the developmental activation of cytokine loci in NKT cells. Nevertheless, Itk-deficient NKT cells are severely impaired in IL-4 protein production. Strikingly, unlike conventional CD4⁺ T cells, Itk-deficient NKT cells also have profound defects in IFN-γ production. Furthermore, both IL-4 and IFN-γ production were markedly impaired following in vivo challenge with α-galactosyl ceramide. Function can be restored in Itk-deficient NKT cells by provision of calcium signals using ionomycin. These results suggest that NKT cells are highly dependent on Itk for IL-4- and IFN-γ-mediated effector function. Thus, the pattern of cytokine genes that are affected by Itk deficiency appears to be cell lineage-specific, likely reflecting differences in activation threshold between immune effectors. The severe defect in NKT cell function may underlie a number of the Th1 and Th2 immune defects in Itk-deficient mice. The Journal of Immunology, 2007, 179: 111–119.

C oordination between multiple cell types is required for successful immunity. Innate immune cell types, including NKT cells, are capable of rapid cytokine production and thus may help to shape an adaptive immune response. NKT cells are lymphocytes that express a TCR along with surface markers expressed on NK cells, such as NK2GD, Ly49G2, Ly49C, Ly49I, and NK1.1 (1). Most NKT cells express a rearranged TCR with surface markers presented in the context of the MHC class I-like molecule CD1d (2). Upon TCR stimulation, NKT cells are able to rapidly secrete both Th1 and Th2 cytokines, possibly due to the presence of constitutive cytokine mRNAs or hyperacetylation of histones associated with the IL-4 and IFN-γ promoters (3, 4). However, the TCR signal requirements for NKT cell activation and cytokine production are not well characterized.

Due to their rapid secretion of immunomodulatory cytokines, NKT cells are thought to influence protective immune responses against some viruses (5, 6) and tumors (7). Recently, it has been shown that NKT cells can recognize glycolipid Ags derived from Sphingomonas and Ehrlichia, two Gram-negative, LPS-negative bacteria (8, 9). Although NKT cells play a beneficial role in several models of Th1 infection, their ability to promote Th2 responses is less clear (10). The role of NKT cells in autoimmunity is also controversial. In type I diabetes, both diabetes-prone NOD mice and human patients have a reduced frequency of NKT cells. Increasing the number of NKT cells in the NOD mouse reduces disease progression and is associated with the induction of a Th2 response (11–13). In multiple sclerosis, a similar reduction in NKT cells has been observed in multiple sclerosis patients (14). However, in the mouse, activation of NKT cells by injection of α-GalCer has been reported to either ameliorate or exacerbate the severity of experimental autoimmune encephalomyelitis by shifting the balance of IFN-γ and IL-4 in the CNS (15, 16). Clearly, although the physiological role of NKT cells in immunity is not well understood, NKT cells are capable of influencing immune responses and represent an important immunoregulatory therapeutic target.

Itk is a member of the Tec family of kinases and is expressed by T cells, mast cells, NK cells, and NKT cells (17, 18). In T cells, Itk is activated downstream of TCR stimulation and modulates the magnitude of the calcium flux via phosphorylation of phospholipase Cγ (PLCγ) (19). Consistent with its role in modulating TCR signal strength, Itk plays an important role in thymic selection of conventional CD4⁺ and CD8⁻ T cells. In itk⁻⁻ mice, the ratio of CD4⁺CD8⁻ cells is reduced in the thymus and, in peripheral organs, there is approximately a 2-fold reduction in the total numbers of CD4⁺ cells (20). Similarly, in the absence of Itk, T cells bearing a low-avidity, MHC class II-restricted transgenic TCR specific for moth cytochrome c peptide (88–103) were less efficiently selected compared with T cells bearing a high-avidity TCR specific for the same peptide (21). Early studies showed that the frequency of CD8⁻ cells in the thymus and periphery were not as severely reduced in the absence of Itk (22). However, additional studies

¹ Abbreviations used in this paper: αGalCer, α-galactosyl ceramide; PLCγ, phospholipase Cγ; SAP, signaling lymphocyte activation molecule-associated protein; PKCθ, protein kinase Cθ; WT, wild type; eGFP, enhanced GFP.

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revealed that positive selection of HY-specific, MHC class I-restricted transgenic T cells was impaired in the absence of Itk, and that many of the CD8+ cells present in \(i^{k/-}\) mice are nonclassical, innate-like MHC class Ib-restricted T cells with a previously activated phenotype (23–25). Thus, it appears that Itk-dependent signals are required for efficient thymic selection of both conventional CD4+ and CD8+ T cell lineages.

Functionally, Itk-deficient CD4+ cells are able to differentiate into IFN-\(\gamma\)-secreting Th1 effectors, but Th2 responses are severely impaired (26–28). Upon further analysis of the \(i^{k/-}\) phenotype, it was shown that under Th2-polarizing conditions, the kinetics and magnitude of IL-4, IL-5, IL-13, and GATA-3 transcript up-regulation during Th2 priming are similar in WT and \(i^{k/-}\) cells, suggesting that Itk is not required for early Th2 differentiation steps and Th2 lineage commitment. However, upon a secondary TCR stimulation, Th2-primed \(i^{k/-}\) cells fail to enhance IL-4 transcript levels, correlating with severe defects in IL-4 protein production both in vitro and in vivo (29). Thus, Itk appears to be required for the implementation, rather than acquisition of Th2 effector function.

Several studies have identified signaling molecules that are required for efficient NKT cell development, such as the Src kinase Fyn, as well as signaling lymphocytic activation molecule-associated protein (SAP), DOCK2, protein kinase C\(\theta\) (PKC\(\theta\)), NF-\(\kappa\)B1, Vav-1, and IKK2 (30–36). Additionally, the decreased proportion of mature NKT cells, coupled with a decline in total NKT cell frequency with age, suggests that Itk also plays an important role in NKT cell development and/or homeostasis (37). The signals for execution of effector function in NKT cells are less well defined than the B1 pathway appears important. In addition, an acquired number of nonconventional innate-like T cells (23, 24).

**Materials and Methods**

**Mice and \(oGalCer\) treatment**

WT B10.D2 mice were purchased from The Jackson Laboratory. \(i^{k/-}\) B10.D2, WT BALB 4\(get\), \(i^{k/-}\) BALB 4\(get\), and TCR Ca+ mice were maintained in the pathogen-free animal care facility at the University of Rochester Medical Center (Rochester, NY). Mice were 6–8 wk of age, unless otherwise stated. For in vivo challenge, mice were injected i.v. with 2 \(\mu\)g \(oGalCer\) (Alexis Biochemicals) in a vehicle consisting of 0.9% NaCl and 0.5% Polysorbate-20 (Bio-Rad).

**Flow cytometry**

Cells were preincubated with blocking anti-FcR mAb 2.4G2 for 15 min at 4°C, followed by staining with PBS-57/Cd1d tetramers (National Institutes of Health tetramer core facility) at 4°C for 45 min. Abs also used in this study include anti-TCR\(\gamma\)/\(\delta\) clone H57-597, anti-CD3\(\varepsilon\) clone 145-2C11, anti-CD122 clone TM-81, anti-CD69 clone H1.2F3 (BD Pharmingen), and anti-NK1.1 clone PK136, anti-CD44 clone IM7 (eBioscience).

**Cell purification and stimulation**

CD4+ cells were enriched from spleen and lymph node by Ab/complex-mediated lysis (26) and sorted (FACSaria; BD Biosciences) for naive CD4+ cells, >99% CD62LhiCD44+Foxp3-. For NKT cell purification, spleen cells were depleted of MHC class II+ and CD24+ cells by Ab complement lysis, followed by sorting for CD1d/PBS-57 tetramer 'CD3e' cells. Irradiated (2500 rad) Itk-sufficient APCs were used in all experiments. Splenocytes were either negatively selected from WT mice for TCR-negative CD1d-tetramer-negative cells by FACS (WT APCs) or isolated from TCR Ca+ mice (TCR Ca+ APCs). We found no difference in the stimulatory capacity of WT and TCR Ca+ splenocytes for WT or \(i^{k/-}\) NKT cell cytokine production. Unless otherwise indicated, NKT cells were stimulated with plate-bound anti-TCR\(\gamma\) coated at a concentration of 0.5 \(\mu\)g/ml or with \(oGalCer\) at a concentration of 100 ng/ml plus APCs.

**Liver lymphocyte isolation**

Livers were first perfused with 5 ml of PBS through the portal vein. Each homogenized liver was then incubated at 37°C with 5 mg of collagenase (Sigma-Aldrich) and 1 mg of DNase I (Sigma-Aldrich) for 45 min. Cells suspensions were then centrifuged in a 21.5% Optiprep solution (Accurate) density gradient. Liver lymphocytes were removed from the interface and washed for FACS analysis.

**Real-time PCR**

Total RNA was extracted from sorted naïve CD4+ CD62LhiCD44+ cells and NKT cells (TCR\(\beta\)/PBS-57/CD1d tetramer +) using TRizol reagent (Invitrogen Life Technologies) and reverse-transcribed (reverse transcriptase for PCR kit; BD Clontech). RNA was also harvested from NKT cells after stimulation with \(oGalCer\) and TCR Ca+ APCs for 24 h for analysis of IL-4, IFN-\(\gamma\), T-bet, and GATA-3 mRNA induction. Real-time PCR was performed using Assays-on-Demand TaqMan primer/probe sets with an Applied Biosystems Prism 7900 sequence detection system (Applied Biosystems). Graphs display transcript levels relative to the endogenous control CD3\(\varepsilon\) and were calibrated relative to unstimulated naïve CD4+ cells.

**Cytokine measurements**

For analysis of IL-4 and IFN-\(\gamma\) production by ELISA, sorted NKT cells were stimulated either with plate-bound anti-TCR\(\beta\) mAb or with \(oGalCer\) and WT APCs for 48 h. For analysis of IL-4 and IFN-\(\gamma\) production by ELISPOT, sorted NKT cells were stimulated with either plate-bound anti-TCR\(\beta\) mAb or with \(oGalCer\) and WT APCs for 24 h and cytokine-producing cells were detected as described (26). APC cultures with \(oGalCer\) in the absence of NKT cells yielded <5 IFN-\(\gamma\) spots and <4 IL-4 spots. The cytokine secretion assay (Milenyi Biotec) was performed as previously described (29). Briefly, splenocytes from vehicle or \(oGalCer\)-treated mice were incubated directly ex vivo in the presence or absence of ionomycin (1 \(\mu\)g/ml; Calbiochem) for 4 h. After this incubation period, cells were washed and labeled with fluorescently labeled antibodies and incubated for 45 min at 37°C. Cells were washed and IL-4 and IFN-\(\gamma\) were detected with PE-conjugated Abs. For intracellular cytokine staining, splenocytes from vehicle- or \(oGalCer\)-treated mice were harvested and incubated for 4 h with brefeldin A (BD Pharmingen) in the presence or absence of 1 \(\mu\)g/ml ionomycin at 37°C. Cells were washed with CD1d/PBS-57 tetramer and anti-CD3\(\varepsilon\), followed by permeabilization with Cytotox/Cytoperm and stained with anti-IL-4 clone BVD4-1D11 or anti-IFN-\(\gamma\) clone XM11.2 (BD Pharmingen).

**Results**

**Absence of Itk results in partial block of NKT cell maturation**

Given the previously described age-related defect in NKT cell numbers in the absence of Itk (37), we first measured the frequency and number of NKT cells in WT and \(i^{k/-}\) mice. To identify NKT cells, we labeled cells from the thymus, spleen and liver with CD1d tetramers loaded with \(oGalCer\) analog PBS-57. The frequency of TCR\(\beta\) tetramer+ NKT cells in \(i^{k/-}\) mice was reduced <2-fold in the thymus, whereas the reduction was more severe in the spleen and liver (5- to 6-fold; Fig. 1A). Total numbers of NKT cells were significantly decreased (Mann-Whitney U test; \(p = 0.02\)) in all three organs (Fig. 1B), suggesting that NKT cells are highly dependent on Itk expression for development and/or survival. In contrast, the number of tetramer TCR\(\beta\) cells (Fig. 1C) and NK cells (NK1.1+ TCR\(\beta\); Fig. 1D) were not significantly reduced in \(i^{k/-}\) mice. However, recent reports suggest that the “conventional” TCR\(\beta\) T cell compartment contains a substantial number of nonconventional innate-like T cells (23, 24).
Previous studies have reported that the frequency of mature NK1.1+CD69+ NKT cells in itk−/− mice is decreased (37), suggesting that Itk is important for NKT cell maturation. However, it is not clear at which stage itk−/− NKT cells are blocked. To identify where the block in itk−/− NKT cell development occurs, we analyzed surface marker and cytokine transcript expression by NK1.1+ CD44low (stage 1) to NK1.1+ CD44high (stage 2) and finally to NK1.1+CD44high (stage 3) (1). Other markers, such as CD69 and CD122, are also up-regulated during the transition from stage 2 to stage 3. Analysis of thymocytes from Itk-deficient mice revealed that itk−/− NKT cells up-regulate CD44, but are impaired in up-regulation of CD122 and also, in agreement with previous studies, CD69 and NK1.1 (37) (Fig. 2A). These results are consistent with earlier studies on the developmental phenotype of Itk-deficient NKT cells (37), and together the studies suggest that in the absence of Itk, NKT cells up-regulate CD44, but are impaired in progression from stage 2 to stage 3, marked by defective up-regulation of CD69, CD122, and NK1.1.

In contrast to conventional CD4+ T cells, NKT cells have been shown to express cytokine mRNA during development. Bicistronic IL-4/enhanced GFP (eGFP) reporter (4get) mice have been used previously to track the induction of IL-4 transcription in conventional CD4+ T cells (39) and also NKT cells (4). During NKT cell maturation, IL-4 transcripts are up-regulated early, peaking in CD44low cells and decreasing upon transition to CD44high cells (4, 40). It is not clear whether the signals for NKT cell development are the same as those signals that induce cytokine transcripts. Given Itk’s role in conventional Th2 cells, we asked whether the absence of Itk affects expression of IL-4 mRNA in NKT cells. Analysis of thymocytes from 3-wk-old 4get mice showed that itk−/− NKT cells express eGFP and CD44 similarly to WT cells.
Itk is required for NKT cell development and effector function

Previously, we described the ability of conventional itk\(^{-/-}\) CD4\(^{+}\) T cells to initiate IL-4 transcription during Th2 differentiation (29). Under Th2-polarizing conditions, itk\(^{-/-}\) CD4\(^{+}\) cells up-regulated IL-4 mRNA and other Th2 cytokines with the same kinetics and to the same magnitude as WT cells. However, upon a secondary stimulation, Th2-primed itk\(^{-/-}\) cells failed to enhance IL-4 transcription, which correlated with a severe defect in IL-4 protein production. Given that Itk is required for conventional CD4\(^{+}\) T cells to exert, but not gain, Th2 effector function, we asked whether itk\(^{-/-}\) NKT cells were similarly functionally impaired. With the reduced frequency of IL-4/eGFP-expressing NKT cells in the periphery of Itk-deficient mice, IL-4-committed eGFP\(^{+}\) NKT cells were sorted from WT and itk\(^{-/-}\) 4get splenocytes for direct comparison of cytokine production and stimulated with either αGalCer and APCs or with plate-bound anti-TCRβ (Fig. 4A). Both WT and itk\(^{-/-}\) NKT cells were stimulated with αGalCer-loaded WT APCs to directly test functional defects intrinsic to Itk deficiency in the NKT cells themselves. Despite similar levels of eGFP expressed by both WT and itk\(^{-/-}\) eGFP\(^{+}\) populations (eGFP mean fluorescence intensity of CD4\(^{+}\) T cells: WT, 93.8 ± 20.8; itk\(^{-/-}\), 95.5 ± 23.3), IL-4 protein was detected only from WT NKT cells; Itk-deficient NKT cells were impaired in IL-4 production following both Ab and αGalCer/APC stimulation (Fig. 4A). Thus, similar to conventional T cells, itk\(^{-/-}\) NKT cells fail to produce IL-4 upon TCR stimulation, despite constitutive expression of IL-4 mRNA.

Although Itk is required for Th2 responses, Th1 responses develop in the absence of Itk both in vitro and in vivo (26–28). Indeed, Th1-primed itk\(^{-/-}\) CD4\(^{+}\) cells produced similar levels of IFN-γ as WT Th1 cells (Fig. 4B). Unexpectedly, in addition to a defect in IL-4, Itk-deficient NKT cells also failed to produce IFN-γ upon TCR stimulation (Fig. 4B). These results suggest that NKT cells, unlike conventional CD4\(^{+}\) Th cells, are highly dependent on Itk for production of both IL-4 and IFN-γ. In the absence of Itk, the magnitude of TCR signaling is attenuated and could contribute to functional defects (19, 27, 28). However, enhancing signal strength by increasing the stimulating dose also failed to bypass the Itk-dependent deficiency in IL-4 and IFN-γ production (Fig. 4C), suggesting a qualitative rather than quantitative role for Itk in TCR signals for cytokine production.

Our previous studies had highlighted a key requirement for Itk signals in transcriptional enhancement of IL-4 on restimulation of constitutive cytokine mRNAs

NKT cells in the periphery of WT mice constitutively express elevated levels of IL-4, IFN-γ, IL-5, and IL-13 mRNA (3, 4), a characteristic thought to allow for the rapid secretion of these cytokines. Our single-cell analysis of 4get lymphocytes showed that up-regulation of IL-4 occurs independently of Itk expression in thymic NKT cells (Fig. 2B). Despite thymic developmental defects, IL-4/eGFP\(^{+}\) NKT cells were present in peripheral organs of itk\(^{-/-}\) 4get mice (Fig. 3, A and B). However, the number of eGFP\(^{+}\) NKT cells in the spleen was reduced and correlated with a decrease in the ratio of GFP\(^{+}\) to GFP\(^{-}\) NKT cells between WT and itk\(^{-/-}\) mice (Fig. 3, A and B). At the population level, constitutive mRNA expression for a variety of cytokines, including both IL-4 and IFN-γ, was elevated in tetramer\(^{+}\) NKT cells of both WT and itk\(^{-/-}\) splenocytes compared with conventional CD4\(^{+}\) T cells (Fig. 3, B and C). Thus, constitutive expression of many effector cytokine transcripts in NKT cells is Itk independent. Interestingly, constitutive T-bet expression was also unperturbed in the absence of Itk (Fig. 3D). Indeed, levels of T-bet were consistently slightly elevated in splenic TCRβ\(^{+}\) tetramer\(^{+}\) Itk-deficient NKT cells compared with WT NKT cells, despite differences in the frequency of mature NKT cells between the two groups (Fig. 2) (37). The failure to see a defect in T-bet expression in the absence of Itk is somewhat surprising given that T-bet has been reported to be required for terminal maturation of NK and NKT cells (41, 42). The maturation defects seen in the absence of Itk cannot therefore be explained by a defect in the expression of T-bet and may suggest that the stable induction of T-bet expression precedes the development of stage 3 “mature” NK1.1\(^{+}\) NKT cells (1).

Itk is required for NKT cell production of both IL-4 and IFN-γ in vitro and in vivo

FIGURE 3. Constitutive expression of cytokine mRNAs by WT and itk\(^{-/-}\) NKT cells. A. Representative histograms of eGFP expression by WT and itk\(^{-/-}\) 4get NKT cells. Histograms are gated on TCRβ\(^{+}\) tetramer\(^{+}\) NKT cells from the spleens or livers of 6- to 8-wk-old WT and itk\(^{-/-}\) 4get mice. Numbers represent the percentage of cells in the indicated histogram gate. Data are representative of at least two independent experiments, three mice per group. B. Number of TCRβ\(^{+}\) tetramer\(^{+}\) cells expressing eGFP. C. Real-time PCR analysis of IL-4, IL-5, IL-13, IFN-γ, and D. T-bet transcript levels. Transcript levels in sorted splenic NKT cells (TCRβ\(^{+}\) tetramer\(^{+}\)) from WT (■) and itk\(^{-/-}\) (□) mice. Values are calculated relative to sorted naive CD4\(^{+}\) T cells (CD4\(^{low}\)CD62L\(^{hi}\)TCRβ\(^{−}\)) and calibrated to the endogenous control, CD3δ. Data are representative of three independent experiments.

(FIG. 2B). However, consistent with a defect in maturation, the loss of GFP expression associated with thymic maturation to CD122\(^{+}\) was attenuated in the absence of Itk. Our results with IL-4 reporter expression, along with surface marker analysis, suggest that NKT cell precursors can progress through the early stages of NKT cell maturation, as itk\(^{-/-}\) NKT cells express IL-4 and up-regulate CD44, but inefficiently transition to the CD44\(^{hi}\)CD122\(^{−}\) stage (stage 3) of maturation. The reduced frequency of immature eGFP\(^{+}\)CD44\(^{lo}\) cells and elevated frequency of eGFP\(^{+}\)CD44\(^{hi}\) thymocytes (Fig. 2B) are consistent with this notion.

Itk is not required for the induction and maintenance of constitutive cytokine mRNAs

NKT cells in the periphery of WT mice constitutively express elevated levels of IL-4, IFN-γ, IL-5, and IL-13 mRNA (3, 4), a characteristic thought to allow for the rapid secretion of these cytokines. Our single-cell analysis of 4get lymphocytes showed that up-regulation of IL-4 occurs independently of Itk expression in thymic NKT cells (Fig. 2B). Despite thymic developmental defects, IL-4/eGFP\(^{+}\) NKT cells were present in peripheral organs of itk\(^{-/-}\) 4get mice (Fig. 3, A and B). However, the number of
Th2-primed CD4+ effector cells that in turn was critical for optimal cytokine production (29). Resting NKT cells express cytokine transcripts at a similar level to resting differentiated Th1 and Th2 cells (4), but it is unclear much these existing transcripts contribute to cytokine protein production on Ag stimulation. On αGalCer stimulation, NKT cells also exhibit marked cytokine transcriptional up-regulation (100- to 1000-fold; Fig. 4D) similar to that seen for Th1 and Th2 effectors (4). Such transcriptional enhancement was attenuated in the absence of Ift (Fig. 4D), with up-regulation of IFN-γ mRNA being most severely affected by the lack of Ift. The pronounced defect in IFN-γ mRNA enhancement on Ag stimulation correlated with a failure to sustain T-bet expression (Fig. 4E). Interestingly, we found that down-regulation of T-bet occurred coordinate with an increase in GATA-3 mRNA (Fig. 4E). Peripheral NKT cells are thought to express both IL-4 and IFN-γ at the single-cell level (4) but the regulation of cytokine production (relative levels of IL-4 vs IFN-γ) by individual cells is not well understood. It is possible that within individual cells GATA-3 plays a negative regulatory role in IFN-γ production, altering the relative levels of IL-4 and IFN-γ production by NKT cells. The altered T-bet/GATA-3 balance in the absence of IL-4 production may account for the more severe defect in IFN-γ protein production compared to IL-4 production (Fig. 4C). Defects in IL-4 production despite GATA-3 expression in ift−/− NKT cells may reflect Ift's role in regulating the calcium flux and the relative importance of NFAT2, over GATA-3, in IL-4 production by NKT cells (43). The WT and ift−/− NKT cell populations differed in their frequency of mature NK1.1+ NKT cells (Fig. 2A), with ift−/− NKT cells containing one-half as many mature NKT cells, raising the possibility that developmental differences accounted for the changes in cytokine responses observed here. However, on analysis of sorted NK1.1+ TCRβ+ tetramer+ NKT cells we observed on in vivo activation, WT and ift−/− mice were injected with 2 μg of αGalCer or vehicle alone. After 2 h, splenocytes were harvested and incubated with brefeldin A for 4 h before intracellular cytokine staining (Fig. 5). Consistent with our in vitro data, NKT cells from ift−/− mice were impaired in production of both IL-4 and IFN-γ protein upon stimulation with αGalCer in vivo. Similar defects were observed following in vivo activation with i.v. anti-CD3 mAb (data not shown and Ref. 44). Thus, despite evidence of developmental activation of both the IL-4 and IFN-γ loci (represented by the elevated levels of cytokine mRNAs before Ag stimulation, “constitutive” cytokine mRNAs, Fig. 3), ift−/− NKT cells...
are severely functionally impaired in their ability to produce effector cytokines in vivo.

Distinct roles for Itk in NKT cell development and effector function?

NKT cells undergo a series of functional changes during thymic development with cells transitioning from a NK1.1\(^{-}\)IL-4\(^{+}\) IFN-\(\gamma\)\(^{-}\) phenotype to a NK1.1\(^{+}\)IL-4\(^{-}\)IFN-\(\gamma\)\(^{+}\) (37, 40, 45). Once in the periphery, further functional changes result in a peripheral NKT cell pool where the majority of mature NK1.1\(^{-}\) NKT cells express both IL-4 and IFN-\(\gamma\) (3, 4). As suggested previously, given the clear role for Itk in NKT cell development, it was possible that the defects in peripheral NKT cell function were a consequence of defects in development. Therefore, we compared cytokine production from phenotypically immature (NK1.1\(^{-}\)) and mature (NK1.1\(^{+}\)) tetramer\(^{+}\) NKT cells following anti-TCR\(\beta\) stimulation in vitro and \(\alpha\)-GalCer stimulation in vivo (Fig. 6). In vitro, tetramer\(^{+}\) splenocytes were sorted into NK1.1\(^{-}\) and NK1.1\(^{+}\) fractions and stimulated with WT APCs and anti-TCR\(\beta\) (Fig. 6A). In vivo, mice were challenged with \(\alpha\)-GalCer as in Fig. 5, and cytokine production was assessed directly ex vivo using the cytokine capture assay in combination with cell surface phenotyping for NK1.1 expression (Fig. 6B). Cytokine production, both IL-4 and IFN-\(\gamma\), remained severely compromised when mature \(\text{itk}^{-/-}\) splenic NK1.1\(^{+}\) NKT cells were compared with WT NK1.1\(^{+}\) NKT cells, suggesting that Itk plays a role in the execution of NKT cell function independent from its role in NKT cell development.

IL-4 production can be restored in Itk-deficient Th2 effector cells by the provision of TCR signals with ionomycin (29). This result suggested that conventional CD4\(^{+}\) T cells differentiated into Th2 cells with similar cytokine potential as WT cells but lack the

FIGURE 5. Impaired IL-4 and IFN-\(\gamma\) production by \(\text{itk}^{-/-}\) NKT cells following in vivo challenge with \(\alpha\)-GalCer. WT and \(\text{itk}^{-/-}\) mice were injected i.v. with 2 \(\mu\)g of \(\alpha\)-GalCer or vehicle alone. After 2 h, splenocytes were harvested and incubated for 4 h at 37\(^{\circ}\)C in the presence of brefeldin A. Cells were then surface stained, fixed, and permeabilized for intracellular cytokine staining. Filled histograms represent CD3\(^{+}\) \(\alpha\)-tetramer\(^{+}\) cells from vehicle-treated mice and open histograms represent CD3\(^{+}\) \(\alpha\)-tetramer\(^{+}\) cells from \(\alpha\)-GalCer-treated mice. Numbers in the table are the percentages of cytokine-expressing NKT cells. Data representative of three independent experiments.

FIGURE 6. Provision of ionomycin restores IL-4 and IFN-\(\gamma\) production by mature NK1.1\(^{+}\) \(\text{itk}^{-/-}\) NKT cells. A, Splenic NKT (CD3\(^{+}\) \(\alpha\)-tetramer\(^{+}\)) cells were sorted into NK1.1\(^{-}\) and NK1.1\(^{+}\) fractions and stimulated for 18 h with plate-bound anti-TCR\(\beta\) mAb in the presence or absence of 1 \(\mu\)g/ml ionomycin for IL-4 and IFN-\(\gamma\) ELISPOT analysis. Graphs represent the number of WT (■) and \(\text{itk}^{-/-}\) (□) cytokine-secreting cells per 5 \(\times\) 10\(^{5}\) NKT cells. Representative of three separate experiments (*, p \leq 0.05 Student’s t test; n.s. (not significant), p \geq 0.05) B, WT and \(\text{itk}^{-/-}\) mice were challenged with \(\alpha\)-GalCer or vehicle alone in vivo, as in Fig. 5. Splenocytes were incubated for 4 h in the presence or absence of 1 \(\mu\)g/ml ionomycin before the cytokine secretion assay. For IFN-\(\gamma\) (top) and IL-4 (bottom) secretion analysis, splenocytes were labeled with the bispecific “catch” reagent, followed by incubation at 37\(^{\circ}\)C for 45 min. Cells were washed and stained with PE-conjugated IL-4 and IFN-\(\gamma\) Abs and anti-NK1.1 mAb. Dot plots are gated on CD3\(^{+}\) \(\alpha\)-tetramer\(^{+}\) NKT cells. Numbers in quadrants are the percentage of total tetramer\(^{+}\) NKT cells. Data representative of three separate experiments.
appropriate activation signals to exert effector function. To define the cytokine-producing potential of Itk-deficient NKT cells, we asked whether ionomycin could similarly rescue cytokine production. Indeed, supplementing αGalCer signals with ionomycin rescued both IL-4 and IFN-γ production by NK1.1−/−itk−/− NKT cells activated in vitro or in vivo (Fig. 6, A and B). Similar restoration of IFN-γ production was noted on PMA/ionomycin stimulation of itk−/−NK1.1+ NKT cells (37). Thus, phenotypically mature itk−/− NKT cells appear functionally mature when given the appropriate activation signals. These results imply that Itk and the calcium flux are dispensable for the gain of cytokine function but are critical for the production of effector cytokines by NKT cells.

In contrast, analysis of the NK1.1+ immature NKT cells revealed an additional effect on the cytokine potential of splenic NKT cells that appears to arise from the Itk-dependent developmental block. The splenic itk−/−NK1.1+ NKT cells produced IL-4 but not IFN-γ on anti-TCRβ stimulation in vitro in contrast to WT NK1.1+ NKT cells that produced both IL-4 and IFN-γ on anti-TCRβ/ionomycin stimulation (Fig. 6A). The difference between Itk-deficient and WT immature NKT cells is consistent with a developmental block in the absence of Itk from stage 2 to 3 (Fig. 2); a stage where IFN-γ potential is usually first established. Interestingly, we consistently observed an increased frequency of IFN-γ producers in splenic WT NK1.1+ cells compared to WT NK1.1+ cells when stimulated with anti-TCRβ and ionomycin. This is in contrast to published studies on thymocyte subpopulations where the NK1.1+ fraction was enriched for IFN-γ producers (37, 39, 43). This may simply reflect differences in the methods of cytokine detection; published studies measured the total amount of cytokine produced, whereas our study detects the frequency of IFN-γ producers. The studies may be compatible if there are quantitative differences between NK1.1+ and NK1.1− NKT cells in the amount of IFN-γ produced per cell. Alternatively, our results from peripheral tetramer NK1.1+ NKT cells may suggest additional functional maturation within the NK1.1+ subset (without acquisition of NK1.1 itself) or possibly the presence within the NK1.1− fraction of contaminating mature NK1.1+ NKT cells that have lost expression of NK1.1 on activation in the periphery.

Once again, similar effects were seen following in vivo αGalCer challenge, NK1.1+itk−/− NKT cells produced both IL-4 and IFN-γ on ex vivo provision of ionomycin. In contrast, the NK1.1− fraction produced IL-4 (of the NK1.1−tetramer+ fraction: 27% of WT cells were IL-4+ and 37% of itk−/− cells were IL-4+ but IFN-γ production was severely attenuated (of the NK1.1−tetramer+ fraction: 65% of WT cells were IFN-γ− but only 8.8% of itk−/− cells were IFN-γ+) (Fig. 6B). The ionomycin-dependent restoration of IL-4 production in both immature and mature NKT cell subsets suggests that Itk plays a direct role in IL-4 effector function, independent of defects in development. In contrast, the IFN-γ defect in itk−/− NKT cells likely arises due to both the reduced frequency of mature (IFN-γ-competent) NKT cells in the periphery and defects in the liberation of effector function in mature NKT cells.

Discussion
The ability to rapidly secrete cytokines provides NKT cells with the potential to influence immune responses. Therefore, much interest is focused on the signals required for NKT cell development and function. Although conventional T cells and NKT cells both express T cell αβ receptors, a number of recent studies suggest that the signals required for NKT cell development and function may be distinct from those for conventional T cells. In this study, we confirm and extend analysis on the role of Itk in NKT cell maturation from thymic stage 2 (CD44highNK1.1−) to stage 3 (CD44highNK1.1+). Surprisingly, despite a partial block in maturation, mature NK1.1+ NKT cells were present in the periphery and constitutively contain mRNAs for several cytokines, including IL-4 and IFN-γ. Strikingly, such itk−/− NKT cells that appear poised for cytokine synthesis failed to produce effector cytokines on stimulation with αGalCer. These results suggest that Itk-dependent signals, although not required for activation of cytokine loci in NKT cells, are required for optimal IL-4 and IFN-γ production upon TCR stimulation in vitro and in vivo. That the functional potential of mature Itk-deficient NK1.1+ NKT cells could be revealed by provision of ionomycin supports a role for Itk in release of NKT cell function independent of Itk effects on NKT cell development.

The severe defect in IL-4 and IFN-γ protein production in the absence of Itk correlates with an impairment in Ag-driven enhancement of IL-4 and IFN-γ transcription. Although NKT cells constitutively express cytokine mRNAs, activated WT NKT cells further enhanced cytokine transcription 100- to 1000-fold and this response was attenuated in the absence of Itk. In conventional CD4+ T cells at least, such transcriptional enhancement is critical for effective IL-4 production by Th2 cells (29). For IL-4 therefore, NKT cells and conventional Th2 cells share a common requirement for Itk to exert effector function. Interestingly, our studies also highlight a marked dependency on Itk for IFN-γ production in NKT cells. Recent studies also suggest that CD8+ T cells may be dependent on Itk for robust IFN-γ production (46). Thus, Itk is not solely a Th2-dependent kinase. These observations support the idea that Itk regulates the availability of signaling components downstream of PLCγ activation, such as NFAT and AP-1, which are commonly used by many cytokine genes. Interestingly, the pattern of cytokine genes affected by Itk deficiency appears to be cell type specific and likely reflects differences in activation thresholds between immune cells.

Notably, NKT cell production of IFN-γ appears more dependent on a number of signaling molecules (PKCθ, Itk) that are dispensable for Th1 cell production of the same cytokine (Fig. 4B and Refs. 26, 36, and 47). Signals during Th1 differentiation may reset activation thresholds in a lineage-specific manner. Indeed, Th1 effectors acquire changes in the TCR-induced calcium flux that results in a more rapid and higher magnitude calcium response compared with naïve and Th2 effectors (48). The removal of Itk, a key regulator of PLCγ activity, leads to a reduced but still measurable calcium flux in Th1 cells (potentially providing sufficient NFATc/ERK activation for IFN-γ transcription) while itk−/− Th2 calcium responses are ablated (B. B. Au-Yeung and D. J. Fowell, unpublished observations). Therefore, akin to the loss of Itk in Th2 cells, the loss of Itk in NKT cells may reduce the calcium flux below a critical threshold for both IL-4 and IFN-γ transcription. The rescue of both IL-4 and IFN-γ protein production in NKT cells by provision of ionomycin in combination with Itk-deficient TCR signals is consistent with such a notion.

These results suggest that execution of NKT cell function may be more tightly regulated than inferred from their ability to rapidly synthesize cytokines upon TCR ligation. Interestingly, a recent study highlighted another potential checkpoint in NKT cell effector function at the level of cytokine secretion. Unlike the defect presented here in the absence of Itk where cytokine mRNA is intact but cytokine synthesis is impaired (as measured by intracellular cytokine staining, Fig. 5), GM-CSF deficiency led to normal cytokine synthesis in mature NKT cells but an inability to secrete these proteins (38). Once again, the defect was restricted to NKT cells, as cytokine secretion from GM-CSF-deficient NK cells and CD4 and CD8 T cells was unimpaired. Itk-deficient NKT
cell thymocytes and splenocytes express WT levels of GM-CSF (B. B. Au-Yeung and D. J. Fowell, unpublished observations), indicating that the two functional defects are distinct. Combined, these studies highlight the extraordinary lineage specificity in signal requirements for production and secretion of a given cytokine in different immune effector cell types.

Itk joins an expanding group of signaling components whose requirements for development and function differ between conventional T cells and NKT cells. For example, in mice lacking SAP, NF-κB1, or PKCθ, conventional T cell numbers were not affected but NKT cell development was impaired (31, 34, 36, 47–51). At the functional level, SAP-deficient and PKCθ-deficient conventional CD4+ T cells show selective defects in Th differentiation, capable of Th1 differentiation and IFN-γ secretion but impaired Th2 responses in vitro and in vivo (47, 49), similar to the phenotype observed in itk−/− mice. Strikingly, NKT cells deficient in PKCθ, SAP, and now Itk exhibit a more severe functional defect with impaired production of both IL-4 and IFN-γ (31, 34, 36, 50). However, the severe reduction in NKT cell number in the SAP-deficient mice may make accurate measurements of functional capacity problematic. The similar defects in cytokine production by NKT cells lacking PKCθ, Itk, SAP suggest that these molecules contribute to common signaling pathways that are critical for NKT cell effector function. Previous studies have linked signaling lymphocytic activating molecule family receptor stimulation with SAP, Fyn, and PKCθ recruitment, leading to activation of the NF-κB pathway (51, 52). How Itk could contribute directly to this pathway is unclear. The absence of Itk phosphorylation in T cells lacking Lck suggests that Fyn does not have a major role in phosphorylation of Itk (53). Currently, there is no evidence to suggest that Itk interacts with SAP. However, association between Itk and PKCθ, possibly resulting in enhanced PLCγ activation, has been observed both in T cell lines and in mast cells (54, 55). This interaction may also occur in NKT cells, promoting transduction of signals important for enhanced cytokine transcription.

Given the potential immunomodulatory effects of NKT cell-derived cytokines, the striking defect in cytokine production (both IL-4 and IFN-γ) by Itk-deficient NKT cells in vivo could significantly impact the magnitude and quality of the developing acquired immune response. It will be interesting to revisit in vivo models previously used to study immune responses in the absence of Itk to determine the relative contributions of distinct Itk-deficient immune cell types to the overall immune response. Our previous work on the role of Itk in Th2 development provided evidence for a CD4−intrinsic defect in Th2 cytokine production through the use of CD4+ T cell adoptive transfers (26). However, these studies did not rule out a contribution to the magnitude of the type 2 response by other immune cell types following infection of Itk-deficient mice. For example, mice lacking Itk have reduced airway hyperreactivity and lower levels of IL-4 and IL-13 in the bronchoalveolar lavage fluid in a model of allergic asthma (56, 57). This phenotype was attributed to reduced T cell infiltration to the lungs and impaired Th2 cytokine production. However, recent evidence suggests that NKT cells are sufficient to induce airway hyperreactivity, a hallmark of asthma, even in the absence of conventional CD4+ T cells (58). Given that Itk is critical for cytokine production by NKT cells and Th2 cells, it would be interesting to re-evaluate how these cell types contribute to attenuated airway hyperreactivity and type 2 cytokine production in itk−/− mice. The original description of immune defects in the absence of Itk demonstrated increased susceptibility of itk−/− mice to Toxoplasma gondii infection despite similar frequencies of IFN-γ-producing immune cells on in vitro restimulation with soluble tachyzoite Ag 30 days after infection (59). The generation of antimicrobial effectors but subsequent death from T. gondii infection may also be explained by a defect in NKT cell function given a number of reports suggest a protective role for NKT cells in limiting T. gondii-induced immune pathology (60, 61).

The Tec kinases appear attractive therapeutic candidates for the modulation of TCR signal strength (62–64). However, the complexity of the signal requirements for individual cytokine genes in distinct immune cell types may make it difficult to predict the outcome of Tec kinase modulation in vivo: attenuation of IL-4 only or IL-4 and IFN-γ. A common and encouraging theme to come of recent studies on Itk is that the kinase appears to be critical, not for the gain of function but, for the execution of effector function in both CD4+ T cells and NKT cells, making it an appealing candidate for the termination of existing cytokine-mediated immunopathology.

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Disclosures

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