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In conventional αβ T cells, the Tec family tyrosine kinase Itk is required for signaling downstream of the T cell receptor (TCR). Itk also regulates αβ T cell development, lineage commitment, and effector function. A well established feature of Itk−/- mice is their inability to generate T helper type 2 (Th2) responses that produce IL-4, IL-5, and IL-13; yet these mice have spontaneously elevated levels of serum IgE and increased numbers of germinal center B cells. Here we show that the source of this phenotype is γδ T cells, as normal IgE levels are observed in Itk−/- mice. When stimulated through the γδ TCR, Itk−/- γδ T cells produce high levels of Th2 cytokines, but diminished IFN-γ. In addition, activated Itk−/- γδ T cells up-regulate costimulatory molecules important for B cell help, suggesting that they may directly promote B cell activation and Ig class switching. Furthermore, we find that γδ T cells numbers are increased in Itk−/- mice, most notably the Vγ1.1+ Vδ6.3+ subset that represents the dominant population of γδ NKT cells. Itk−/- γδ NKT cells also have increased expression of PLZF, a transcription factor required for αβ NKT cells, indicating a common molecular program between αβ and γδ NKT cell lineages. Together, these data indicate that Itk signaling regulates γδ T cell lineage development and effector function and is required to control IgE production in vivo.

Results

γδ T Cells Promote the Hyper IgE and Enriched Germinal Center Phenotype Seen in Itk−/- Mice. In an effort to identify the cell type producing Th2 cytokines and driving IgE class-switching and secretion in unimmunized Itk−/- mice, we considered γδ T cells. To test this possibility, Itk−/- mice were crossed to Tcrd−/- mice (20) that lack γδ T cells. As shown in Fig. 1A and reported previously (3, 4), Itk−/- mice have elevated concentrations of serum IgE compared with WT controls. Strikingly, in Itk−/-Tcrd−/- double-deficient mice, serum IgE levels drop significantly compared with Itk−/- mice. Similar results were seen upon analysis of the proportion of germinal center phenotype B cells (B220+ peanut agglutinin [PNA]+) (Fig. 1B). Individual cohorts of mice were tested at 2 months of age, 3.5 months of age, and 5 months of age, with similar results.

The authors declare no conflict of interest.


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Itk−/− Mice Have Increased Numbers of γδ T Cells. The finding that elevated serum IgE levels in Itk−/− mice were dependent on γδ T cells suggested that the γδ T cells present in Itk−/− mice might be altered relative to their WT counterparts. We first examined γδ T cell numbers. As seen in Fig. 1C, Itk−/− mice have increased proportions of γδ T cells in the thymus, the spleen, the mesenteric lymph nodes, and the liver, but not in the intestinal intraepithelial lymphocyte (iIEL) compartment. A summary of data indicates significant increases in the proportion and absolute numbers of γδ T cells in both the thymus and the spleen, as well as an increase in the percentage of γδ T cells in the liver, in the absence of Itk (Fig. 1 D–F).

Itk−/− Mice Contain Increased Proportions of γδ T Cells Subsets Expressing Vγ1.1/Vδ6.3+, CD4+, and NK1.1. Analysis of TCRγ chain repertoire indicated an increased proportion in Vγ1.1+ γδ T cells in Itk-deficient versus WT mice, and a concomitant decrease in the other major subsets (Vγ2+ and Vγ5+) (Fig. 2A and B). Further, the majority of Vγ1.1+ γδ T cells in the thymus and liver of Itk−/− mice also expressed Vδ6.3 (Fig. 2C). Overall, we find a significant increase in the absolute numbers of this γδ T cell subset in thymus and spleen of Itk−/− mice compared with controls (Fig. 2 D and E), as well as an increased proportion of Vγ1.1+Vδ6.3+ γδ T cells in Itk−/− liver (Fig. 2F). Interestingly, Vγ1.1+Vδ6.3+ γδ T cells are the predominant subset of γδ NKT cells, and readily produce effector cytokines (17, 21, 22).

In contrast to WT γδ T cells, the Itk−/− γδ T cell population shows a striking increase in the proportion of CD4+ cells in the thymus, spleen and liver (Fig. 3 A and B). Itk−/− mice also had elevated proportions of thymic NK1.1+ γδ T cells (Fig. 3 C and D). Interestingly, the majority of the thymic Itk−/− CD4+ γδ T cells and virtually all of the thymic Itk−/− NK1.1+ γδ T cells express high levels of the memory cell marker CD44 (Fig. 3 E), as well as the activation marker CD69. As previously reported, the majority of γδ T cells in the liver of WT mice are NK1.1+ (i.e., γδ NKT cells) (17), and this is also the case in Itk−/− liver (Fig. 3 C and D).

Focusing on the Vγ1.1+Vδ6.3+ subset of γδ T cells, we find that WT and Itk−/− mice each have a similar proportion of NK1.1+ CD4+ cells, whereas NK1.1+ CD4+ cells are increased substantially in Itk−/− mice [supporting information (SI) Fig. S1]. Furthermore, outside the thymus, the Vγ1.1+Vδ6.3+ subset is found predominantly in the liver, rather than the spleen (Fig. 3 F and G), as has been reported previously for γδ NKT cells (17). The γδ T cells not expressing Vγ1.1+Vδ6.3 correspond to the “conventional” NK1.1+ CD4−CD8− subset; this conventional γδ T cell subset is not altered in numbers in Itk−/− versus WT mice, indicating that ITK controls the generation of “nonconventional” CD4+ or NK1.1+, but not conventional, γδ T cells. Expanded populations of CD4+ γδ T cells have been previ-
uously demonstrated in mice lacking expression of both TCRβ and CD5 (14). We therefore examined CD5 expression on Itk−/− γδ T cells, to determine if altered levels of CD5 might be contributing to the increased number of CD4+ γδ T cells in these mice, but detected no differences between WT and Itk−/− mice. However, like a previously-reported subset of activated γδ T cells specific for self-ligands, the MHC class Ib molecules, T10/T22 (23), the NK1.1+ subset of thymic Itk−/− γδ T cells are all CD122-positive.

To determine whether the alterations we observed in Itk−/− mice are intrinsic to the hematopoietic cells or to the environment, we generated bone marrow chimeras using WT or Itk−/− bone marrow cells. These experiments demonstrated that the increased proportions of both γδ T cells and germinal center B cells seen in Itk−/− mice are intrinsic to Itk−/− hematopoietic cells (Fig. S2). Consistent with these data, we also find that the predominant Tec kinase expressed in WT γδ T cells is Itk (Fig. S3).

**Enhanced Expression of IL-4 and PLZF in Itk-Deficient γδ T Cells.** To determine the basis for the altered function of Itk−/− γδ T cells, we examined mRNA levels for T-bet, Eomesodermin, and GATA-3 in WT versus Itk−/− γδ T cells. We found that Itk−/− γδ T cells consistently expressed higher levels of GATA-3 mRNA and protein compared with WT γδ T cells, while the NK1.1+ subset of thymic Itk−/− γδ T cells have reduced levels of mRNA for both T-bet and Eomesodermin (Fig. S3). These findings suggested that Itk−/− γδ T cells might produce a distinct pattern of cytokines compared with WT γδ T cells. We first examined mRNA levels for the signature cytokines, IL-4 and IFNγ, after γδ T cell activation in vitro. As shown in Fig. 4, in response to γδ TCR stimulation, Itk−/− γδ T cells had constitutively elevated levels of IL-4 mRNA before stimulation and exhibited dramat-
ically induced expression of IL-4 mRNA at 10 hours and 20 hours poststimulation compared with WT γT cells. Basal levels of IFNγ mRNA were similar between the WT and Itk−/− γT cells; after stimulation, both cell types produced IFNγ, although WT γT cells showed higher levels of IFNγ mRNA compared with γT cells lacking Itk.

Based on the high proportion of Vγ1.1 Vδ6.3+ γT cells present in Itk−/− mice and the previous association of this γT cell subset with dual production of IL-4 and IFNγ (21, 22), we also examined WT and Itk−/− γT cells for expression of the transcription factor PLZF. PLZF has recently been found to be critical for the development and effector function of TCRβ+ NKT cells, where it promotes the simultaneous production of IL-4 and IFNγ (18, 19). Interestingly, we found that splenic Itk−/− γT cells express substantially higher amounts of PLZF mRNA than do WT γT cells (Fig. 4B). Furthermore, among Itk−/− γT cells, the NK1.1+ fraction expresses particularly high levels of PLZF mRNA. These findings suggest the conclusion that γT cell development is altered in the absence of Itk.

To assess levels of cytokine protein secretion, individual subsets of Itk−/− and WT γT cells were purified and stimulated. As previous studies have found that NK1.1+ γT cells and CD4+ γT cells produce the highest levels of cytokines, particularly “Th2” cytokines (24–28), we compared NK1.1+ to NK1.1−, and CD4+ to CD4− γT cell subsets. After 72 hours of stimulation, WT NK1.1+ γT cells secreted more IL-4, IL-10, and IL-13 than their NK1.1− counterparts; in contrast, both NK1.1+ and NK1.1− subsets of WT γT cells secreted large amounts of IFNγ (Fig. 4C). Consistent with the analysis of cytokine mRNA levels, we observed elevated secretion of the Th2 cytokines IL-4, IL-10, and IL-13 by the Itk−/− NK1.1+ γT cells when compared with WT NK1.1+ cells; furthermore, Itk−/− NK1.1+ γT cells secreted much higher amounts of these cytokines per cell relative to the Itk−/− NK1.1− subset. In addition, we found that both subsets of Itk−/− γT cells secreted IFNγ, but at a lower level than the WT cells.

Comparison of the CD4+ versus CD4− subsets of γT cells confirmed previous data (27, 28) that γT cells expressing CD4 are the major cytokine-producing population, particularly for the “Th2” cytokines. As shown in Fig. 4D, Itk−/− CD4+ γT cells produce elevated levels of IL-4 and IL-13 compared with WT CD4+ γT cells, but produce similar levels of IL-10. None of these cytokines were detected in supernatants of stimulated CD4− γT cells from either Itk−/− or WT mice. As noted above, WT γT cells (both CD4+ and CD4− subsets) secrete higher levels of IFNγ than Itk−/− γT cells.

On a population basis, Itk−/− γT cells secreted both IL-4 and IFNγ after stimulation. To determine whether individual cells were dual producers of both effector cytokines, we stimulated WT and Itk−/− γT cells in vitro with phorbol 12-myristate 13-acetate (PMA) and ionomycin and then examined IL-4 and IFNγ production by intracellular cytokine staining (Fig. 4E). As expected, a larger proportion of WT γT cells produce IFNγ in response to stimulation compared with those that produce IL-4, and few cells produce both cytokines. In contrast, Itk−/− γT cells include a significantly larger population that produces IL-4 than is seen in the WT γT cell subset (Itk−/−, 6.9 ± 1.1; WT, 3.5 ± 0.8; n = 7; P = 0.04); additionally, a greater proportion of Itk−/− γT cells produces both IL-4 plus IFNγ compared with WT γT cells (Itk−/−, 3.9 ± 0.8; WT, 1.7 ± 0.3; n = 7; P = 0.02). Because this pharmacological stimulation bypasses the need for ITK in TCR-mediated signaling, these data indicate that a larger proportion of Itk−/− γT cells are programmed to produce IL-4, as well as IL-4 plus IFNγ, before their activation. This latter finding, together with the data demonstrating increased numbers of CD4+ and NK1.1+ γT cells in the Itk−/− mice, strongly suggests that γT cell development is altered in the absence of Itk leading to a striking increase in a PLZF-positive, IL-4-producing γT cell population.

**Itk−/− γT Cells Up-Regulate Surface Receptors That Promote B Cell Help.** We next examined γT cells for the expression of co-stimulatory molecules that provide B cell help, such as CD40L, CD70, OX40, and ICOS (15). For these experiments, WT and Itk−/− thymocytes and splenocytes were evaluated directly ex vivo and, in addition, were cultured for 24 hours in the presence of stimulatory anti-TCRβ antibodies. Although analysis of splenic γT cells from WT and Itk−/− mice did not reveal any major changes in co-stimulatory markers, we did see a small increase in the proportion of constitutively ICOS-positive γT cells in the spleens of Itk−/− mice. Inasmuch as Itk−/− mice have a two-fold increase in the absolute number of total γT cells in the spleen compared with WT, this difference amounts to a ~10-fold increase in ICOS-positive splenic γT cells, and thus could account for the enhanced B cell activation observed in Itk−/− mice.

More strikingly, levels of ICOS were increased on a large proportion of the thymic Itk−/− γT cells compared with controls, but remained unaltered following stimulation (Fig. 5A). Evaluation of the ICOS+ fraction of Itk−/− thymic γT cells indicated that nearly all of these cells were CD4+, and a substantial proportion were also NK1.1+ (Fig. 5B). Little to no difference was seen in the basal levels of CD40L and OX40 when comparing thymic Itk−/− γT cells to WT γT cells directly ex vivo (Fig. 5A). However, after 24 hours of in vitro stimulation on anti-TCRβ-coated plates, Itk−/− cells up-regulated both CD40L and OX40, whereas WT γT cells did not. This up-regulation of CD40L and OX40 was detected on all subsets of Itk−/− γT cells (Fig. 5B). Finally, we could not detect expression of CD70 on either WT or Itk−/− thymic γT cells.

**Discussion.** Overall, our data indicate that γT cell development is significantly altered in the absence of Itk, yielding increased numbers of γT cells and a shift in the major effector functions mediated by these cells. Most strikingly, Itk−/− mice have elevated numbers of γT cells expressing CD4 and NK1.1. Furthermore, unlike the γT cells in WT mice, the Itk−/− γT cells secrete large quantities of the Th2 cytokines, IL-4, IL-10, and IL-13, in addition to the IFNγ typically secreted by activated WT γT cells, correlating with high levels of the transcription factor, PLZF. These findings strongly suggest that Itk signaling plays a key role in regulating γT cell lineage development.

Surprisingly, these altered γT cells are responsible for promoting significant levels of spontaneous IgE secretion in Itk−/− mice. Based on the findings presented here, it is likely that the high levels of IL-4 and IL-13 produced by the activated Itk−/− γT cells are a major factor in this response. Our data indicate that activated Itk−/− γT cells produce elevated amounts of B cell co-stimulatory molecules, such as ICOS, CD40L, and OX40, further suggesting that the γT cells may be directly providing help to the B cells, leading to B cell activation and Ig class switching.

In humans, a variety of studies have implicated γT cells in allergic airway inflammation (29, 30) and, specifically, in promoting B cell activation and IgE class switching (31, 32). One interesting clinical report found that IL-4-producing γT cells were likely responsible for a case of hyper IgE syndrome (33). Studies performed in vitro with human γT cells showed that these cells, in combination with IL-4, can induce B cell activation, Ig isotype switching, and secretion of IgE (34). Further, these findings correlate well with observations that human γT cells can express ICOS, CD40L, OX40, and CD70 (15). Our data indicate that the Tec family tyrosine kinase, Itk, plays a key role in regulating this potentially highly detrimental function of γT cells.
Recently, γδ T cells have also been implicated in the elevated IgE concentrations seen in mice carrying mutations in additional T cell signaling proteins. For instance, in the absence of the E3-ubiquitin ligase, Itch, γδ T cells secrete IL-4 and promote IgE production in nonimmunized mice (29). More strikingly, mice expressing a mutant allele of the adapter protein linker of activated T cells (LAT), which lacks the three c-terminal tyrosines, succumb to a fatal lymphoproliferative disorder that is mediated by γδ T cells (35). In these LAT mutant mice, the γδ T cells accumulate to large numbers, and show a phenotype remarkably similar to those lacking Itk. For instance, the LAT mutant γδ T cells secrete IL-4, rather than IFN-γ, and many of them express the CD4 co-receptor; in addition, the mice also have elevated levels of serum IgE. As this mutant LAT protein does not support αβ T cell development, these altered γδ T cells are the only source of T cell help for B cell activation and IgE class switching. As Itk and LAT interact in a TCR-dependent signaling complex in αβ T cells, the similarities in the γδ T cell phenotype in these two lines of mice strongly suggest that these proteins are also in the same signaling pathway downstream of the γδ TCR, and furthermore, that this pathway regulates the development and effector function of γδ T cells. As Itk has previously been shown to suppress the development of innate αβ lineage T cells and to promote the development of conventional αβ T cells (36), a similar function for Itk may be required in γδ T cells; thus, in the absence of Itk, enhanced development of innate (e.g., PLZF, NK1.1+) γδ T cells occurs, leading to increased numbers of effector cytokine-producing γδ T cells in Itk−/− mice.

Interestingly, a recent report by Jensen et al. demonstrates that γδ T cells, like αβ T cells, are found as both naïve and effector subsets (23). Effector-type γδ T cells express higher levels of CD44, NK1.1, and CD122 relative to the naïve subset and in addition, show an altered cytokine secretion profile. Furthermore, the presence of the effector γδ T cell population correlated with the expression of the TCR ligand for these γδ T cells, indicating that ligand recognition was responsible for their activated phenotype. As a large population of Itk−/− γδ T cells exhibit a similar effector cell phenotype and produce effector cytokines such as IFN-γ and IL-4; these findings suggest that ligand recognition by Itk−/− γδ T cells may contribute to their activation in vivo and their role in promoting IgE production in nonimmunized mice.

These findings have substantial relevance to the potential effects of small molecule inhibitors of Itk. Given the importance of Itk in Th2 development and cytokine production by CD4+ αβ T cells, this kinase is currently being targeted for the development of drugs to treat asthma and other allergic diseases (5, 37). It would be unfortunate if Itk inhibition also led to the aberrant activation of γδ T cells and thus to enhanced production of IgE. Elevated levels of serum IgE would, in turn, lead to up-regulation of the FceR1 on mast cells (38), promoting hyperresponsiveness of these cells to IgE-mediated receptor aggregation. In light of the findings presented here, further studies on the role of Itk in human γδ T cells are clearly warranted.

Materials and Methods

Mice. Itk−/− mice (39) are on the C57BL/10 strain. Tcrd−/− mice (20) on the B6SJL/J background (Jackson Laboratories) were crossed to Itk−/− mice to obtain Itk−/−Tcrd−/− mice. Wild-type mice were Itk−/−Tcrd−/− littersmates or C57BL/10 mice (Jackson Laboratories). All mice used were 2–3 months of age and were maintained at the University of Massachusetts Medical School under specific pathogen-free conditions after institutional animal care and use committee approval.

Cell Preparations, Antibodies, and Flow Cytometry. Liver lymphocytes were isolated by collagenase digestion of minced liver followed by Ficoll gradient centrifugation; iEILs were isolated by incubation of cleaned intestine followed by Ficoll gradient centrifugation. The following antibodies were purchased from BD Pharmingen: TCRβ(GL3)-FITC, Vγ2-FITC, Vγ3-IFITC, Vβ6.2/6.3-PE, TCRβ-alkaline-phosphocyanin, TCRβ-PE, TCRβ-biotin (bio), CD4-alkaline-phosphocyanin, CD4-PE, CD8e-PerCP-Cy5.5, NK1.1-Cy7, CD5-CyChrome, IL-4-PE, IFNγ-alkaline-phosphocyanin, B220-alkaline-phosphocyanin, streptavidin (stre-)alkaline-phosphocyanin, and OX40-biotin. TCRβ-alkaline-phosphocyanin, ICOS-PE, and CD40L-alkaline-phosphocyanin were purchased from ebioscience. PNA-FITC was purchased from Vector Laboratories. Strep-Cascade Yellow was purchased from Intron Molecular Probes. Vγ1.1-bio was a kind gift from Lynn Puddington (University of Connecticut Health Center, Storrs, CT). Cells (500,000–2,000,000 events) were collected on a LSRII (BD Biosciences) flow cytometer. Data were analyzed using FlowJo software (Tree Star).

Quantitative Real-Time PCR. RNA and cDNA were prepared from sorted cells as previously described (40). Real-time PCR was also performed as previously described (6) on an i-Cycler (Bio-Rad). A CDNA clone encoding PLZF was a kind gift from Albert Bendelac (University of Chicago, Chicago).

In Vitro Stimulation Assays. Wild-type and Itk−/− TCRγ−/−NK1.1−, TCRγ−/−NK1.1−, TCRγ−/−CD4−, and TCRγ−/−CD4− subsets were activated in vitro with 10 μg/ml
of anti-TCR+ biotin (BD Pharmingen) for 72 hours, and supernatants were collected and IL-4, IL-10, IL-13, and IFN-γ were measured by cytometric bead array (CBA) (BD Pharmingen). Cells used for quantitative PCR were stimulated for 10 and 20 hours and examined for IL-4 and IFN-γ mRNA. For intracellular cytokine staining, cells from WT and Itk−/− mice were stimulated as previously described (6). Cells were then surface stained for anti-TCRγ and anti-NK1.1, fixed, and permeabilized using a Cytofix/Cytoperm kit (BD Pharmingen) and stained for IL-4 and IFN-γ. For ICOS, CD40L and OX40 expression on γδ T cells, cells were stimulated for 24 hours with 10 μg/ml anti-TCRγ. Cells were then surface stained with anti-ICOS, anti-CD40L, and anti-OX40 antibodies.

**Serum Analysis.** Blood was collected from WT, Tcrd−/−, Ikt−/−, and Ikt−/− Tcrd−/− mice. Serum was obtained by spinning the blood at 5000 rpm for 5 minutes and removing the supernatant. Supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) for IgE.

**Statistical Analysis.** InStat software (GraphPad) was used to perform two-tailed nonparametric Mann-Whitney tests.

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