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The Tec Kinase ITK Regulates Thymic Expansion, Emigration, and Maturation of γδ NKT Cells

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The Tec family tyrosine kinase, Itk, regulates signal transduction downstream of the TCR. The absence of Itk in CD4+ T cells results in impaired Th2 responses along with defects in maturation, cytokine production, and survival of iNKT cells. Paradoxically, Itk−/− mice have spontaneously elevated serum IgE levels, resulting from an expansion of the Vγ1.1/Vδ6.3+ subset of γδ T cells, known as γδ NKT cells. Comparisons between γδ NKT cells and αβ iNKT cells showed convergence in the pattern of cell surface marker expression, cytokine profiles, and gene expression, suggesting that these two subsets of NKT cells undergo similar differentiation programs. Hepatic γδ NKT cells have an invariant TCR and are derived predominantly from fetal progenitors that expand in the thymus during the first weeks of life. The adult thymus contains these invariant γδ NKT cells plus a heterogeneous population of Vγ1.1/Vδ6.3+ T cells with diverse CDR3 sequences. This latter population, normally excluded from the liver, escapes the thymus and homes to the liver when Itk is absent. In addition, Itk−/− γδ NKT cells persistently express high levels of Zbh16 (PLZF) and Il4, genes that are normally downregulated in the most mature subsets of NKT cells. These data indicate that Itk signaling is required to prevent the expansion of γδ NKT cells in the adult thymus, to block their emigration, and to promote terminal NKT cell maturation. The Journal of Immunology, 2013, 190: 2659–2669.

One unexpected phenotype observed in Itk−/− mice was the spontaneous elevation in levels of serum IgE (10, 11). On the basis of the data that indicated that Itk−/− conventional CD4+ T cells were unable to generate Th2 effector responses (12) and the fact that Itk−/− αβ TCR+ iNKT (αβ iNKT) cells were also defective in producing IL-4 (13, 14), the source of type 2 cytokines responsible for promoting B cell isotype switching to IgE was initially unclear. However, recent studies showed that the hyper-IgE syndrome seen in Itk−/− mice was dependent on γδ TCR+ T cells (15). Subset analysis of γδ T cells in Itk−/− mice identified the Vγ1.1/Vδ6.3+ subset, expressing the transcription factor PLZF (hereafter referred to as VδT cells) (16–18), as greatly increased in number in Itk−/− mice. Functional studies showed that these cells secreted high levels of Th2 cytokines (15). In addition to the shared expression of PLZF linking γδ to αβ NKT cells (19, 20), transcriptome analysis substantiated a common molecular program among these two cell lineages (21).

Elegant studies have demonstrated that the adult thymus contains a mixed population of γδ NKT cells. One subpopulation originates from fetal progenitors, undergoes substantial expansion in early neonatal life, and localizes to the liver; these cells predominantly express an invariant TCR sequence that is characterized by the absence of junctional diversity, consistent with their fetal/neonatal origin. In contrast, a second subpopulation is derived from adult precursors and remains as a largely thymic resident population (22–24).

αβ iNKT cells are an innate subset of αβ T cells that reside in the thymus, spleen, and liver, are rapid producers of cytokines such as IFN-γ, IL-4, and IL-10 (25, 26), and predominantly express an invariant TCR (27). αβ iNKT cells develop in the thymus from CD4+CD8+ double-positive precursors and are dependent on the presence of the nonclassical MHC class I molecule CD1d (28–30). Following their initial positive selection, αβ iNKT cell precursors undergo additional stages of differentiation in the thymus. Starting with the earliest detectable population (CD24 (HSA)+, CD4+CD8+ iNKT cells), these cells proceed to downregulate CD24 (stage 1) and then upregulate CD44 (CD44+...
CD44hiNK1.1lo; stage 2). Finally, at the terminal maturation stage, the cells upregulate the IL-2/IL-15R β-chain (CD122) and NK cell receptors, such as NK1.1 (CD44hiCD44loNK1.1hi; stage 3). Transition through these stages is also accompanied by a change in cytokine profile. At the intermediate stage, αβ NKT cells are potent producers of IL-4 but make little IFN-γ. As they transition to stage 3, αβ NKT cells produce abundant amounts of IFN-γ but less IL-4, and PLZF expression decreases (31).

Previous studies have shown that Itk−/− mice have reduced numbers of αβ NKT cells and that those present have a defect in maturation (13, 14, 32). Although γδ T cells develop in the thymus, they develop from CD4+CD8− progenitors rather than from CD4+CD8+ double-positive thymocytes (22, 33, 34), and their thymic selection requirements are not known. Thus, the exact extent of overlap in γδ and αβ NKT cell developmental programs, and the impact of TCR signaling in γδ NKT cell differentiation is unclear. In this study, we show that CD4+γδ NKT thymocytes follow a maturation sequence composed of three stages ascribed to αβ NKT cells. Itk−/− γδ NKT cells are impaired in their maturation, leading to increased export of PLZFhi IL-4−producing γδ NKT cells from the thymus. TCR8 sequence analysis indicated that, unlike the γδ NKT cells in livers of wild-type (WT) mice that are exclusively derived from fetal progenitors, Itk−/− hepatic γδ NKT cells include a subset derived from adult progenitors. These data indicate that Itk normally functions to prevent the expansion, as well as the export, of adult-origin γδ NKT cells and that Itk plays a parallel role in the functional and phenotypic maturation of αβ and γδ NKT cells.

Materials and Methods

**Mice**

Itk−/− mice (35) are on the C57BL/6 strain. γδ T cells were isolated from WT-4Get and Itk−/−/4Get mice (36) were crossed to 4Get mice. MHCI- and MHCII−/− mice were obtained from The Jackson Laboratory or Taconic Laboratories and are on a C57BL/6 background. C57BL/6 mice were used as controls. Mice were used between 2 and 3 mo of age and were maintained at the University of Massachusetts Medical School under specific pathogen-free conditions in accordance with institutional animal care and use committee guidelines.

**Cell preparations, Abs, and flow cytometry**

To isolate lymphocytes from the liver, livers were first perfused with 5 ml PBS through the portal vein, followed by collagenase digestion of minced liver. Lymphocytes were then isolated by Percoll gradient centrifugation. The following Abs were purchased from BD Pharmingen: rat anti-mouse IgG1-FITC, Vγ1-Vδ6.3+ liver. Lymphocytes were then isolated by Percoll gradient centrifugation.

**Sample preparation for microarray analysis**

Samples were prepared for microarray analysis as described previously (21). Briefly, thymocytes were pooled from 4 to 30 mice and enriched for γδ T cells by depletion of CD8+ cells with magnetic beads and an autoMACS. Cells were then stained and sorted with a FACSAria (∼2 × 10^6–3 × 10^6 cells; >95% pure) directly into TRIzol (Invitrogen). Independent triplicates were sorted unless indicated otherwise (complete sorting details available from the ImmGen Project). Population labels correlate with ImmGen (http://immgen.org) populations as follows: mature WT thymic V6 (MatV6), WT and Tgd.vg1+vd6+4Get.Th; total V6 cells from Itk−/− thymus (TotalV6. Itk−/−), Tgd.vg1+vd6+Th. Itk−/− mature WT CD4+CD8+ thymocytes (MatCD4, TISP24-Th; mature WT CD4+CD8+ thymocytes (MatCD4, TISP24-Th; mature WT thymic stage 0–1 NKT cells (NKT.44, NK1.1) [sorted in duplicate], NKT.44-NK1.1-Th; WT thymic stage 2 NKT cells (NKT.44NK1.1), NKT.44-NK1.1-Th; and WT thymic stage 3 NKT cells (NKT.44N1.1), NKT.44-NK1.1-Th. Note that all V6 cells in Itk−/− mice are CD24hi. Microarray data are available on the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo), accession number GSE15907.

**Tcr cloning and sequencing**

Total RNA was isolated from WT and Itk−/−Vγ1.1Vδ6.3+ liver γδ T cells. RNA was also isolated from WT-4Get and Itk−/−/4Get mature (HASaKb) Vγ1.1Vδ6.3+ thymic γδ T cells that were GFPCD122+, GFPCD122+, and GFPCD122+. Reverse transcription of 0.2 μg RNA was performed with qScript DNA Supermix (Quanta). TCR8 V region sequences were amplified from cDNA by PCR using published primers (37). Amplified sequences were cloned into pCR2.1-TOPO TA vector by TOPO TA cloning (Invitrogen). Microarray data were analyzed and visualized as described previously (21). Briefly, data were analyzed with modules of the GenePattern genomic analysis platform. ConsolidatedProbeSets, a custom GenePattern module written by S. Davis (Harvard Medical School), was used for the consolidation of multiple probe sets into a single mean probe-set value for each gene. Genes with differences in regulation were identified with the Multiplot module of GenePattern through the use of the average of all replicates. Genes were considered to be regulated differently if they differed in expression by >2-fold, had a coefficient of variation among replicates <0.5, had a Student t test p < 0.05, and had a mean expression value >120 in at least one subset in the comparison. Heat maps were generated by hierarchical clustering (HierarchicalClustering module of GenePattern) of data on the basis of gene (row) and subset (column) with the Pearson correlation for distance measurement. Data were log transformed and clustered with pairwise complete linkage. Data were centered on rows before visualization with the HeatMapViewer module of GenePattern. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was used for functional classifications through the Database for Annotation, Visualization, and Integrated Discovery (DAVID) portal.

**Results**

γδ NKT cells in the spleens of Itk−/− mice display an immature phenotype

We have previously shown that the absolute number and proportion of peripheral γδT cells are increased in Itk−/− mice. This defect can be attributed to a specific subset of γδT cells expressing the Vγ1.1Vδ6.3 TCR (hereafter referred to as V6) (Fig. 1A) (15). As numerous similarities between V6 cells and αβ NKT cells have been observed, we performed a side-by-side comparison of αβ NKT cells and V6 cells in the spleens of both WT and Itk−/− mice. Consistent with previous reports, αβ NKT cells from Itk−/−

In vitro T cell activation

WT and Itk−/− thymocytes were stimulated as described previously (15). Cells were surface stained for anti-TCR8, anti-Vγ1.1, anti-Vδ6.3+, or CD1d-PBS77 tetramer, fixed, and permabilized using fixation/permeabilization kit (eBioscience) and stained for IL-4, IFN-γ, and PLZF.
mice were reduced ~2-fold in both numbers and percentages (13, 14, 32). To focus on the later stages of NKT cell maturation, we analyzed CD24lo subsets of V6 cells and ab iNKT cells and found that the majority of these cells expressed the maturation markers CD44 and CD122. Furthermore, as previously described for ab iNKT cells (19, 20), maturation of WT V6 cells is associated with downregulation of CD4 and PLZF (Fig. 1B). Examination of the CD24lo V6 population from Itk2/2 mice revealed a drastically altered phenotype when compared with WT controls. Although the majority of Itk2/2 V6 cells expressed CD44 and CD122, similar to WT cells, they also aberrantly retained expression of PLZF and CD4, indicating incomplete maturation (Fig. 1B). A similar trend, although less dramatic, was observed when comparing WT versus Itk2/2 iNKT cells in the spleen, as previously reported, including modestly impaired upregulation of CD122 and downregulation of CD4 on Itk2/2 iNKT cells (14).

To address the potential of these cells to produce cytokine, we first used IL-4 reporter mice (4Get (36)), which express GFP from a bicistronic IL-4 mRNA. For these experiments, we compared the proportion of V6 and iNKT cells expressing GFP in the presence or absence of Itk. As shown in Fig. 1C, the vast majority of V6 cells from the spleens of Itk2/2 mice were GFP+, compared with V6 cells from WT controls. Furthermore, the cell surface phenotype of GFP+ V6 cells correlated with that of PLZF-expressing V6 cells in both WT and Itk2/2 mice (Fig. 1B, 1C). Interestingly, analysis of 4Get mice showed that the Itk2/2 iNKT cell population included fewer cytokine-producing cells than the WT iNKT cell subset, again consistent with previous published data (13, 14, 32). Taken together, these data indicate that the majority of V6 cells from the spleens of Itk2/2 mice display a more immature phenotype than their WT counterparts, a phenotype shared with Itk2/2 iNKT cells.
Increased proportions of PLZF- and CD4-expressing γδ NKT cells in the livers of Itk−/− mice

Unlike conventional T cells, NKT cells preferentially home to the spleen and liver following thymic egress; furthermore, αβ iNKT cells continue to mature in the liver relative to their phenotype in the spleen (38). To determine whether a similar change was occurring with V6 cells and whether Itk−/− liver V6 cells were also impaired in this maturation, we examined liver NKT cells. As shown in Fig. 2, we found that fewer WT αβ iNKT cells and WT V6 cells in the liver expressed high levels of PLZF compared with these populations in the spleen (Figs. 1B, 2A). A comparison of WT liver cells with those from Itk−/− mice showed that iNKT cells were similar; in contrast, Itk−/− liver V6 cells had a substantial increase in the proportion that expressed PLZF and CD4 (Fig. 2A). Consistent with these data, an increased proportion of Itk−/− liver V6 cells also constitutively expressed IL-4 mRNA (Fig. 2B).

These data suggested that peripheral γδ NKT cell maturation was impaired in Itk−/− mice. Alternatively, we considered the possibility that adult Itk−/− mice might be generating unusually large numbers of PLZF+ V6 cells in the thymus and that ongoing emigration of these cells was contributing substantially to the V6 population in the liver. This is in contrast to what is seen in WT mice, where the V6 cells found in the adult liver are predominantly derived from fetal/neonatal progenitors (22, 39). To address this latter possibility, we examined the rearranged TCRγ chain sequences in V6 cells from the livers of WT and Itk−/− mice. As expected, liver V6 cells from WT mice exhibited extremely limited diversity in their TCRγ sequences, with 100% of the sequences identical to the canonical sequences previously found in V6 cells isolated from the fetal thymus and distinguished by their absence of the D61 and N region nucleotides (22). In contrast, TCRγ sequences from Itk−/− liver V6 cells included a fraction of sequences that were clearly of adult origin, in addition to those with the canonical TCRγ chain rearrangements (Fig. 2C, Supplemental Fig. 1). These data indicate that, in the absence of Itk, adult-derived thymic V6 cells are able to emigrate to the liver.

Altered thymic development of γδ NKT cells in Itk−/− mice

To address the changes in V6 cell development that might account for the presence of adult-derived γδ NKT cells in the liver of Itk−/− mice, we examined thymocytes from WT and Itk−/− mice and characterized iNKT cell and V6 cell populations. As previously observed, the percentage of thymic iNKT cells in Itk−/− mice was decreased compared with WT (Fig. 3A) (13, 14, 32). In contrast, Itk−/− thymi contained a substantial increase in the proportion of γδ T cells, an increase that was largely accounted for by expanded numbers of V6 cells (Fig. 3A).

Several stages of αβ iNKT cell differentiation have been characterized based on a variety of cell surface markers, cytokines, and transcription factors. One notable example is the up-regulation of CD44 and CD122, which indicate a transition to the terminal maturation stage (40, 41). Similar to αβ iNKT cells, HSAab V6 cells upregulate CD44 while concurrently upregulating CD122 (Fig. 3B). Analysis of Itk−/− thymic V6 cells indicated modest changes in the percentages of CD44+ and CD122+ cells compared with WT V6 cells, leading to a reduction in the proportion of CD44+Itk−/− V6 cells that had upregulated CD122. One difference between αβ iNKT cells and V6 cells was the expression of CD4. Whereas WT αβ iNKT cells remained largely CD4+ in the thymus as they matured, WT thymic V6 cells downregulated CD4 coincident with the upregulation of CD44 and CD122. Strikingly, the majority of V6 cells in the thymus of Itk−/− mice remained CD4+ (Fig. 3B). Taken together, these data suggest that γδ NKT cells in Itk−/− mice have a modest defect in the transition to terminal maturation, similar to the impaired maturation seen with thymic Itk−/− iNKT cells (Fig. 3B) (14).

A major transcription factor important in the development of αβ iNKT cells is PLZF (19, 20). As reported, PLZF levels decline as αβ iNKT cells mature; thus, the iNKT cells expressing the highest levels of CD44 or CD122 express less PLZF than their less mature counterparts (Fig. 3B) (19, 20). A parallel pattern of expression was evident for thymic V6 cells (Fig. 3B), as has also recently been reported (42). For V6 cells, those expressing the highest levels of PLZF expressed less CD44 and CD122 than the PLZFlow subset. As shown,
we observed an overall increase in the total numbers of both PLZFhi and PLZFint V6 populations among Itk2/2 thymocytes (Fig. 3D).

Another feature of αβ iNKT cell terminal maturation is a reduction in IL-4 production (32). To determine whether a similar pattern is observed for V6 cells, we analyzed thymocytes from WT- and Itk2/2-4Get mice. V6 cells from WT mice showed graded GFP expression, such that the lowest levels of GFP correlated with the highest expression of maturation markers, CD44 and CD122. In addition, GFP-negative WT thymic V6 cells were also CD4 negative. Itk2/2 thymic V6 cells showed a similar pattern; however, the proportion of cells retaining high levels of GFP was dramatically increased compared with WT (Fig. 3C, 3D).

**Cytokine production by γδ NKT cells correlates with PLZF expression**

As αβ iNKT cells undergo terminal maturation, they switch from being single IL-4 producers to being dual producers of both IL-4 and IFN-γ, a change that correlates with reduced PLZF expression.

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**FIGURE 3.** γδ NKT cells in the thymus of Itk2/2 mice have the characteristics of less mature αβ iNKT cells. Thymocytes from WT and Itk2/2 mice or WT-4Get and Itk2/2-4Get mice were stained and analyzed by flow cytometry. (A) Total thymocytes were analyzed for TCRβ versus TCRδ (left panels), and TCRδ-positive cells were gated on and examined for Vγ1.1 and Vδ6.3 expression (second panels). V6 cells were further gated on HSA (third panels). iNKT cells were identified by staining with CD1d tetramer and anti-TCRβ (fourth panels). iNKT cells were further gated on HSA expression (right panels). (B) HSA− V6 γδ T cells and iNKT cells in the thymus were analyzed for the expression of CD44, CD122, CD4, and intracellular PLZF. Dot plots show representative data; compilations of data from all experiments are shown in the graphs below. Statistically significant differences are shown with p values. (C) HSA− V6 γδ T cells and iNKT cells from WT-4Get and Itk2/2-4Get mice were stained and analyzed for CD44 versus GFP, CD122 versus GFP, and CD4 versus GFP expression. Data are representative of at least two independent experiments. (D) PLZFhi and PLZFint V6 γδ T cell and iNKT cell populations were gated as shown in (B), and absolute numbers were calculated (left two panels). The percentages and absolute numbers of GFP− cells in the HSA− V6 γδ T cell and iNKT cell populations are shown (right two panels). Statistically significant differences are shown with p values.
To determine whether V6 cells expressing high versus intermediate levels of PLZF showed a similar pattern of cytokine production, we stimulated WT and Itk−/− thymocytes with PMA and ionomycin and examined IFN-γ and IL-4 production. Similar to αβ NKT cells, V6 cells were capable of producing both cytokines upon stimulation, although the proportion of V6 cells producing IFN-γ and IL-4 simultaneously is less than seen for αβ NKT cells. Surprisingly, there was little difference in the frequency of total Itk−/− V6 cells capable of producing IFN-γ and/or IL-4 compared with WT V6 cells (Fig. 4A).

We then examined cytokine production in cells stained for PLZF expression. As can be seen in Fig. 4B and 4C, only the PLZFint subset of iNKT cells was capable of producing IFN-γ, whereas both PLZFhi and PLZFint NKT cells produced IL-4. A similar pattern was seen for V6 cells and was shared between WT and Itk−/− thymocytes. These results indicate that PLZFhi cells in Itk−/− mice are functionally competent, despite their delayed progression toward terminal maturation.

The gene signature of Itk−/− γδ NKT cells resembles immature WT iNKT cells

The data presented thus far suggest that the terminal maturation of V6 cells is impaired or delayed in the absence of Itk. To examine this issue at a molecular level, we performed gene expression microarray analysis. V6 cells were isolated from the thymus of Itk−/− mice, and their gene expression profile was compared with that of multiple populations of WT thymocytes. As shown in Fig. 5, 71 genes were upregulated by ≥2-fold in Itk−/− V6 cells compared with WT CD24hi V6 cells, and 124 genes were downregulated (Fig. 5A, Supplemental Table 1). Using the DAVID analysis program, we identified several KEGG pathways that appeared to be disregulated in Itk−/− V6 cells (Fig. 5B). These pathways were involved in cytokine signaling, some of which are required for the development of T cells, as well as effector functions, including molecules frequently associated with αβ NKT and NK cells. To confirm these findings at the protein level, we examined the expression of several NK cell receptors that can mediate effector function in αβ NKT cells. As shown, this analysis revealed that Itk−/− V6 cells have decreased or absent expression of CD122, NK1.1, 2B4, Ly49E/F, NKG2A/C/E, and CD94 compared with WT V6 cells (Fig. 6A, 6B). As acquisition of NK cell receptors occurs at the final stage of αβ NKT cell maturation (30, 32, 44), these data suggest that this developmental program is conserved for γδ NKT cells as well.

Given the similarity between αβ NKT cells and V6 cells, we then examined the relationship between Itk−/− V6 cells and several isolated subsets of iNKT cells as well as conventional CD4 and CD8 thymocytes, using the αβ NKT gene signature list generated previously (21). For this analysis, we compared the list of genes that were upregulated in αβ NKT cells versus naive conventional CD4 and CD8 thymocytes, along with genes that were downregulated in αβ NKT cells versus CD4 and CD8 thymocytes, to the 124 genes that were downregulated in Itk−/− V6 cells relative to WT V6 cells. Approximately 40% of the genes (51 genes) that were decreased in Itk−/− versus WT V6 cells are shared with the subset of genes normally upregulated in αβ NKT cells relative to conventional CD4 and CD8 T cells (Fig. 5C). In contrast, only ~5% of the genes (six genes) that were decreased in Itk−/− V6 cells are common to the genes normally downregulated in αβ NKT cells. These data indicate that the WT V6 cells are similar to WT αβ NKT cells and further that Itk−/− V6 cells are lacking a subset of the mature αβ NKT cell signature. Using cluster analysis and comparing the 51 genes that WT αβ NKT and Itk−/− V6 cells share in Fig. 5C, the heat map shows that WT HSAlo V6 cells are similar to mature WT αβ NKT cells, whereas the Itk−/− V6 cells are most similar to immature (NK1.1+) WT αβ NKT cells (Fig. 5D, Supplemental Table 2). Taken together with the phenotypic analysis, these data confirm that Itk−/− V6 cells are impaired in their terminal maturation in the thymus.

Itk−/− thymic γδ NKT cells are derived from both fetal and adult progenitors

Previous studies have demonstrated that peripheral V6 cells in WT mice arise from fetal progenitors that expand in number in the neonatal thymus and migrate out to populate peripheral tissues (22, 24). In contrast, the V6 cells found in adult Itk−/− liver included a subset that was clearly derived from adult progenitors. These findings suggested that Itk might be required to specifically limit the numbers of adult progenitor–derived V6 cells in the thymus; for example, Itk might be required to promote a TCR-dependent cell death signal, akin to the negative selection of conventional T cells. If this were the case, we would predict that thymic V6 cells in adult Itk−/− mice would be greatly enriched for the adult progenitor–derived population. In addition, we considered the possibility that PLZFhi versus PLZFint V6 cells might represent distinct V6 subsets. For instance, it was possible that PLZFint V6 cells represented the continued presence of fetal progenitor–derived V6 cells expressing the invariant TCR δ sequence, whereas the PLZFhi V6 cells might represent V6 cells developing in the adult thymus from adult progenitors.

To address these issues, we analyzed three populations of V6 cells from the thymus of WT and Itk−/− mice. To facilitate the
Gene expression microarray analysis indicates impaired terminal maturation of \( \text{Itk}^{-/-} \) γδ NKT cells. (A) Scatter plot showing expression of consolidated probe sets by CD24(HSA)\(^{lo}\) V6 γδ T cells in thymocytes from adult WT and \( \text{Itk}^{-/-} \) mice. Nomenclature is according to ImmGen (http://immgen.org): MatV6.WT = CD24(HSA)\(^{lo}\) V6 γδ T cells from adult WT thymus; TotalV6.Itk\(^{-/-}\) = total V6 γδ T cells from adult \( \text{Itk}^{-/-} \) thymus, which are 95–98% CD24\(^{lo}\). Each dot represents one gene (mean of all probe sets and mean of replicates); red and blue indicate genes with expression increased or decreased, respectively, by >2-fold \((p < 0.05, \text{Student } t \text{ test; coefficient of variation} < 0.5; \text{mean expression value} > 120 \text{ in one subset})\); numbers in parentheses above plot indicates total number of these genes. The numbers within the plot indicate the number of upregulated (red) or downregulated (blue) genes in the comparison. Data are from three independent experiments with 4–30 mice each. (B) The 124 genes increased in MatV6.WT versus TotalV6.Itk\(^{-/-}\) cells were classified into functional pathways using the KEGG analysis program (DAVID). The top five pathways enriched in the data set are shown along with the number of genes, the gene names, the \( p \) value, and the Benjamini \( p \) value for each pathway. (C) Venn diagram (Figure legend continues)
FIGURE 6. Decreased surface expression of NK cell markers on Itk−/− V6 cells. (A) Thymocytes from WT and Itk−/− mice were stained for expression of NK1.1 along with CD122, CD44, and CD4. (B) Thymocytes from WT and Itk−/− mice were stained for expression of NK cell markers CD49a (ITGA1), 2B4 (CD244), Ly49E/F (KLRA5/6), NKG2A/C/E (KLRC1/2/3), CD94 (KLRD1), and CD122. Dot plots show gated CD24loV6+ cells. Results are representative of two experiments with at least three mice per group.

isolation of distinct subsets, we used WT-4Get and Itk−/−-4Get thymocytes and sorted GFP+/CD122+ (stage 1), GFP+/−/CD122+ (stage 2), and GFP−/CD122− (stage 3) populations, taking advantage of the fact that GFP expression is correlated with PLZF expression in V6 cells (see Fig. 3). TCRδ sequence analysis indicated the presence of a highly diverse repertoire in each of the subsets and in both WT and Itk−/− mice (Supplemental Fig. 1). Furthermore, a significant proportion of the sequences from both WT and Itk−/− thymocytes of each subset used D61 and contained N region additions, indicating that these adult thymic V6 cells were not derived from fetal progenitors. Nonetheless, the invariant TCRδ sequence found in WT liver V6 cells was detected in each of the adult thymic V6 subsets, confirming the persistence of these fetal-derived cells in the adult thymus, as reported previously (22).

A detailed inspection of the data from WT thymocytes showed that, among the more mature V6 subsets (CD122+ stages 2 and 3), there was a reduced proportion of these fetal progenitor–derived sequences relative to their abundance in the less mature GFP+/−/CD122− subset, stage 1 (p < 0.0001 and p = 0.002, respectively; Fig. 7). This finding indicated selection against this invariant TCR during the maturation of adult thymic V6 cells. Although overall the pattern of TCRδ sequences among adult thymic Itk−/− V6 cells was similar to that seen in the WT adult thymus, we did observe an apparent lack of selection against the invariant fetal progenitor–derived sequence as Itk−/− V6 cells mature. These data indicated that the numerical increase in V6 cells in the Itk−/− thymus was not due to a preferential expansion of adult progenitor–derived cells but instead was the result of expansion of both fetal- and adult-derived V6 cell subsets. Furthermore, these data demonstrated that, in the WT thymus, V6 subsets distinguished by high or low PLZF expression and/or CD122 expression were not derived from different progenitors; instead, each population contained a mixture of cells of both origins. As a result, these data support the conclusion that V6 stages 1→2→3 represent a developmental lineage of γδ NKT cells.

Discussion
To understand the basis for the γδ T cell–dependent hyper-IgE syndrome that develops in the absence of Itk, we performed a detailed analysis γδ NKT cells in Itk−/− mice. Our previous studies demonstrated that this γδ T cell subset, expressing the Vγ1.1/Vδ6.3 (V6) TCR and the transcription factor PLZF, was highly expanded in Itk−/− mice and could secrete large amounts of type II cytokines, such as IL-4 and IL-13 (15). Using a combination of phenotypic, functional, and molecular analysis of V6 cells in the thymus, spleen, and liver, we have determined a number of important features of these cells. First, we show that the increased V6 population in Itk−/− mice is generally accounted for by cells expressing high levels of PLZF and constitutively expressing IL-4 mRNA. Second, based on a comparison with NKT cells, we conclude that Itk−/− V6 cells do not fully mature and cannot transit to the stage associated with high level IFN-γ production. Third, we provide evidence that Itk−/− V6 cells arising from adult progenitors are migrating to the liver, a process that is not permitted for WT V6 cells. Taken together, these findings reveal alterations in the V6 γδ NKT cell population present in Itk−/− mice could account for the hyper-IgE syndrome seen in these mice.

We found a number of striking parallels between V6 cells and γδ NKT cells that suggest a common developmental program shared between these two cell lineages. Recently, we found that mature WT thymic V6 cells and thymic γδ NKT cells express a common NKT cell gene expression signature (21). By examining changes in cell surface marker expression, PLZF levels, and cytokine

comparing the list of genes that were increased or decreased in γδ NKT (NKT-44′NKT1.1′) cells versus MatCD4 (CD24+), MatCD8 (CD24+) single-positive thymocytes (identified previously in Ref. 21) with the list of 124 genes downregulated in TotalV6.Itk−/− versus MatV6.WT cells. Roughly 40% of the genes decreased in TotalV6.Itk−/− versus MatV6.WT cells are normally increased in NKT-44′NKT1.1′ cells versus mature single-positives. (D) Heat map showing relative expression of the 51 genes identified in (C) that were decreased in TotalV6.Itk−/− versus MatV6.WT cells and increased in NKT-44′NKT1.1′ cells versus MatCD4 and MatCD8 single-positive thymocytes in populations of NKT and MatCD4 cells from WT mice and V6 cells from WT or Itk−/− mice. Data were log transformed, centered by gene row, and hierarchically clustered by gene and subset. The clustering dendrogram for samples is shown. Color-coding reflects relative expression levels of a given gene in each subset and does not provide information about absolute expression levels of each gene.
thermore, our TCR regulate CD4, and are dual producers of IFN-activation (CD44, CD122, and a host of NK receptors), down-relative to WT V6 cells. In addition, possibility is that competition exists between expand in the thymus during the first 2 wk after birth (22). These cells undergo a maturation program similar to maturing in the thymus of adult mice. Similar to WT V6 cells, preferentially home to the liver; however, increased numbers of V6 cells are found in the absence of Itk. A second wave of adult precursor-derived development program for these two subsets of V6 cells have a defect in terminal maturation and retain high expression of PLZF and IL-4 of a reduction in V6 cells also develops in increased numbers (blue).

FIGURE 8. A model of V6 development in WT and Itk−/− mice. In WT mice (left panel), V6 cells initially develop from fetal precursors (pink) and expand in the thymus during the first 2 wk after birth (22). These cells undergo a maturation program similar to αβ NKT cells before emigrating to the periphery, with a preference for homing to the liver (bold pink). As WT mice age, a second wave of V6 cells develops from adult precursors (blue) and matures prior to emigrating to peripheral lymphoid organs. A small number of fetal-derived and adult-derived V6 cells are present and continuously maturing in the thymus of adult mice. Similar to WT V6 cells, Itk−/− V6 cells (right panel) also initially develop from fetal precursors (pink) and preferentially home to the liver; however, increased numbers of V6 cells are found in the absence of Itk. A second wave of adult precursor-derived Itk−/− V6 cells also develops in increased numbers (blue). Itk−/− V6 cells have a defect in terminal maturation and retain high expression of PLZF and IL-4 relative to WT V6 cells. In addition, Itk−/− V6 cells arising from adult precursors are able to emigrate to the liver (green). E20, Embryonic day 20.
high levels of CCR9, whereas in WT mice expressing the self-ligand, a majority of these cells are CCR9− and CD122+. These findings provide a clear precedent for intrathymic TCR signaling-mediated maturation of γδ T effector subsets and support our conclusion that Itk−/− γδ NKT cells escape negative selection, do not efficiently upregulate CD122, and fail to downregulate CCR9 because of impaired TCR signaling. Our efforts to define a potential TCR-dependent ligand for V6 cells in the thymus have, to date, been largely unsuccessful. We find that V6 cells are not dependent on classical or nonclassical MHC class I or class II molecules for their development (Supplemental Fig. 2). Nonetheless, additional studies of genetically modified mice support the conclusion that V6 cell development is strongly influenced by TCR signaling. Specifically, mice deficient for Kruppel-like factor 2 (47) or inhibitor of DNA binding 3 (Id3) (17, 48), as well as mice expressing a mutant form of the SLP-76 adaptor protein (SLP76-Y145F) that is unable to bind to ITK (17, 49), have been analyzed. In each of these cases, the mice exhibit a similar phenotype to Itk−/− mice, characterized by a dramatic increase in the numbers of thymic V6 cells. Taken together, these data are consistent with the model that γδ NKT cells normally undergo negative selection because of robust TCR signaling in the thymus. In the absence of Itk, adult thymic γδ NKT cells are able to emigrate from the thymus. It is also possible that the invariant fetal-derived V6 cells also continue to emigrate from the thymus in adult Itk−/− mice (Fig. 8), although we cannot resolve this issue based on our current analysis. One possible explanation for the deregulation of V6 cell migration in the absence of Itk is a failure of Itk−/− V6 cells to downregulate chemokine receptors that may promote T cell trafficking to peripheral organs. Consistent with this notion, our microarray data indicate that, in addition to CCR9, CCR2 is more highly expressed in Itk−/− V6 cells compared with WT controls. Although CCR9 is one of the receptors that can induce homing to the gut as well as to other peripheral tissues, it is only modestly expressed on γδ NKT cells in the liver. In contrast, CCR2 is highly expressed in γδ NKT cells isolated from the liver and, interestingly, is most abundant in the CD4+ fraction of this population (http://www.immgen.org). These findings suggest that a failure to downregulate CCR2 as adult thymic γδ NKT cells mature could account for their continued ability to emigrate from the thymus and home to the liver.

Our findings also clarify some of the inconsistent data regarding the development of V6 cells in Id3−/− and Itk−/− mice. Previous reports demonstrated that similar to Itk−/− mice, Id3−/− mice had an increase in V6 cells (17, 48). We previously showed that reconstitution of WT mice with Itk−/− bone marrow cells resulted in donor-specific generation of the expanded V6 population (15), whereas reconstitution of WT mice with Id3−/− bone marrow failed to do so (18). This difference might arise if the Id3 deficiency selectively affected the expansion of the fetal progenitor-derived V6 cell population, whereas the lack of Itk led to an expansion of both V6 cell populations. In support of this interpretation, analysis of TCRβ sequences among adult thymic V6 cells from Id3−/− mice indicates that 84% of the cells are derived from fetal progenitors, a substantially higher proportion than seen in the adult Itk−/− thymus (18).

Recently, Lin28b has been found to play an essential role in programming fetal liver and fetal thymic hematopoietic progenitor cells to generate unique subsets of innate-like B and T lymphocytes (50). In particular, mice transplanted with Lin28b-transduced adult bone marrow stem cells developed a >10-fold increase in the numbers of PLZF+ αβ and γδ NKT cells relative to mice reconstituted with WT bone marrow cells. These important findings raise the formal possibility that Lin28b expression is dysregulated in mice that show expanded populations of PLZF+ innate T cells, such as Id3−/− mice. In the case of Itk−/− mice, altered regulation of the Lin28b pathway is unlikely to fully account for the increased number of V6 cells in the adult Itk−/− thymus, because we see a concomitant decrease in the numbers of αβ INKT cells. Thus, although there are likely distinct progenitor-specific programs controlling fetal versus adult NKT cell differentiation, Itk-dependent TCR signaling is an arbiter of the size of NKT cell populations and is required for their terminal maturation.

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Disclosures

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