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Subtle Defects in Pre-TCR Signaling in the Absence of the Tec Kinase Itk

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αβ T cell development in the thymus is dependent on signaling through the TCR. The first of these signals is mediated by the pre-TCR, which is responsible for promoting pre-T cell proliferation and the differentiation of CD4<sup>+</sup>CD8<sup>+</sup> (DN) thymocytes into CD4<sup>+</sup>CD8<sup>-</sup> (DP) cells. In many cases, T cell signaling proteins known to be essential for TCR signaling in mature T cells are also required for pre-TCR signaling in DN thymocytes. Therefore, it came as a surprise to discover that mice lacking the Tec kinases Itk and Rlk, enzymes required for efficient activation of phospholipase C-γ1 in mature T cells, showed no obvious defects in pre-TCR-dependent selection events in the thymus. In this report, we demonstrate that DN thymocytes lacking Itk, or Itk and Rlk, are impaired in their ability to generate normal numbers of DP thymocytes, especially when placed in direct competition with WT DN thymocytes. We also show that Itk is required for maximal pre-TCR signaling in DN thymocytes. These data demonstrate that the Tec kinases Itk and Rlk are involved in, but are not essential for, pre-TCR signaling in the thymus, suggesting that there is an alternative mechanism for activating phospholipase C-γ1 in DN thymocytes that is not operating in DP thymocytes and mature T cells. The Journal of Immunology, 2007, 179: 7561–7567.

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ultiple independent signaling pathways regulate T cell development in the thymus. The most important of these are signals mediated by the TCR and cytokine receptors. The first developmental checkpoint that requires signaling through the TCR is the progression of CD4<sup>+</sup>CD8<sup>-</sup>CD3<sup>-</sup> (DN) thymic progenitor cells to CD4<sup>+</sup>CD8<sup>+</sup> (double positive (DP)) immature thymocytes, a required step in the development of αβ<sup>+</sup> T cells. Following TCRβ chain gene rearrangement and expression, DN thymocytes assemble a "pre-TCR" complex consisting of TCRβ, the invariant pre-γ chain, and the signaling modules CD3ε, CD3γ, and TCRζ. Signals through this pre-TCR complex promote cell survival, proliferation, differentiation to the DP stage, and allelic exclusion of the TCRβ locus (for review, see Ref. 1).

To date, a number of proteins have been shown to be critical for optimal pre-TCR signaling. These include the Src family tyrosine kinases Lck and Fyn (2–6), as well as the Syk family kinases Zap-70 and Syk (7). Downstream of these kinases are several important adapter proteins, such as SLP-76 (8, 9), LAT (10), and Gads (11), which promote the activation of the Ras-MAPK and phospholipase C (PLC) γ pathways. Although induction of Ras-MAPK activation is sufficient to signal the survival, proliferation, and differentiation of DN thymocytes (12), this pathway has no apparent role in allelic exclusion (13). In contrast, activation of PLCγ appears to be critical for all pre-TCR-mediated events, as demonstrated by the profound block at the DN stage of thymocyte development observed in LAT knock-in mice that express a LAT protein lacking the phosphorylation site required for PLCγ binding (14, 15).

In mature T cells as well as thymocytes, efficient activation of PLCγ requires the Tec family tyrosine kinases Itk and Rlk (16–18). In light of these data and the evidence that PLCγ activity is important for pre-TCR signaling (14, 15), a role for Tec family kinases in pre-TCR signaling seems likely. Consistent with this notion, analysis of Tec kinase gene expression indicates that these genes are expressed in thymic progenitor cells. For instance, early studies performed using Northern blot analysis demonstrated that both Itk (19) and Rlk (20) mRNA can be detected in the thymus at day 14 of fetal development, a time at which the thymus is comprised solely of DN1 and DN2 precursor cells. However, preliminary analyses of thymic development in itk<sup>−/−</sup>, rlk<sup>−/−</sup>, and itk<sup>−/−</sup>rlik<sup>−/−</sup> mice surprisingly revealed normal proportions of both DN and DP thymocytes, suggesting that progression to the DP stage of development is not significantly altered by the absence of these Tec family proteins (18, 21).

In this report, we provide a more comprehensive analysis of early thymocyte development in mice lacking Itk or Itk and Rlk. Using a variety of approaches, we demonstrate that itk<sup>−/−</sup> and itk<sup>−/−</sup>rlik<sup>−/−</sup> DN thymocytes have subtle impairments in pre-TCR signaling. As a consequence, the efficiency with which these Tec kinase-deficient thymocytes progress from the DN to the DP stage of development is reduced compared with wild-type (WT) cells, particularly when itk<sup>−/−</sup> and itk<sup>−/−</sup>rlik<sup>−/−</sup> thymocytes are competing with WT cells to populate the DP compartment. Overall, however, the modest nature of these defects indicate that DN thymocytes have an alternative mechanism for PLCγ activation that plays a more important role at this early stage of thymocyte development than it does in DP thymocytes or mature peripheral T cells.
Materials and Methods

Mice

Iκκ-deficient mice were generated in our laboratory previously (16). Iκκ−/−/κκ−/− mice were a gift from Dr. P. Schwartzberg (National Institutes of Health, Bethesda, MD). Iκκ−/−/κκ−/− mice were backcrossed onto the C57BL/J10 (B10) background for more than nine generations. Iκκ−/−/κκ−/− mice were backcrossed onto the C57BL/6 (B6) background for more than five generations. WT mice used in these studies were Iκκ−/−/κκ−/− littermates of Iκκ−/−/κκ−/− mice or B10 mice purchased from The Jackson Laboratory. CD45.1 (B6) congenic mice were purchased from Charles River Laboratories at National Cancer Institute-Fredrick Animal Production Area (Fredrick, MD). Rag2−/− mice were purchased from The Jackson Laboratory. All mice used were between 6 and 12 wk of age and were maintained at the University of Massachusetts Medical School facility under specific pathogen-free conditions following Institutional Animal Care and Use Committee review.

Abs and flow cytometry

The following Abs and secondary reagents were purchased from BD Pharmingen: CD4-PE, CD8-Chrome (Cy), CD8-allophycocyanin, CD44-FITC, CD44-Cy, CD25-PE, CD3-biotin (bio), CD4-bio, CD6-bio, B220-bio, IgM-bio, Ter119-bio, Pan-NK(DX5)-bio, Gr1(Ly6G)-bio, Mac1(CD11b)-bio, CD11c-bio, streptavidin (streap-allophycocyanin). BrdU-FITC was purchased from BD Biosciences. To characterize the DN thymocyte subsets, lineage-positive cells were stained with biotinylated Abs to CD3, CD4, CD8, B220, IgM, Ter119, Pan-NK(DX5), Gr1(Ly6G), Mac1(CD11b), and CD11c, followed by staining with anti-CD25, antiCD44, and streap-allophycocyanin. All streap-allophycocyanin-positive events were gated out and the CD25 vs CD44 profiles of the remaining cells were determined. Cells (100,000–500,000 events) were collected on a FACS-Calibur (BD Biosciences) flow cytometer. Data were analyzed using both CellQuest (BD Biosciences) and FlowJo (Tree Star) programs.

Sorting of thymocyte subsets, mRNA isolation, and cDNA production

For isolation of subsets based on CD4 and CD8 expression, thymocytes were stained with anti-CD4-PE and anti-CD8-Cy and sorted into DN, DP, CD4+ single-positive (SP), and CD8+ SP populations. For DN1–DN4 subsets, thymocytes from 25 C57BL/6 mice were pooled and depleted with complement-fixing anti-CD4 and anti-CD8 Abs, followed by incubation with rabbit complement (Cedarlane Laboratories). Live cells were isolated on a Lympholyte-M (Cedarlane Laboratories) gradient and sorted as described above into DN1–DN4 subsets on a high-speed MoFlo cell sorter (DakoCytabm). RNA and cDNA were prepared from sorted cells (see previous published work) (22).

Real-time PCR

Real-time quantitative PCR was performed as described previously (22). All samples and standards were run in triplicate. The values of Itk, Rlk, Btk, Tec, and Schwartzberg were normalized to β-actin by dividing the above copy number of the respective transcript by the average copy number of β-actin transcripts in each sample. The PCR conditions were as follows: templates were initially denatured at 95°C for 10 min followed by 40 cycles of 95°C for 10 s, (62°C, β-actin), (60°C, Itk), (61.7°C, Rlk), (60°C, Btk), or (54°C-Tec) for 20 s and 72°C for 20 s. Primers were: Itk-1, 5′-CTC CGAT CCA GTT TTC TTC C-TCC-3′; Itk-2, 5′-GTC CTT GGT GAG CCA GTA GCC C-3′; Rlk-1, 5′-TCA ATA CCA AGG AGG C-3′; Rlk-2, 5′-CTG TCA GTG CCA A-3′; Tec-1, 5′-GTT TGG AGT GGT GAG GTCT T-3′; Tec-2, 5′-GAT AAC GAT GTA GAG GCT G-3′; Btk-1, 5′-CCG CTC TCT TCA GTG CCA A-3′; Tec-1, 5′-GTT TGG TGT AGT GTG GAG GCT T-3′; Tec-2, 5′-GAT AAC GAT GTA GAG GCT G-3′; Btk-1, 5′-CCG GGT TGG TGT GTG GCT GCA GAG GCT T-3′; Btk-2, 5′-GGT AAT ACT GGC TCT GGT G-3′; β-actin 1, 5′-CCA ACC CCA GCA GAG AGG AGG C-3′; and β-actin 2, 5′-CCA GGT TGG TGC TTA GGG TTC AGG GC-3′. The distribution of the four major DN thymocyte subsets (DN1: lin−CD44−CD25−; DN2: lin−CD44+CD25−; DN3: lin−CD44−CD25+; and DN4: lin−CD44+CD25+) was comparable for thymocytes derived from WT, itk−/−, and itk−/−/κκ−/− BM. In addition, we used cells containing cDNA clones of Btk, Rlk (gift from P. Schwartzberg, National Institutes of Health), Btk (gift from M. Schmidt, National Institutes of Health), and 23.9 ml ddH2O) and incubated at room temperature for 10–30 min. Cells were then washed and incubated in 50 μl of a 1/10 dilution of anti-BrdU-FITC for 30 min at room temperature and analyzed by flow cytometry.

Injection of rag2−/−mice with anti-CD3 Ab

Iκκ−/−rag2−/− and Iκκ−/−/κκ−/−rag2−/− mice were injected i.p. with 10 μg of purified anti-CD3ε (2C11) Ab from ebioscience. Thymocytes were analyzed at days 4 and 6 following injection for CD4, CD8, CD25, and CD44 expression by flow cytometry.

Results

Analysis of DN thymocyte subsets in WT, itk−/−, and itk−/−/κκ−/− mice

Initial studies of thymocytes in itk−/− and itk−/−/κκ−/− mice indicated that T cell development proceeds relatively normally in the absence of these kinases with the exception of an altered CD4:CD8 ratio (Fig. 1A), as previously reported (16, 18, 21, 23). In addition, previous studies also revealed a small, but reproducible reduction in total DP thymocyte numbers (~30% reduction in itk−/−, and ~20% reduction in itk−/−/κκ−/− DP thymocytes compared with WT, respectively), but no difference in DN thymocyte numbers (18). These findings were somewhat surprising in light of the predicted role of Tec family kinases in pre-TCR signaling. Therefore, we chose to perform a more detailed analysis of the early stages of αβ T cell development in mice lacking Itk or Itk and Rlk.

To examine the development of thymocytes lacking Itk or Itk and Rlk, we generated BM chimeras in which CD45.2 WT BM cells were mixed with 5×105 CD45.1 WT, itk−/−, or itk−/−/κκ−/− BM cells. Itk−/− and itk−/−/κκ−/− BM were used to reconstitute irradiated WT-congenic mice (CD45.1+). This approach was used to eliminate potential caveats introduced by Itk- or Itk/Rlk-deficient non-hemopoietic cells. Thymocytes from these BM chimeric mice were then used to analyze the early stages of T cell maturation. For this analysis, lineage-positive cells were first excluded with a panel of Abs (see Materials and Methods for details) and the remaining CD45.2+ thymocytes were analyzed for CD44 and CD25 expression. As shown in Fig. 1B, the distribution of the four major DN thymocyte subsets (DN1: lin−CD44−CD25−; DN2: lin−CD44+CD25−; DN3: lin−CD44−CD25+; and DN4: lin−CD44+CD25+) is comparable for thymocytes derived from WT, itk−/−, and itk−/−/κκ−/− progenitors. These findings indicate that, at a first approximation, both Itk and Rlk are largely dispensable for pre-TCR signaling.

To further assess the efficiency of pre-TCR signaling, we examined the up-regulation of CD5 on DN thymocytes from intact itk−/− and itk−/−/κκ−/− mice. CD5 is a cell surface receptor that negatively regulates TCR signaling; previous studies have demonstrated that CD5 is up-regulated following pre-TCR signaling and that the magnitude of CD5 up-regulation is correlated with the strength of TCR signaling (24, 25). As shown in Fig. 1C, WT thymocytes up-regulate CD5 following the transition from the DN3 to the DN4 stage; in contrast, itk−/− or itk−/−/κκ−/− thymocytes are impaired in their ability to up-regulate CD5 at the DN4 stage. This defect is not greatly exacerbated by the loss of Rlk in addition to Itk. Overall, these findings strongly suggest that pre-TCR signaling is reduced in the absence of Itk.
Expression of Itk, Rlk, Tec, and Btk in adult DN thymocyte subsets

In light of the somewhat surprising finding that the early stages of thymocyte development appear to proceed normally in the absence of both Itk and Rlk, we investigated the expression levels of the four Tec family kinases found in lymphocytes (Itk, Rlk, Tec, and Btk). Due to the scarcity of the DN thymocyte populations, which makes direct protein analysis difficult, we examined this issue using real-time quantitative RT-PCR to assess Itk, Rlk, Tec, and Btk mRNA levels in purified DN thymocytes.

Analysis of the four major thymocyte subsets, as defined by CD4 and CD8 expression, demonstrated that Itk, Rlk, and Tec mRNA are detectable in all subsets, Btk mRNA is not detectable in any thymocyte subset except for the DN population, where it is found to be barely above the level of detection (Fig. 2A). Itk mRNA shows the highest level of expression, followed by Rlk, while Tec mRNA is substantially less abundant (~40-fold lower than Itk and Rlk mRNA). The levels of transcripts for each kinase are comparable in DP thymocytes and the two SP subsets, but with the exception of Tec, appear slightly reduced in the DN subset. However, in this analysis, the sorted DN cells were not depleted of all lineage-positive cells and, thus, are not comprised solely of T-lineage cells. Therefore, the levels of Itk and Rlk mRNA in this subset may be artificially reduced by the presence of non-T-lineage cells that do not express these kinases.

To more carefully assess the expression of Itk, Rlk, Tec, and Btk in thymic progenitor cells, we purified the four subsets of DN thymocytes, as defined above. Real-time PCR analysis indicated that Itk, Rlk, and Tec mRNA are expressed in each DN subset, while Btk mRNA is only detected in the DN1 subset, possibly due to the fact that DN1 cells are not yet committed to the T cell lineage (Fig. 2B). In these thymocyte subsets, Itk mRNA is also the most abundant of the four Tec kinases analyzed. Overall, these data indicate that Itk, Rlk, and Tec transcripts are present in all thymocyte subsets based on expression of CD4 and CD8. Levels of Itk, Rlk, Tec, and Btk mRNA were determined by real-time quantitative PCR. Data are normalized to $\beta$-actin mRNA levels in each sample. Results shown are representative of three independent experiments.

T-cells are generated by the interaction of the T cell receptor (TCR) with an appropriate antigen-presenting cell (APC). The TCR is a heterodimeric protein composed of two polypeptide chains, a and $\beta$, and these chains are expressed on the surface of T cells. The TCR recognizes and binds to specific peptides presented by the MHC molecule, which is important for the activation and differentiation of T cells.

Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ thymic progenitor cells demonstrate impaired developmental potential when competing with WT progenitor cells

To assess more rigorously the ability of Tec kinase-deficient thymocyte progenitors to generate normal numbers of DP thymocytes, we examined the developmental potential of these cells when placed in direct competition with WT progenitor cells. For this experiment, mixed BM chimeras were generated with a 50:50 mix of WT (CD45.1$^+$) and Itk$^{-/-}$ (CD45.2$^+$) BM cells or WT (CD45.1$^+$) and Itk$^{-/-}$Rlk$^{-/-}$ (CD45.2$^+$) BM cells. As controls, we generated chimeras with homogeneous populations of BM cells (i.e., WT, Itk$^{-/-}$, and Itk$^{-/-}$Rlk$^{-/-}$). To assess the mixing of the BM in the WT plus Itk$^{-/-}$ (WT:Itk$^{-/-}$) chimeras and the WT plus Itk$^{-/-}$Rlk$^{-/-}$ (WT:Itk$^{-/-}$Rlk$^{-/-}$) chimeras, chimeraizm in the B cell compartment was examined, and found to be close to 50:50 (see Fig. 3 legend). The individual thymocyte subsets were then normalized to the proportion of B cells of that genotype in each individual mouse (i.e., for each WT:Itk$^{-/-}$ chimera, the percentage of WT DN thymocytes was divided by the percentage of WT B cells, and the percentage of Itk$^{-/-}$ DN thymocytes was divided by...
the percentage of \( \text{itk}^{-/-} \) B cells; a similar analysis was done for \( \text{WT:itk}^{-/-} \) chimeras.

As shown in Fig. 3A, WT and the \( \text{itk}^{-/-} \) B cells contribute equally to the DN compartment in mixed WT:itk\((/-) \) chimeric mice. However, as cells transition from DN4 to the DP stage, the proportion of \( \text{itk}^{-/-} \) cells decreases relative to the WT cells present in the same thymus. These data indicate that Itk plays some role in signaling the transition from the DN to the DP stage. Analysis of the WT:itk\((/-) \) chimeras is more complex, as the \( \text{itk}^{-/-} \) cells show a strong advantage over WT cells in populating the DN compartment (Fig. 3B). This advantage of the \( \text{itk}^{-/-} \) cells persists through the DN4 stage. Nonetheless, as cells progress from the DN4 to the DP stage, the \( \text{itk}^{-/-} \) cells show an impaired ability to populate the DP compartment, as their relative ratio to WT cells decreases. Thus, the \( \text{itk}^{-/-} \) cells, similar to the \( \text{itk}^{-/-} \) cells, are less efficient at generating DP thymocytes from the starting population of DN cells present than are the WT cells. These findings indicate that Itk and Rlk are required for efficient generation of DP thymocytes from early thymic progenitors, suggesting a role for these kinases in pre-TCR signaling. Interestingly, this function seems to be largely dependent on Itk, as the developmental defect seen in mixed BM chimeras made with \( \text{itk}^{-/-} \) cells is no more severe than that seen with \( \text{itk}^{-/-} \) cells.

**FIGURE 3.** Reduced contribution of \( \text{itk}^{-/-} \) and \( \text{itk}^{-/-} \) \( \text{itk}^{-/-} \) } chimeras were generated with 50:50 mixtures of WT (CD45.1\(^{+}\)) and \( \text{itk}^{-/-} \) BM (A) or WT (CD45.1\(^{+}\)) and \( \text{itk}^{-/-} \) BM (B). Thymocytes were stained with Abs to CD4 and CD8 to define the subsets of CD4\(^{+}\)CD8\(^{-}\) (DN), CD4\(^{+}\)CD8\(^{+}\) (DP), and CD4\(^{-}\)CD8\(^{+}\) (CD4) cells and the proportion of CD45.1\(^{+}\) and CD45.1\(^{-}\) cells in each subset were determined. To determine the DN2/DN3 and DN4 subsets, Lin\(^{-}\) cells and the proportion of CD45.1\(^{+}\) were analyzed for CD25 and CD45.1 expression. Chimerism in the B cell compartment was determined by staining splenocytes and was near 50:50. Specifically, for the WT:itk\((/-) \) chimeras, the mean percentage of WT cells was 57 \( \pm \) 1.9\%, \( n = 18 \); for the WT:itk\((/-) \) \( \text{itk}^{-/-} \) \( \text{itk}^{-/-} \) \( \text{itk}^{-/-} \) B cells were analyzed for CD25 and CD45.1 expression. Chimerism in the B cell compartment was determined by staining splenocytes and was near 50:50.

**FIGURE 4.** Proliferation of thymocytes in mixed BM chimeras. BM chimeras were generated with 50:50 mixtures of WT (CD45.1\(^{+}\)) and \( \text{itk}^{-/-} \) BM (A) or WT (CD45.1\(^{+}\)) and \( \text{itk}^{-/-} \) BM (B). Mice were injected i.p. with 2 mg of BrdU and thymocytes were harvested 90 min later. After harvest of thymocytes, cells were stained with Abs to CD45.1, CD4, and CD8, fixed, permeabilized, treated with DNase, and then stained with anti-BrdU Ab and analyzed by flow cytometry. Dot plots show gated BrdU\(^{+}\) cells of WT (CD45.1\(^{+}\)) and \( \text{itk}^{-/-} \) (CD45.1\(^{-}\)) origin (A) or WT (CD45.1\(^{+}\)) and \( \text{itk}^{-/-} \) (CD45.1\(^{-}\)) origin (B) analyzed for CD4 vs CD8 expression. Data are representative of 9 WT:itk\((/-) \) and 10 WT:itk\((/-) \) mixed BM chimeras analyzed.

**Impaired accumulation of DP thymocytes in the absence of Itk and Rlk**

As a more direct measure of the consequences of pre-TCR signaling, we examined the extent of proliferation induced in DN and DP thymocytes in the mixed BM chimeric mice. For these experiments, WT:itk\((/-) \) and WT:itk\((/-) \) chimeric mice were injected with BrdU and thymocytes were harvested 90 min later. As shown in Fig. 4 and Table I, BrdU\(^{+}\) thymocytes of WT origin were predominantly DP, with relatively few cells in the immature compartments (DN and immature SP (CD3\(^{-}\)CD8\(^{-}\))). In contrast, BrdU\(^{+}\) thymocytes lacking Itk or Itk and Rlk consisted of a higher proportion of immature cells and a reduced proportion of DP cells. Independent staining of thymocytes from BrdU-injected mice confirmed that BrdU\(^{+}\) CD4\(^{-}\)CD8\(^{-}\) thymocytes are TCR/CD3 negative and, thus, not mature CD8 SP cells (data not shown). When data from 9 WT:itk\((/-) \) and 10 WT:itk\((/-) \) mixed BM chimeras were averaged, we found a 2-fold difference in the ratio of immature BrdU\(^{+}\) (DN + immature SP) thymocytes to BrdU\(^{+}\) DP thymocytes when comparing WT to Tec kinase-deficient cells (Table I).

These data indicate one of several defects in the maturation and expansion of \( \text{itk}^{-/-} \) and \( \text{itk}^{-/-} \) progenitor thymocytes. First, it may be that the rate of proliferation of Tec kinase-deficient cells in response to pre-TCR signaling is reduced compared with WT cells; alternatively, \( \text{itk}^{-/-} \) and \( \text{itk}^{-/-} \) DN thymocytes may have a defect in differentiation to the DP stage. Finally, it may be that the \( \text{itk}^{-/-} \) and \( \text{itk}^{-/-} \) DN thymocytes that progress to the DP stage have impaired survival. Because we detected no increase in the proportion of \( \text{itk}^{-/-} \) or \( \text{itk}^{-/-} \) DP thymocytes showing evidence of programmed cell death compared with WT DP cells (data not shown), we do not favor the latter explanation.
Instead, these data indicate that \textit{itk} \textsuperscript{−/−} and \textit{itk} \textsuperscript{−/−} \textit{rlk} \textsuperscript{−/−} thymocytes undergo fewer rounds of proliferation as they progress to the DP stage, or that these cells are impaired in their ability to differentiate into DP cells. At the current time, we are unable to distinguish between these two possibilities due to the variability in absolute thymocyte cellularity and variability in overall BrdU incorporation between individual chimeric mice.

Pre-TCR signaling in vivo is impaired in the absence of Itk

To directly assess pre-TCR signaling in the absence of Itk, we used a well-established assay in which \textit{rag2} \textsuperscript{−/−} thymocytes are induced to expand and differentiate into DP cells following in vivo injection of anti-CD3 Ab (26). For these experiments, \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} and \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} mice were injected with 10 μg of anti-CD3e Ab and then analyzed on days 4 and 6 following injection. As shown in Fig. 5, \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} mice develop fewer DP thymocytes than \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} mice in response to anti-CD3 stimulation in vivo. Upon analysis of DP thymocyte percentages, as well as absolute numbers, we find that this difference is statistically significant on day 4 (Fig. 5B). By day 6 postinjection, the DP thymocyte numbers are more variable between individuals of the same genotype and differences between the \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} and the \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} mice are not statistically significant. Analysis of the DN thymocyte subsets using CD44 vs CD25 staining show a modest increase in the percentage of DN4 cells in the anti-CD3-injected \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} mice compared with the \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} mice, further suggesting a slight block in the transition from the DN4 to the DP stage when Itk is absent. These data directly demonstrate that pre-TCR signaling is modestly impaired in the absence of Itk.

**FIGURE 5.** Reduced response of \textit{itk} \textsuperscript{−/−} DN thymocytes to direct pre-TCR triggering. \textit{Itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} and \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} mice were injected with 10 μg of anti-CD3 Ab and analyzed 4 and 6 days later. A. Dot plots show CD4 vs CD8 (top row, total thymocytes) and CD25 vs CD44 (bottom row, gated on CD4+CD8− cells) staining of thymocytes analyzed 4 days postinjection compared with an un.injected \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} control. Numbers indicate the percentage of DN and DP thymocytes (top row) and DN1 through DN4 cells (bottom row). B. DP thymocyte percentages (left panel) and absolute numbers (right panel) for all \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} and \textit{itk} \textsuperscript{−/−} \textit{rag2} \textsuperscript{−/−} mice analyzed at days 4 and 6 postinjection are shown. The numbers of mice in each group are indicated below. Uninjected controls had no detectable DP thymocytes. Statistical significance was tested using the Mann-Whitney non-parametric t test. Asterisks denote statistically significant differences, \( p < 0.05 \).
Discussion

Previous studies have implicated PLCγ in pre-TCR signaling and have indicated a role for this pathway in all TCRβ-mediated selection processes, including survival, proliferation, differentiation, and allelic exclusion (14, 15). Because the Tec family kinases Itk and Rlk are critical for PLCγ activation in DP thymocytes and mature T cells, a role for Itk and Rlk in pre-TCR signaling seemed logical. However, initial characterization of the DN, DP, and SP thymocyte subsets in both itk−/− and itk−/−/rlk−/− mice indicated no obvious defects in T cell development before the stages of TCR repertoire selection and CD4/CD8-lineage commitment (16–18, 21). This seemed surprising given the fact that thymocytes deficient in Lck, LAT, or SLP-76, all of which are required for Itk activation, show a developmental block at the DN3 to DN4 transition (5, 6, 8–10, 27).

Based on the lack of overt defect in pre-TCR signaling in itk−/− mice, we then considered whether the absence of Itk leads to defects similar to those seen in vav−/− mice, which have a defect at the DN to DP transition without a complete block at the DN3 stage. For instance, vav−/− mice have a 2-fold decrease in the DP subset, resulting in a 2-fold reduction in thymic cellularity compared with WT mice (28, 29). In addition, detailed analysis of DN subsets in vav−/− mice revealed an increased ratio of DN3:DN4 thymocytes, suggesting a mild impairment in pre-TCR signaling (28). Previous analysis by Schaeffer et al. (18) also had demonstrated that itk−/− mice have a reduction in the total number of DP thymocytes, although the magnitude of this reduction is substantially smaller than that seen in vav−/− mice. Another point of distinction is that itk−/−/rlk−/− thymocytes, unlike vav−/− thymocytes, show no obvious alterations in the proportions of cells comprising the four DN thymocyte subsets.

We considered the possibility that the surprising absence of alterations in the DN thymocyte subsets in mice lacking Itk could be due to compensation by additional Tec family kinases expressed in these cells. Based on quantitative real-time PCR analyses, we found that both Rlk and Tec transcripts are present in all four DN thymocyte subsets. Similar to peripheral T cells (30), DN thymocytes have high levels of Itk mRNA, intermediate levels of Rlk mRNA, and very low levels of Tec mRNA. Because Rlk seemed most likely to compensate for the absence of Itk, we also examined the early stages of T cell development in mice lacking both Itk and Rlk. Similar to the findings in itk−/− mice, DN thymocyte subsets appear totally normal in itk−/−/rlk−/− mice. In fact, in contrast to the effects of a double deficiency in Itk plus Rlk on TCR signaling in DP thymocytes and peripheral T cells, the loss of Rlk does not appear to further impair the modest defects in pre-TCR signaling seen in the absence of Itk.

We then addressed this issue by generating mixed BM chimeras. Lethally irradiated WT mice were reconstituted with a 1:1 mixture of WT and itk−/− or itk−/−/rlk−/− BM cells. In this approach, a modest defect in pre-TCR signaling in the absence of Tec family kinases might be revealed when the cells are placed in competition with WT cells. Preliminary analysis of the contribution of each BM type to the DN and DP subsets revealed that significantly fewer cells of itk−/− origin contributed to the DP compartment compared with WT cells, suggesting that the DN to DP transition is less efficient in the absence of Itk; however, we still did not observe any differences in the DN subsets present when comparing the two types of progenitors present in the same chimeric animal. Analysis of the WT and itk−/−/rlk−/− chimeras was more complicated due to a reproducible advantage of itk−/−/rlk−/− cells over WT cells in reconstituting the DN compartment. Although we currently have no explanation for the enhanced progenitor activity of itk−/−/rlk−/− BM cells, these experiments nonetheless indicated that the itk−/−/rlk−/− cells in the mixed chimeras showed a relative deficit in contributing to the DP compartment compared with their contribution at the DN stage. Thus, the defect in thymocyte development caused by the absence of Itk is most evident in cells at the DN4 to DP stage of maturation.

Additional evidence supports the conclusion that itk−/− and itk−/−/rlk−/− cells are receiving weaker pre-TCR signals during β selection, a process that is initiated at the DN3 stage of development. First, CD5 up-regulation at the DN4 stage is impaired in itk−/− and itk−/−/rlk−/− thymocytes relative to WT cells. Similarly, BrdU incorporation data indicate that fewer of the proliferating itk−/− and itk−/−/rlk−/− thymocytes have progressed to the DP stage of development, again compared with WT cells in the same individual animal. Finally, and most importantly, anti-CD3 Ab injection into itk−/−/rag2−/− mice produces fewer DP thymocytes than are seen in itk−/−/rag2−/− mice.

Interestingly, our data indicate that the overall extent of proliferation is normal following pre-TCR signaling in the absence of Tec family kinases (Table I), suggesting that reduced accumulation of DP thymocytes results from a defect at a subsequent stage. Given the nature of the BrdU assay, it is impossible to determine whether the alterations in thymocyte subsets seen by examining BrdU+ cells is due to altered proliferation, survival, or differentiation. Previous studies have shown that the DN to DP transition requires sustained signaling in order for cells to complete β selection and that the process takes 24 h (31). Because Tec kinase-deficient thymocytes are unable to maintain a sustained Ca2+ response to TCR stimulation (18), it is possible that the sustained signaling required to complete differentiation does not occur in as many developing cells in the absence of Itk and Rlk.

Overall, these findings raise a question about the mechanism of PLCγ1 activation in DN thymocytes. If Itk and Rlk are both dispensable for this process, it is likely that an alternative tyrosine kinase is providing this function. One possibility is Tec, the third Tec family kinase expressed in T cells. Although tec−/− mice have no overt defects in thymic development (32), it still remains possible that Tec can compensate for the absence of Itk and Rlk. Alternatively, PLCγ activation in DN thymocytes may occur by a distinct pathway that is less tyrosine phosphorylation dependent or is mediated by another tyrosine kinase, such as a member of the Src or Syk family. Finally, it is possible that pre-TCR signaling is less dependent on PLCγ activation than signaling in mature T cells. Our efforts to detect PLCγ activation in WT DN thymocytes triggered by anti-CD3 Ab cross-linking were unsuccessful, despite a readily detectable signal in peripheral T cells (data not shown). It is likely that this latter issue will only be resolved by the generation of a T cell-specific conditional knockout of PLCγ1.

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Disclosures

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