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The Absence of Itk Inhibits Positive Selection Without Changing Lineage Commitment

Julie A. Lucas, Luana O. Atherly, and Leslie J. Berg

The Tec family tyrosine kinase Itk is critical for efficient signaling downstream of the TCR. Biochemically, Itk is directly phosphorylated and activated by Lck. Subsequently, Itk activates phospholipase C-γ1, leading to calcium mobilization and extracellular signal-regulated kinase/mitogen-activated protein kinase activation. These observations suggested that Itk might play an important role in positive selection and CD4/CD8 lineage commitment during T cell development in the thymus. To test this, we crossed Itk-deficient mice to three lines of TCR transgenics and analyzed progeny on three different MHC backgrounds. Analysis of these mice revealed that fewer TCR transgenic T cells develop in the absence of Itk. In addition, examination of multiple T cell development markers indicates that multiple stages of positive selection are affected by the absence of Itk, but the T cells that do develop appear normal. In contrast to the defects in positive selection, CD4/CD8 lineage commitment seems to be intact in all the TCR transgenic itk−/− lines tested. Overall, these data indicate that altering TCR signals by the removal of Itk does not affect the appropriate differentiation of thymocytes based on their MHC specificity, but does impact the efficiency with which thymocytes complete their maturation process. The Journal of Immunology, 2002, 168: 6142–6151.

The development of mature T cells from thymic precursors is mediated in large part by signals downstream of TCR complexes. The largest population of immature T cells in the thymus are double positive (DP) for the coreceptors CD4 and CD8, have productive TCR α- and β-chain gene rearrangements, and therefore express a mature TCR signaling complex on their surface. At this stage in development, thymocytes must undergo two concurrent, but distinct, developmental processes to become functional and tolerant mature T cells. One of these processes is known as repertoire selection, which ensures that mature T cells can recognize foreign peptides presented by self-MHC molecules, but will not become activated when they encounter self-peptide/self-MHC complexes. The second process, lineage commitment, is the process by which thymocytes that recognize peptide in the context of MHC class I become CD8+ cytotoxic T cell precursors, and thymocytes that recognize peptide in the context of MHC class II molecules become CD4+ Th cell precursors. Both these processes are known to be dependent on TCR signaling; however, it has often been debated how the same initial signals can give rise to these different and opposing developmental outcomes.

Current theories propose that the strength of the signal received by a DP thymocyte through its TCR and/or coreceptor can determine the developmental fate of the thymocyte with respect to both selection and lineage commitment. In the case of selection, it is believed that the avidity of the TCR for self-peptide/self-MHC complexes in the thymus will influence the intensity of the TCR signal. Thus, thymocytes that weakly bind self-peptide/self-MHC complexes receive weak signals that induce positive selection and the generation of T cells that would be useful to the individual. In contrast, if the thymocytes bind strongly to self-peptide/self-MHC complexes, a strong signal would be transduced, leading to negative selection and the elimination of potentially autoreactive cells. Consistent with this idea, there is extensive evidence that both TCR avidity for self-peptide/self-MHC and TCR signal strength influence the repertoire selection process (1). Further, it has recently been demonstrated that there is a clear link between TCR-MHC/peptide avidity and the ensuing strength of the TCR signal (2, 3).

The events or signals that direct thymocytes into a specific lineage have been more difficult to dissect. Initial studies focused on the CD4 and CD8 coreceptors, including efforts to identify unique signaling molecules/events downstream of each of these coreceptors; however, little progress was made from these approaches. More recent data indicate that alterations in the activities of certain signaling molecules downstream of the coreceptor/TCR may influence lineage commitment. Although still somewhat controversial, it has been shown that both the activity of Lck (4, 5) and the extracellular signal-regulated kinase (Erk)/mitogen-activated protein kinase (MAPK) signaling pathways (6–8) affect lineage commitment, with high levels of both of these signals leading to CD4 commitment and low levels of these signals leading to CD8 commitment. These studies are consistent with early data indicating that the cytoplasmic tail of CD4 binds more efficiently to Lck that of CD8 (9–12). Putting these observations together, one popular model of lineage commitment suggests that when thymocytes recognize class II MHC/peptide complexes, engaging both the TCR and CD4, stronger Lck signals would be induced compared with engagement of TCR and CD8 during recognition of MHC class I/peptide complexes. In addition, it has been shown that agonist signals through the TCR induce CD4 development, whereas antagonist signals induce CD8 development (13, 14). Thus, similar
to repertoire selection, lineage commitment may also be dependent on the strength of TCR plus coreceptor signaling.

In this study we were interested in determining the role of the Tec family tyrosine kinase, Itk, in both the processes of TCR repertoire selection and lineage commitment. Work from our laboratory and others has suggested that Itk is important for efficient TCR signaling. Studies in peripheral T cells have determined that cross-linking of the TCR in Itk-deficient cells results in reduced PLC-γ phosphorylation, reduced inositol trisphosphate generation, no sustained Ca2+ flux, and reduced translocation of NFAT into the nucleus (16). In addition, Itk has been suggested to be important for full activation of the Erk/MAPK signaling pathway (17). From these studies it appears that although Itk is not required for the activation of these signaling pathways, in its absence, signals downstream of the TCR are reduced. Therefore, it seemed likely that both selection and lineage commitment would be altered in the absence of Itk.

The analysis of itk−/− mice crossed to either a class I- or a class II-restricted TCR transgenic line revealed that virtually no TCR transgenic T cells developed in the absence of Itk (18). Based on this original observation and recent studies with these same TCR transgensics crossed to both itk−/− and rlk−/− itk−/− mice, which lack an additional Tec family member, it has been proposed that Tec family kinases play a role in setting thresholds for thymocyte development (19). To better understand the role of Tec family members in thymocyte development, we crossed itk−/− mice to additional TCR transgenic lines that express TCRs with different avidities for their selecting ligands in the thymus. These mice would allow us to determine whether the requirement for Tec family signals is influenced by the avidity of the TCR on the developing thymocyte. In addition, these mice provide the opportunity to determine whether signaling thresholds for both selection and lineage commitment are shifted in the absence of Itk.

Materials and Methods

Mice

Itk-deficient mice were generated in our laboratory previously (15). These mice were crossed to 2B4 (20), 5C.C7 (21), and AND (22) TCR transgenic mice, all of which have been on the B10.BR (H-2d) genetic background. The itk−/− mice crossed to the TCR transgenic mice had a mixed B10 × B10 genetic background (H-2d). Since the 129 strain carries two superantigens that delete Vβ3-expressing T cells, a PCR screen was designed to distinguish mice that carried at least one SAγ from those that were SAγ negative. SAγ-negative mice were used for all experiments. The nontransgenic itk−/− and itk+/− mice in all figures were backcrossed to B10 mice at least eight times. Mice were analyzed between 6 and 12 wk of age, and all TCR transgenic control mice were itk−/− littermates. Neonates were analyzed 22–40 h postbirth. When examined, there were no significant phenotypic differences in the lymphocyte profiles of TCR transgenic itk−/− and itk+/− mice (data not shown). Mice were breed and maintained in a specific pathogen-free facility.

Antibodies

The following mAbs, purchased from BD Pharmingen (San Diego, CA), were used for staining cells: anti-CD4-PE (H129.19), anti-CD4-CyChrome (anti-CD4-Cy; H129.19), anti-CD8e-FTTC (53-6.7), anti-CD8e-Cy (53-6.7), anti-CD8e-allophycocyanin (53-67), anti-TCRαβ-Cy (H57-597), anti-CD3e-PE (K25), anti-CD4-Cy (53-7.3), anti-CD45-bio (H1.2F3), anti-CD24 (heat-stable Ag (HSA))-bio (M1/69), purified hamster anti-mouse Bcl-2 (3F11), and purified hamster anti-trinitrophenol (107.3). Hamster Abs were detected by goat F(ab′)2 anti-hamster IgG (H+L)-FITC (Caltag Laboratories, Burlingame, CA). In most cases the 2B4 TCR transgenic was detected by staining with the A2B4-2 Ab (23), which was purified and conjugated to FITC in our laboratory. Biotinylated Abs were detected using streptavidin-allophycocyanin (BD Pharmingen).

Cell preparation, staining, and flow cytometry

Lymphocyte cell suspensions were made from thymus, spleen, and lymph nodes (LNs; pooled inguinal, axillary, brachial, and cervical) by dissociation between two frosted slides in RPMI and 10% FCS. RBC were lysed by incubation in Tris-ammonium chloride for 5 min at room temperature. Cells were washed, resuspended in FACS buffer (1 × HBSS, 2% FCS, and 0.01% NaN3), and plated in microwell staining plates at 5 × 105 to 2 × 106 cells/well. Biotinylated Abs were added, and cells were incubated on ice for 15–30 min. The cells were washed and incubated with a mixture of directly conjugated Abs and strepavidin-allophycocyanin on ice for 30 min. After washing, cells were fixed by the addition of 30 μl 4% paraformaldehyde. Fixed cells were analyzed 12–36 h later on a FACSCalibur (BD Biosciences, Mountain View, CA) flow cytometer. In general, 10,000–50,000 live (based on forward vs side scatter profiles) events/sample were collected for the analysis of CD4, CD8, and TCR staining in LN, spleen, and thymus preparations. For marker analysis on thymic subsets 100–200,000 events/sample were collected. Data were analyzed using CellQuest software (BD Biosciences).

Intracellular Bcl-2 staining

Cells were prepared and stained with Abs to cell surface molecules as described above. After extracellular staining, the cells were washed and fixed/permeabilized by incubation in 100 μl Cytofix/CytoPerm (BD Pharmingen) on ice for 20 min. The samples were then washed with Perm/ Wash buffer (BD Pharmingen), and a hamster mAb to Bcl-2 was added. Duplicate cell samples were also stained with an irrelevant hamster Ab (anti-trinitrophenol). Cells were incubated on ice for 30 min. After washing, the hamster Abs were detected by incubation with a goat anti-hamster Ab conjugated to FITC on ice for 30 min. Cells were again washed and analyzed immediately. For samples stained with anti-Bcl-2, 200,000 events/sample were collected; 50,000 events/sample were collected for samples stained with the control hamster Ab.

Results

T cell development is altered in Itk-deficient mice

The examination of itk−/− mice generated in our laboratory (15) suggested that Itk plays a role in T cell development. As previously reported (18, 19), we observed altered CD4+ and CD8+ T cell populations in the thymus, spleen, and LN of itk−/− mice (Fig. 1). Although we did not observe significant differences in lymphocyte cellularity in these organs, the proportions of CD4+ and CD8+ T cells differ from those in control mice (itk+/+ or itk+/−). In the thymus, itk−/− mice have a slight reduction in the percentage of CD4+CD8+ (SP) cells and at least a 2-fold increase in the percentage of CD8+ SP cells (Fig. 1A). However, in the periphery the percentage of CD4+ cells is, on the average, half that in control mice, while the percentage of CD8+ T cells is essentially normal (Fig. 1B).

Based on this phenotype we hypothesized that the development of CD4+ T cells is more affected by the absence of Itk. In addition, we were intrigued by the observation that there was a consistent increase in both the percentage and number of CD8+ T cells in the thymus of itk−/− mice. One potential explanation for this phenotype is that CD4/CD8 lineage commitment is altered in the absence of Itk, leading to the development of MHC class II-restricted DP thymocytes into CD8+ SP cells. To better understand the defects in itk−/− mice and the role of Itk during selection and lineage commitment, we crossed itk−/− mice to several lines of TCR transgenic mice.

Development of T cells expressing 2B4, 5C.C7, or AND TCR in H-2d mice is affected by the absence of Itk

We crossed itk−/− mice to three different lines of TCR transgensics, specifically, 2B4 (20), 5C.C7 (21), and AND (22) transgenic mice. Each of these transgensics expresses a TCR that uses Vα11 and Vβ3 gene segments and is specific for a moth cytochrome c peptide (88–103) bound to the MHC class II molecule I-Eα. However, each TCR is thought to have a different avidity for selecting ligand(s) in the thymus based on differences in the efficiency of positive selection (2B4 < 5C.C7 < AND) (24). Thus, the use of these TCR transgenic lines would allow us to determine whether the absence of Itk would differentially affect the development of...
The thymus. Itk-deficient mice were crossed to the 2B4, 5C.C7, and AND TCR transgenics (all on an H-2k background). CD4 vs CD8 profiles of thymocytes (A) and LN (B) cells were shown for live gated cells (left) and live TCRhigh gated cells (right). Each set of itk+/- and itk-/- are 8-wk-old littermates. Cells from nontransgenic mice were stained with CD4-PE, CD8-Cy, and CD3-allophycocyanin. Cells from transgenic mice were stained with an FITC-conjugated Ab that detected the transgenic TCR (anti-Vα11.1b,d or A2B4-2) along with anti-CD4-PE and anti-CD8-Cy.

cells bearing TCRs presumed to have varying avidities for selecting ligands. We chose to use class II-restricted TCR transgenics because the development of CD4+ T cells seemed to be more affected by the absence of Itk. In addition, it has been proposed that signaling strength may be a potential mechanism for T cell lineage commitment (25) and that CD8+ T cells develop as a result of weaker signals. Thus, we were interested in determining whether the absence of Itk would lead to lineage switching in one or more of these TCR transgenic lines. This outcome would be visible as the development of MHC class II-restricted itk-/- thymocytes into CD8+ T cells instead of CD4+ T cells, due to reduced signaling in the absence of Itk.

Analysis of the 2B4, 5C.C7, and AND H-2K itk-/- mice revealed that the ability of the different TCR transgenic thymocytes to develop and populate the periphery in the absence of Itk varies in a manner consistent with the proposed avidity of each TCR for its selecting ligand(s) in the thymus. In the 2B4 and 5C.C7 transgenic itk-/- mice, the percentages and absolute numbers of CD4+ SP cells in the thymus are dramatically reduced compared with the corresponding itk+/+ TCR transgenic (Fig. 1A and Table I). In the AND itk-/- mice, the average percentage of CD4+ SP cells in the thymus is reduced. However, as a reflection of the slight increase in thymic cellularity in AND itk-/- mice, the absolute numbers of CD4+ SP cells are comparable to that seen in AND itk+/+ mice (Table I).

The hierarchy of developmental defects seen in the thymus is also reflected in the peripheral T cell populations in all three TCR transgenic lines (Fig. 1B), and correlates with the presumed avidity

Table I. Thymocytes and LN cells from itk+/+ and itk-/- TCR transgenic mice

<table>
<thead>
<tr>
<th></th>
<th>Thymus</th>
<th>Lymph Nodes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% DP</td>
</tr>
<tr>
<td>2B4+/+</td>
<td>4</td>
<td>72 ± 5.5</td>
</tr>
<tr>
<td>2B4-/-</td>
<td>8</td>
<td>62 ± 2.0</td>
</tr>
<tr>
<td>5cc7+/+</td>
<td>10</td>
<td>29 ± 2.6</td>
</tr>
<tr>
<td>5cc7-/-</td>
<td>9</td>
<td>66 ± 2.4</td>
</tr>
<tr>
<td>AND+/+</td>
<td>7</td>
<td>30 ± 4.3</td>
</tr>
<tr>
<td>AND-/-</td>
<td>3</td>
<td>42 ± 4.9</td>
</tr>
<tr>
<td>AND+/+</td>
<td>1</td>
<td>25 ± 1.9</td>
</tr>
<tr>
<td>AND-/-</td>
<td>4</td>
<td>45 ± 4.6</td>
</tr>
<tr>
<td>AND+/+</td>
<td>4</td>
<td>35 ± 1.8</td>
</tr>
<tr>
<td>AND-/-</td>
<td>6</td>
<td>60 ± 3.4</td>
</tr>
</tbody>
</table>

*The percentage and absolute numbers of T cell subsets in the thymus and lymph nodes are given. The values are the mean of 3–10 animals for each genotype with SEMs. Absolute numbers are given in millions. Similar analyses were performed on splenocytes with comparable results (data not shown).
of each TCR for selecting ligands. In itk$^{-/-}$ mice that express the 2B4 TCR, which is thought to be a low avidity TCR, there are very few CD4$^{+}$ cells that express the transgenic TCR. In contrast, there are many more TCR transgenic CD4$^{+}$ T cells in the itk$^{-/-}$ mice that express a high avidity TCR, such as 5C.C7 or AND. Itk-decient mice and wild-type controls are more substantial in all three transgenic lines. This is indicative of the lower percentages of CD4$^{+}$ SP being generated in the thymus of these mice. As these mice age, the numbers of peripheral CD4$^{+}$ transgenic T cells in AND itk$^{-/-}$ mice increase to numbers equivalent to those found in and$^{-/-}$ mice; however, this is not the case for the 2B4 and 5C.C7 itk$^{-/-}$ mice. These observations suggest that age is not the only factor that allows the AND itk$^{-/-}$ mice to accumulate as many CD4$^{+}$ transgenic T cells as wild-type AND mice (see below).

Development of AND itk$^{-/-}$ T cells is affected by the strength and density of selecting ligands expressed by APC in the thymus

Based on the analysis of 2B4, 5C.C7, and AND itk$^{-/-}$ mice, it appears that the presumed avidity of the TCR for its selecting ligand(s) in the thymus plays an important role in the development of T cells in the absence of Itk. However, it is known that the development of T cells in each TCR transgenic line can be somewhat idiosyncratic, due in part to the fact that transgene expression can affect development before thymocytes reach the DP stage (26). Therefore, it is possible that the developmental efficiency of thymocytes in each of the transgenics is not solely based on their given avidities for the selecting ligands. To address this concern, we chose to analyze itk$^{-/-}$ mice that express one of the transgenic TCRs on different MHC backgrounds in which varying levels of two different selecting ligands are expressed.

The AND TCR can be positively selected on both I-E$^{b}$ and I-A$^{b}$ molecules (27). However, the generation of mature CD4$^{+}$ Vα11$^{high}$ T cells is greatly reduced in mice that only express the I-A$^{b}$ molecule. These observations strongly suggest that the AND TCR has a higher avidity for I-E$^{b}$ vs I-A$^{b}$ MHC molecules plus the self peptide(s) that mediates its selection. Therefore, we decided to compare the selection of AND itk$^{-/-}$ T cells on the H-2$^{b}$, H-2$^{b}$, and H-2$^{b}$ MHC backgrounds.

Analysis of lymphocytes from wild-type AND mice on all these backgrounds confirms the selection hierarchy: AND$^{b/b} <$ AND$^{b/b}<$ AND$^{b/b}$ (Fig. 2). The percentage and perhaps the absolute number of CD4$^{+}$ cells in the thymus of wild-type AND$^{b/b}$ mice is slightly lower than those in wild-type AND$^{b/b}$ mice (Fig. 3A and Table I), but the AND$^{b/b}$ mice have more peripheral CD4$^{+}$ Vα11$^{high}$ cells (Fig. 3B and Table I). A probable explanation for this phenotype is that, as previously suggested (27), the interaction between AND thymocytes and their selecting ligand(s) in the thymus of AND$^{b/b}$ mice is of such high avidity that some of the cells are actually deleted in these mice. This is supported by the fact that AND$^{b/b}$ mice generally have lower total thymocyte numbers and fewer DPs compared with AND$^{b/b}$ or AND$^{b/b}$ mice (Table I).

The phenotype of AND$^{b/b}$ itk$^{-/-}$ mice has been reported previously (18, 19), and our analysis is in agreement with these reports. Specifically, the development of transgenic T cells in these mice is dramatically reduced compared with that in AND$^{b/b}$ itk$^{-/-}$ mice. Despite the significant numbers of CD4$^{+}$ SP cells in both the thymus and LNs of the AND$^{b/b}$ itk$^{-/-}$ mice (Fig. 2), very few of the CD4$^{+}$ cells in the periphery express high levels of the transgenic TCR, as demonstrated by staining for Vα11 (Fig. 2B). In contrast, AND itk$^{-/-}$ mice that express I-E$^{b}$ molecules have significant numbers of CD4$^{+}$ Vα11$^{+}$ T cells in both the thymus and periphery. As shown in the previous section, the generation of transgenic T cells in the thymus of AND$^{b/b}$ itk$^{-/-}$ mice appears to be slightly less efficient than in AND$^{b/b}$ itk$^{-/-}$ mice, but the percentage and number of peripheral transgenic T cells are comparable. Although time may lead to the accumulation of transgenic T cells in the itk$^{-/-}$ mice, it is also likely that the AND$^{b/k}$ itk$^{-/-}$ mice develop as many peripheral cells as wild-type AND mice, because the deletion of some of the AND thymocytes that takes place in wild-type AND$^{b/k}$ mice does not occur, or occurs to a lesser extent, in the absence of Itk.

![FIGURE 2](https://example.com/figure2.png)

FIGURE 2. The density and avidity of the selecting ligands present in the thymus affect the development of Itk-deficient AND transgenic T cells. Itk-deficient AND TCR transgenic mice were bred onto H-2$^{b}$, H-2$^{b}$, and H-2$^{b/b}$ backgrounds. Thymocytes (A) and LN (B) cells from 8-wk-old itk$^{-/-}$ and itk$^{-/-}$ AND transgenic mice were stained with anti-Vα11.1$^{high}$-FITC, anti-CD4-PE, and anti-CD8-Cy. CD4 vs CD8 profiles are shown for live gated (left) and live Vα11$^{high}$ gated (right) cells.
of Itk. Therefore, we were interested in determining whether the survival factor, Bcl-2 is properly up-regulated in \( \text{Itk}^{-/-} \) thymocytes.

It has been shown that Bcl-2 is up-regulated in a subset of TCR\(^{\text{high}}\) DP thymocytes and remains high in SP cells (28). This expression pattern correlates with cells that have received or are receiving positive selection signals. Fig. 3 depicts intracellular staining for Bcl-2 in \( \text{Itk}^{-/-} \) thymocytes compared with their wild-type littermates. In non-TCR transgenic \( \text{Itk}^{-/-} \) cells, the levels of Bcl-2 are up-regulated in both the DP TCR\(^{\text{high}}\) and CD4\(^+\) SP populations to the same extent as in thymocytes from a wild-type littermate (Fig. 3A). The same is true in the analogous populations of TCR transgenic thymocytes on all the selecting backgrounds (Fig. 3B and data not shown). These data indicate that \( \text{Itk}^{-/-} \) cells in which the early stages of positive selection are properly initiated, as determined by up-regulation of TCR, also properly up-regulate the survival factor Bcl-2. However, within the entire DP population from ANDk\(^b\) \( \text{Itk}^{-/-} \) thymi, a greater proportion of DP cells have background (very low) levels of Bcl-2. This is probably due to fewer cells in these mice receiving the initial signals for selection.

Analysis of the Bcl-2 levels in cells undergoing selection and in cells that have completed selection suggested that \( \text{Itk}^{-/-} \) thymocytes properly up-regulated this important survival factor and thus would have comparable viability as their wild-type counterparts. Therefore, we were interested in determining whether the regulation of additional markers is normal in \( \text{Itk}^{-/-} \) thymocytes undergoing selection; this analysis would allow us to identify the stage(s) of selection in which \( \text{Itk}^{-/-} \) thymocytes are defective. Fig. 4A depicts the stages of positive selection based on the expression of the TCR, CD69, HSA, and the co-receptors, CD4 and CD8.

This analysis revealed that a smaller percentage of DP have up-regulated TCR in the absence of Itk in all TCR transgenic lines (data not shown), suggesting that fewer \( \text{Itk}^{-/-} \) DP cells are receiving a strong enough signal to initiate selection. Next we wanted to determine whether Itk-deficient thymocytes would also be impaired at later stages of positive selection. Thus, we examined the pattern of CD69 expression on TCR\(^{\text{high}}\) DP and SP thymocytes (Fig. 4B). Thymocytes that have up-regulated CD69 are in either stage C (TCR\(^{\text{high}}\) DP) or stage D (SP) (29). Comparing the percentage of cells in these intermediate stages of selection from wild-type AND mice on each selecting background revealed that there are fewer CD69\(^{\text{high}}\) cells in the presence of the strongly selecting JE\(^b\) ligand. One interpretation of these data is that thymocytes receiving stronger signals up-regulate CD69 and then progress through stages C and D more efficiently than cells receiving weaker signals. If this interpretation is correct, we would expect that a higher percentage of TCR\(^{\text{high}}\) DP and SP thymocytes in AND \( \text{Itk}^{-/-} \) mice would have high levels of CD69 compared with their wild-type littermates. As shown in Fig. 4B, this is, in fact, the case, suggesting that in the absence of Itk, thymocytes do not progress through these stages as efficiently as cells that express Itk.

Finally, we examined HSA levels on the CD4\(^+\) SP cells from these mice (Fig. 4C). HSA is down-regulated in the final stage of thymocyte selection and differentiation, such that the most mature cells are HSA\(^-\) (30). Although more of the CD4\(^+\) SP thymocytes in AND \( \text{Itk}^{-/-} \) mice are still CD69\(^{\text{high}}\) compared with those in control mice, it appears that the same proportion of the CD4\(^+\) SPs have begun to down-regulate HSA in both \( \text{Itk}^{-/-} \) and \( \text{Itk}^{-/-} \) mice. This suggests that the down-regulation of HSA can take place while TCR signaling is still occurring and that this happens normally even in the absence of Itk.
The expression of CD5, a marker for TCR signal strength, is lower on thymocytes from itk<sup>−/−</sup> mice

CD5 surface expression is regulated throughout T cell development and correlates with the strength of the TCR signal received by the developing thymocyte (31). The studies of peripheral itk<sup>−/−</sup> T cells (15) and thymocytes (32) indicated that T cells get a weaker signal through their TCR in the absence of Itk; thus, we would expect that CD5 levels would be lower on thymocytes from itk<sup>−/−</sup> mice. This prediction was first verified by staining thymocytes from non-TCR transgenic itk<sup>−/−</sup> mice, which revealed that DP thymocytes have significantly lower levels of CD5 compared with DPs from itk<sup>+/+</sup> mice (Fig. 5A).

We also examined CD5 levels on DP and CD4<sup>+</sup> SP cells from all the TCR transgenic mice to compare T cells that all express the same TCR (Fig. 5B). Similar to the non-TCR transgenic mice, all the transgenic mice lacking Itk had lower surface levels of CD5 on their DP thymocytes with the exception of AND<sup>bb</sup> itk<sup>−/−</sup> mice. Comparison of AND<sup>kk</sup>, AND<sup>bb</sup>, and AND<sup>bb</sup> mice shows that as the strength of the selecting background increases, the differences in CD5 surface expression between DPs that express Itk and those that do not is diminished. Interestingly, the CD5 surface levels do not seem to vary significantly among the wild-type AND mice with different selecting backgrounds. Unlike the non-TCR transgenic itk<sup>−/−</sup> mice, all the TCR transgenic itk<sup>−/−</sup> mice have lower levels of CD5 on their CD4<sup>+</sup> SP thymocytes, further indicating that the strength of the signal through the same TCR is lower in cells that lack Itk. Staining with the anti-V<sub>α</sub>11 Ab confirms that the differences in CD5 expression are not due to lower TCR levels on itk<sup>−/−</sup> thymocytes (Fig. 5B).

Lineage commitment is not affected by reduced TCR signaling in the absence of Itk

In light of increasing evidence that the strength of the TCR signal influences CD4/CD8 lineage commitment, and the suggestion that Tec family kinases are important for regulating TCR signaling thresholds (19), we were interested in determining whether lineage commitment would be affected in itk<sup>−/−</sup> mice. This model of lineage commitment proposes that strong signals downstream of the TCR induce DPs to differentiate into CD4<sup>+</sup> T cells, while weak signals downstream of the TCR cause DPs to differentiate into CD8<sup>+</sup> T cells (25). The TCR transgenic itk<sup>−/−</sup> mice that we generated to examine the role of Itk in positive selection also provided us with an ideal system to examine how lineage commitment would be affected in the absence of Itk, as these mice represent a
range of TCR avidities for selecting ligands. Specifically, we anticipated that lineage switching might occur in some of the TCR transgenics, particularly those with low selection efficiencies. In such an instance, the loss of transgenic CD4+ SP cells would be compensated for by an increase in transgenic CD8+ SP cells. This expectation would be consistent with the increased percentage of CD8+ SP cells seen in non-TCR transgenic Itk−/− mice.

As shown in Figs. 1 and 2 there were a small number of CD8+ SP cells in the thymus and LN of all TCR transgenic mice. The percentage and absolute number of these cells that express high levels of the transgenic TCR are shown in Table I. In virtually all cases there was no significant increase in the absolute number of transgenic TCRhigh CD8+ SPs in the absence of Itk as determined by Student’s t test (p < 0.05). The only exception was in the LN of ANDv/h mice, in which the number of CD8+ cells was slightly increased. Therefore, the data from the TCR transgenic mice suggest that although the selection of CD4+ T cells is reduced, lineage commitment occurs normally.

If this is indeed the case, the issue of why non-TCR transgenic Itk−/− mice have such a large number of CD8+ SP cells in the thymus and an altered CD4:CD8 ratio in the periphery remained unresolved. To address this question we analyzed the expression of multiple thymocyte and T cell markers on the CD8+ SP cells in Itk−/− mice (Fig. 6a). The phenotype that we observe is that the majority of the CD8+ cells in the thymus of Itk−/− mice appeared to be mature CD8+ T cells rather than newly developed CD8+ SP cells. For instance, the vast majority of CD8+ SP thymocytes in Itk−/− mice are HSAlow/−, and thus resemble peripheral T cells rather than maturing thymocytes (Fig. 6a). Likewise, the analysis of Bcl-2 (Figs. 3a and 6) and CD5 (Figs. 5a and 6a) expression on the CD8+ SPs of Itk−/− mice demonstrates that the majority of these cells have high levels of both of these proteins, similar to the levels found on the most mature thymocytes or peripheral T cells.

Although the analysis of these markers suggests that a significant number of CD8+ SPs in the thymus of Itk−/− mice have a mature phenotype, we are unable to determine whether these cells are peripheral cells that have returned to the thymus or cells that have fully matured, but are not exported from the thymus normally. It has been shown that activated peripheral T cells can re-enter the thymus (33), so we decided to analyze the expression of activation markers on these CD8+ SPs. The analysis of CD69, which is expressed on activated T cells, revealed that there was no difference in the proportion of CD8+ SPs that were CD69+ between wild-type and Itk−/− mice. We also examined the activation/memory marker CD44. The expression of CD44 is up-regulated on cells as they become activated, but, unlike CD69, it remains high, and thus is often used as a marker for memory T cells (34). As shown in Fig. 6a, the majority of CD8+ SP thymocytes in Itk−/− mice are CD44high, while in wild-type mice the majority of these cells are CD44low. Based on these results it appears that the CD8+ SP thymocytes in Itk−/− mice are not activated, but may have been previously. These data leave open the possibility that activated CD8+ cells recirculate from the periphery to the thymus in Itk−/− mice, but in a steady-state analysis show up largely as previously activated cells.

**FIGURE 6.** The majority of CD8+ SP thymocytes in the thymus of Itk-deficient mice have a mature phenotype. A. Thymocytes were stained with Abs to CD4 and CD8 along with either an Ab to CD5, Bcl-2, HSA, or CD44. The expression level of each of these thymocyte markers on live CD4+ and CD8+ SP thymocytes from 8-wk-old nontransgenic Itk+/+ (solid line) and Itk−/− (dotted line) mice are shown. For Bcl-2 staining, nonspecific staining with the irrelevant hamster Ab is also shown (filled histograms). B. Thymocytes from neonatal (1–1.5 days old) mice were stained with Abs to CD4 and CD8 along with either CD44 or HSA. CD4 vs CD8 profiles for live (left panel) and live TCRhigh (right panel) cells are shown. The expression levels of HSA and CD44 on the CD8+ SP population from nontransgenic Itk+/+ (solid line) and Itk−/− (dotted line) mice are also shown. The genotyope of the neonates was determined from DNA isolated from liver tissue. Data are representative of two separate experiments and a total of eight or nine neonates of each genotype.
Alternatively, it is possible that the CD8+ SP cells acquire this phenotype in the thymus due to irregular development. To address this, we analyzed CD8+ SP thymocytes from neonatal Itk-deficient mice to determine whether these cells have a mature phenotype before CD8+ T cells have populated the periphery. The CD4 vs CD8 profiles from neonatal itk+/− and itk−/− mice show that there are significantly fewer CD4+ SPs in the Itk-deficient mice (Fig. 6B). However, in contrast to adolescent mice, there does not appear to be an increase in CD8+ SPs, a phenotype that is more obvious when examining the profiles of mature TCRhigh cells. In addition analysis of CD44 and HSA expression on the CD8+ SPs indicate that the first few CD8+ SPs that are developing in itk−/− mice have a normal phenotype, as shown by comparison with itk+/− littermates. Thus, if the CD8+ SPs in Itk-deficient mice acquire the phenotype shown in Fig. 6A while still in the thymus, it must occur postnatally during later waves of CD8+ T cell maturation.

**Discussion**

The analysis of mice that are deficient in various TCR signaling molecules has provided significant insight into the role of these molecules in T cell development. In this study we use Itk-deficient mice crossed to several TCR transgenic lines to determine how altering the level of TCR signaling affects the processes of positive selection and CD4/CD8 lineage commitment. Biochemical studies with Itk-deficient thymocytes and T cells indicate that this kinase is important for efficient PLC-γ1 activation leading to sustained Ca2+/H11002 mobilization and Erk/MAPK activation in response to TCR stimulation (15, 19). Although these pathways are reduced by the absence of Itk, signaling is not completely abolished, possibly due to weak compensation by other Tec family kinases, such as Rlk/Txk (19). Thus, itk−/− mice provide an ideal opportunity to determine how decreased activation of these signaling pathways affects T cell development.

For our studies we crossed itk−/− mice to three lines of MHC class II-specific TCR transgenics on three different MHC backgrounds. A previous study has addressed the role of Tec kinases in setting the signaling threshold between positive and negative selection using a single MHC class I TCR transgenic system. Here we address the effect of decreased TCR signaling on positive selection over a range of TCR avidities as well as on CD4 vs CD8 lineage commitment. Our results, based on analyses of thymocytes, show that the efficiency of positive selection is reduced in all cases in the absence of Itk. Interestingly, we did not observe some TCR/MHC combinations in which positive selection was completely abolished and others that were unaffected by the absence of Itk.

These studies extend previous reports of thymic selection in Itk-deficient mice by substantiating the conclusion that reduced CD4+ T cell numbers result from defective positive selection, as opposed to reduced CD4+ cell survival. First, we demonstrated that the survival factor, Bcl-2, is up-regulated to an equivalent degree in DP thymocytes, SP thymocytes, and peripheral T cells from itk−/− mice compared with control mice. In addition, the expression of other surface markers that define the final stage of thymocyte maturation, such as HSA, CD69, CD44, and CD62 li-gand, is similar on cells generated in wild-type vs itk−/− TCR transgenic mice. In the course of this analysis we also observed that wild-type AND TCR transgenic mice expressing the highly selecting ligand, IL3, had fewer thymocytes with high levels of CD69. Thus, in an environment where thymocytes are receiving strong selection signals, there are fewer cells in the CD69high transition stage, and there is an increase in the more mature CD69− CD4+ SP subset. In the Itk-deficient AND mice there is a decrease in the overall number of TCRhigh thymocytes; however, among those cells, there is a dramatic increase in the proportion of CD69high transition cells. This suggests that thymocytes receiving weaker signals during positive selection may require longer to complete the selection process. Together, these findings support the conclusion that in the absence of Itk, fewer thymocytes undergo positive selection, and those that do take longer to become fully mature CD4+ SP cells.

Overall, these data reinforce the view of positive selection as an inherently stochastic process, where only a fraction of thymocytes with appropriate TCRs receive sufficient signals to undergo selection before they succumb to programmed cell death (death by neglect). Positive selection is thought to take 1.5−2 days of continuous low level signaling through the TCR (35), and it has been proposed that there are a limiting number of niches that are present in the thymus that will support selection (36). This concept has been used to explain why, in TCR transgenic mice in which all developing thymocytes express an appropriate TCR, the majority of cells do not actually get selected. Thus, one explanation for the less efficient development of itk−/− thymocytes is that these cells take longer to receive sufficient signals, and due to the limiting niches, fewer cells are successfully selected before undergoing cell death. This probabilistic model of positive selection is also consistent with the outcomes we observed in itk−/− mice with a range of avidities of TCR-MHC interactions (e.g., AND6b vs AND8b or AND8c), where the higher the TCR avidity, the higher the probability that even cells lacking Itk will achieve sufficient signals to promote maturation and survival.

Signals downstream of TCR complexes on thymocytes are also important for developmental processes in addition to positive selection. At the pro- to pre-T cell transition, signals downstream of the pre-TCR are required for proliferation, survival, differentiation, and allelic exclusion. Recent evidence suggests an important role for PLC-γ in pre-TCR signaling (37). This observation suggests a role for Itk in this signaling pathway, as Itk is important for full activation of PLC-γ1 in peripheral T cells (15). In fact, we did observe an increase in the percentage of CD4+ CD8+ thymocytes in a number of the TCR-transgenic itk−/− mice (Figs. 1A and 2A). However, initial characterization of the CD4+ CD8+ CD3+ (TN) thymocyte populations in non-TCR transgenic Itk-deficient mice indicates that there is no overt defect at this stage of development, as determined by CD25 and CD44 analysis (J. Lucas and L. Berg, unpublished observations). Therefore, we believe the increase in TN thymocytes is more reflective of a loss of DP and/or CD4+ SPs in these mice. Consistent with this is the fact that a significant increase in the percentage of TN cells is only observed in itk−/− TCR transgenic mice that have an overall reduction in thymic cellularity.

This study also demonstrated that the majority of CD8+ thymocytes in itk−/− mice have a phenotype that is not consistent with newly generated CD8+ SPs, but instead resembles peripheral T cells with a memory phenotype (CD44high). Corroborating this observation, we found that the majority of CD8+ T cells in the periphery of itk−/− mice are also CD44high (data not shown). One interpretation of these data is that the CD44high CD8+ SP thymocytes in itk−/− mice are previously activated peripheral cells that have reentered the thymus. An alternative possibility is that these cells could be the products of homeostatic expansion, although this seems unlikely, as itk−/− mice are not lymphopenic. A third possibility is that the CD8+ SP thymocytes acquire this mature phenotype in the thymus and accumulate there before being exported to the periphery. We have begun to address this by looking at the phenotype of the CD8+ SPs in neonatal mice. The examination of the very earliest CD8+ SPs suggest that they do not acquire the...
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mature phenotype early in development. A more detailed kinetic analysis of CD8+ SP maturation and thymocyte emigration is ongoing and should help shed light on the nature of these cells. However, at this point we believe that our initial hypothesis that CD4 development is more affected by the absence of Itk may need revision, as the defects in CD8+ T cell development may have been masked by expansion (in the thymus and/or the periphery) of the few cells that are selected.

The most unexpected result that we observed is the lack of effect on CD4/CD8 lineage commitment in Itk-deficient mice. The initial analyses of non-TCR transgenic itk−/− mice revealed that T cells of both lineages develop, but that the normal ratio of CD4+ to CD8+ cells is altered in favor of increased CD8+ cells. An attractive hypothesis to explain this observation was that stronger selection signals induce CD4+ differentiation, and that in the absence of Itk, class II-specific thymocytes would develop into CD8+ T cells due to weaker selection signals. This potential explanation for the phenotype of itk−/− mice was strengthened by recent studies identifying signaling molecules that appear to influence CD4/CD8 lineage decisions in the thymus. For instance, the level of Lck activity in developing thymocytes has been shown to directly affect the outcome of lineage commitment (4). As Itk is directly downstream of Lck, alterations in Lck activity should be reflected in concomitant changes in Itk activity. More importantly, the activity of the Erk/MAPK signaling pathway has also been shown to influence CD4/CD8 lineage commitment and as stated above, itk−/− T cells have reduced phosphorylation of Erk1/2 following CD3 cross-linking (19). Therefore, because molecules that are thought to be both upstream and downstream of Itk signaling are implicated in CD4/CD8 lineage commitment, it seemed likely that Itk would also be involved in this process.

Nonetheless, our data argue that Itk is not involved in CD4/CD8 lineage commitment. In non-TCR transgenic itk−/− mice the bulk of CD8+ SP thymocytes present do not represent newly differentiating cells. Furthermore, in all five different TCR transgenic/MHC backgrounds that we examined we never observed a substantial increase in the number of CD8+ cells developing in the absence of Itk. It is possible that we did not see a difference because the alteration in the strength of the TCR signal in these cases was either too large or too small without Itk, such that development of the transgenic T cells was nearly abolished (too large) or was reduced, but not enough to induce lineage commitment (too small). However, with the number of combinations examined, we do not think that this explanation is likely. Rather, we believe that the activity of Itk is not required for the signal that induces commitment to either specific lineage.

These observations support the interesting possibility that unique signals downstream of the coreceptor are responsible for lineage commitment and that Itk is not involved in this signaling pathway. Consistent with this idea, previous studies have shown that altering the coreceptor, Lck activity, or Erk/MAPK signaling can have an effect on lineage commitment, but that simply reducing TCR signaling does not (25). An alternative view of lineage commitment has proposed that CD4/CD8 lineage decisions are influenced by the kinetics/duration of TCR/coreceptor signaling (38, 39), rather than the strength, with CD4 lineage commitment requiring sustained TCR signals. Thus, it is possible that we did not observe lineage switching in the class II-restricted TCR transgenic itk−/− mice because these thymocytes may have received sustained signals for a sufficient period of time to ensure proper differentiation. Finally, it will be interesting to determine how development of the CD8 lineage is altered in thymocytes that lack Itk. Although we did not observe lineage switching, the CD8+ SPs in itk−/− mice have an unusual phenotype, which may suggest some alteration in the maturation of these cells. Therefore, we are currently crossing itk−/− mice to class I-restricted TCR transgenic mice to further examine this issue.

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