Immunity 25, 93–104, July 2006 ©2006 Elsevier Inc. DOI 10.1016/j.immuni.2006.05.011

Altered Development of CD8$^+$ T Cell Lineages in Mice Deficient for the Tec Kinases Itk and Rlk

Christine Broussard,[1,4,5] Christine Fleischecker,[2,4] Reiko Horai,[1,4] Madeva Chetana,[1] Ana M. Venegas,[1] Leslie L. Sharp,[3] Stephen M. Hedrick,[3] B.J. Fowlkes,[2] and Pamela L. Schwartzberg[1,*]
[1] National Human Genome Research Institute
[2] National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland 20892
[3] Division of Biological Sciences
University of California, San Diego
La Jolla, California 92093

Summary

Mutations affecting the Tec kinases Itk and Rlk decrease T cell receptor-induced Ca^{2+} mobilization and Erk kinase activation and impair both positive and negative thymic selection. *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice also have decreased CD4:8 T cell ratios, suggestive of altered CD4:8 lineage commitment. Nonetheless, we find that CD8 single-positive (SP) thymocytes and peripheral CD8$^+$ T cells in these mice do not resemble conventional CD8$^+$ T cells. Instead, these cells express memory markers, rapidly produce interferon-γ, and can be selected on hematopoietically derived cells, similar to MHC class Ib-restricted "innate-type" lymphocytes. Itk deficiency also greatly increases the number of cells selected by MHC class Ib. Expression of a hypersensitive Erk2 mutant partially corrects the CD8$^+$ T cell phenotypes in *Itk*$^{-/-}$ mice, arguing that altered signaling permits development of this innate-type CD8$^+$ cell population. Our results suggest that Tec kinases differentially regulate development of conventional versus nonconventional lymphocytes.

Introduction

Development of mature T cell receptor (TCR) $\alpha\beta$-positive cells in the thymus is regulated in large part by signals they receive from the TCR, with weak or negligible signals leading to death by neglect, strong signals, as from agonist peptides, causing deletion, and moderate signals leading to positive selection and the development of mature CD4 and CD8 SP thymocytes (Starr et al., 2003). Data argue that the differentiation choice between CD4 and CD8 SP T cells and their development in the thymus are also influenced by the strength or duration of signals from the TCR, with short or interrupted signals leading to CD8 and sustained signals to CD4 SP cell development (Germain, 2002; Singer and Bosselut, 2004). In particular, experiments suggest that the extent of Lck and Erk activation by the TCR helps dictate CD4$^+$ over CD8$^+$ T cell lineage development (Bommhardt et al., 1999; Hernandez-Hoyos et al., 2000; Legname et al., 2000; Wilkinson and Kaye, 2001).

However, in addition to these conventional TCR$\alpha\beta$ expressing cells, a number of other lineages, including NKT cells, H2-M3-restricted cells, CD8$\alpha\alpha$ intraepithelial cells (IELs), and CD4$^+$CD25$^+$ regulatory cells, are also selected in the thymus and play important roles in regulating normal immune responses (reviewed in Baldwin et al. [2004]). Several of these atypical lineages, including NKT cells, which recognize glycolipids in the context of CD1d (Bendelac et al., 1995; Gapin et al., 2001), H2-M3-restricted cells, which recognize formyl-methionine-modified peptides (found in bacterial and mitochondrial encoded proteins) in the context of H2-M3 (Loveland et al., 1990), and CD8$\alpha\alpha$ IELs, are positively selected on peptides and/or lipids associated with the less polymorphic MHC class Ib molecules (Bendelac, 1995; Gangadharan and Cheroutre, 2004; Gapin et al., 2001; Lindahl et al., 1997; Yamagata et al., 2004). Intriguingly, MHC class Ib-restricted cells share characteristics with cells of the innate immune system, including expression of memory markers and rapid expression of cytokines (Das et al., 2001; Yoshimoto and Paul, 1994). Evidence suggests that class Ib-restricted cells, like innate immune cells, are involved in early immediate responses to infections (Kerksiek et al., 1999; Yoshimoto and Paul, 1994). Nonetheless, the signals involved in development of these nonconventional T cell lineages in the thymus are not well understood. For NKT and H2-M3-restricted cells, thymic selection can occur on hematopoietic cells (Bendelac et al., 1994; Bix et al., 1993; Ohteki and MacDonald, 1994; Urdahl et al., 2002). Moreover, NKT cell development requires the tyrosine kinase Fyn (Eberl et al., 1999; Gadue et al., 1999), the adaptor molecule SAP (Chung et al., 2005; Nichols et al., 2005; Pasquier et al., 2005), and protein kinase C-θ (Stanic et al., 2004), molecules that are not required for conventional TCR$\alpha\beta$ cell development. These observations suggest there are unique requirements or patterns of TCR signaling leading to development of nonconventional T cell lineages.

Moreover, it is clear that other signaling pathways contribute to thymic lineage decisions. In particular, IL-7 and IL-15 may provide second signals for the development of CD8$^+$ T cells (Brugnera et al., 2000; Yu et al., 2003). IL-15 is also required for differentiation and homeostasis of NKT cells and CD8$\alpha\alpha$ IELs (Kennedy et al., 2000; Ohteki, 2002). Nonetheless, TCR signals may coregulate these signals by influencing expression of components of cytokine or other pathways. Indeed, termination of TCR signaling increases expression of IL-7Rα, allowing a second signal for CD8$^+$ T development (Brugnera et al., 2000). Again, how TCR signals contribute to the generation of other cell lineages remains poorly understood.

The Tec family tyrosine kinases Itk and Rlk are required for full TCR-induced activation of PLC-γ, Ca^{2+} mobilization, and Erk activation (Liu et al., 1998; Schaeffer et al., 1999, 2000). We have previously reported that mutation of *Itk* or *Itk* and *Rlk* leads to

*Correspondence: pams@nhgri.nih.gov
[4] These authors contributed equally to this work.
[5] Present address: Department of Biology, University of La Verne, La Verne, California 91750.

reductions in both positive and negative selection (Schaeffer et al., 2000). Both *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice also have decreased CD4:8 cell ratios in the thymus and periphery, suggestive of altered lineage commitment. Given that Lck is required for activation of Tec kinases, which in turn contribute to Erk activation (Berg et al., 2005), the Tec kinases are potential candidates for regulation of CD4:8 lineage development. Nonetheless, a recent study demonstrated that MHC class II-restricted TCR transgenics on an *Itk*$^{-/-}$ background fail to develop increased numbers of CD8$^+$ T cells, arguing that Itk does not affect CD4:8 lineage commitment (Lucas et al., 2002). Moreover, CD8$^+$ T cells in *Itk*$^{-/-}$ mice were found to express CD44, a memory cell marker, suggesting they might not represent normal CD8$^+$ TCR$\alpha\beta$ lineage cells.

To better understand the nature and ontogeny of the CD8$^+$ T cells in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice, we have characterized their phenotype, chronology of appearance, and the signals required for their development. We find that these cells do not resemble conventional CD8$^+$ T cells and instead have distinct properties, including memory marker expression, rapid production of interferon-γ (IFN-γ), and selection on hematopoietically derived cells, similar to MHC class Ib-restricted innate-type lymphocytes. Moreover, deficiency of Itk greatly increases the number of cells selected by MHC class Ib. Both the increased numbers and altered phenotypes of SP CD8$^+$ cells are partially rescued by expression of a hypersensitive mutant of Erk, suggesting that altered signaling in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice specifically permits development of this distinct cell population. Our results demonstrate that the absence of Tec kinases both prevents conventional CD8$^+$ T cell development and leads to the generation of a large population of nonconventional innate-type CD8 T cells, providing potential insight into the signaling pathways required for regulating the balance between adaptive and innate lymphocyte development.

Results

Altered CD8$^+$ T Cell Development in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ Mice

We have previously reported that mutation of *Itk* or combined mutation of *Itk* and *Rlk* impairs both positive and negative selection of conventional CD4 and CD8 T cells in the thymus (Schaeffer et al., 2000). Additionally, both *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice show decreased CD4:8 ratios, with increased numbers of CD8 SP cells in the thymus, suggestive of altered lineage development (Figure 1 and Tables S1 and S2 available in the Supplemental Data with this article online).

Because CD8 SP cells in the thymus include immature CD8 SP cells en route to DP cells, we initially evaluated the increased CD8$^+$ cell population that develop in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ animals by characterizing the expression of surface maturation markers. We have previously reported that mature SP cells in the thymus of *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice express slightly lower amounts of TCR (Schaeffer et al., 2000), which may correlate with their selection under conditions of impaired signaling. Despite this, SP thymocytes from *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice, especially CD8$^+$ cells, exhibited lower HSA (CD24) than SP cells in wild-type (wt) mice, suggesting that these cells were more mature than normal SP cells (Figure 1A). Moreover, in agreement with recent observations in *Itk*$^{-/-}$ mice (Lucas et al., 2002), we found that CD8 SP thymocytes in both *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice showed increased expression of the memory marker CD44 (Figure 1A and Table S3). These CD8 SP cells also showed increased expression of CD122, also known as the IL-2Rβ subunit, which is another marker associated with a subset of activated or memory CD8$^+$ cells (Judge et al., 2002). CD4 SP cells also showed slightly increased expression of CD44 and decreased expression of CD62L, patterns associated with CD4 memory T cell status (Dutton et al., 1998), although this phenotype was much less pronounced (Figure 1A and data not shown). Increased expression of memory markers was similarly noted in the periphery; most CD8$^+$ T cells expressed CD122 and CD44 in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice (Figure 1B and Table S3). Approximately half of the CD4$^+$ T cells in the periphery of these mice also expressed CD44 and decreased CD62L (Figure 1B and data not shown). Thus, although both lineages express memory markers, CD8$^+$ T cells are more profoundly affected than CD4$^+$ T cells, particularly in the thymus.

Altered CD8$^+$ T Cells Arise Early in Thymic Development

In mice, activated and memory T cells traffic to specific locations, including the thymus (Agus et al., 1991; Reinhardt et al., 2001). Thus, the presence of activated or memory cells in the thymi of *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice could be secondary to altered homeostasis of mature peripheral CD8$^+$ and CD4$^+$ T cells, which then migrate back to the thymus. Nonetheless, experiments in which *Rlk*$^{-/-}$*Itk*$^{-/-}$ CD44^{hi} peripheral T cells were transferred into naïve wt recipients failed to provide evidence for homing of these cells to the thymus (data not shown). Moreover, BrdU incorporation studies demonstrated that the CD8 SP cells were not more actively cycling in the thymi of *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice compared to wt mice, as might be expected from homeostatically expanding cells (data not shown and Atherly et al. [2006]).

To better evaluate the ontogeny of these cells, we examined the appearance of CD8$^+$ and CD4$^+$ cells in the thymus and the periphery during the first week of postnatal life, when SP cells first develop in the thymus (Figure 2). Although the appearance of CD4$^+$ SP cells in the thymus was somewhat delayed in the *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice (data not shown), increased percentages of CD8 SP cells as well as decreased CD4 SP cells were observed by postnatal days 5–7, coincident with the appearance of mature CD8 SP cells in the thymus and prior to the existence of a substantial population of CD8$^+$ T cells in the spleen (Figure 2 and Figure S1). These results suggest that the increased CD8$^+$ T cell development occurs intrathymically and is not the result of recirculation. Moreover, cells with a "memory" (CD122$^+$CD8$^+$) phenotype were detected shortly after CD8 SP cells appeared in the thymus, coincident with the appearance of CD8$^+$ cells in the periphery. Notably, although the few CD8$^+$ T cells in the periphery of both wt and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice expressed CD44 in the first postnatal week, consistent with previous reports of homeostatic proliferation in newborn mice (Min et al., 2003), the percentage

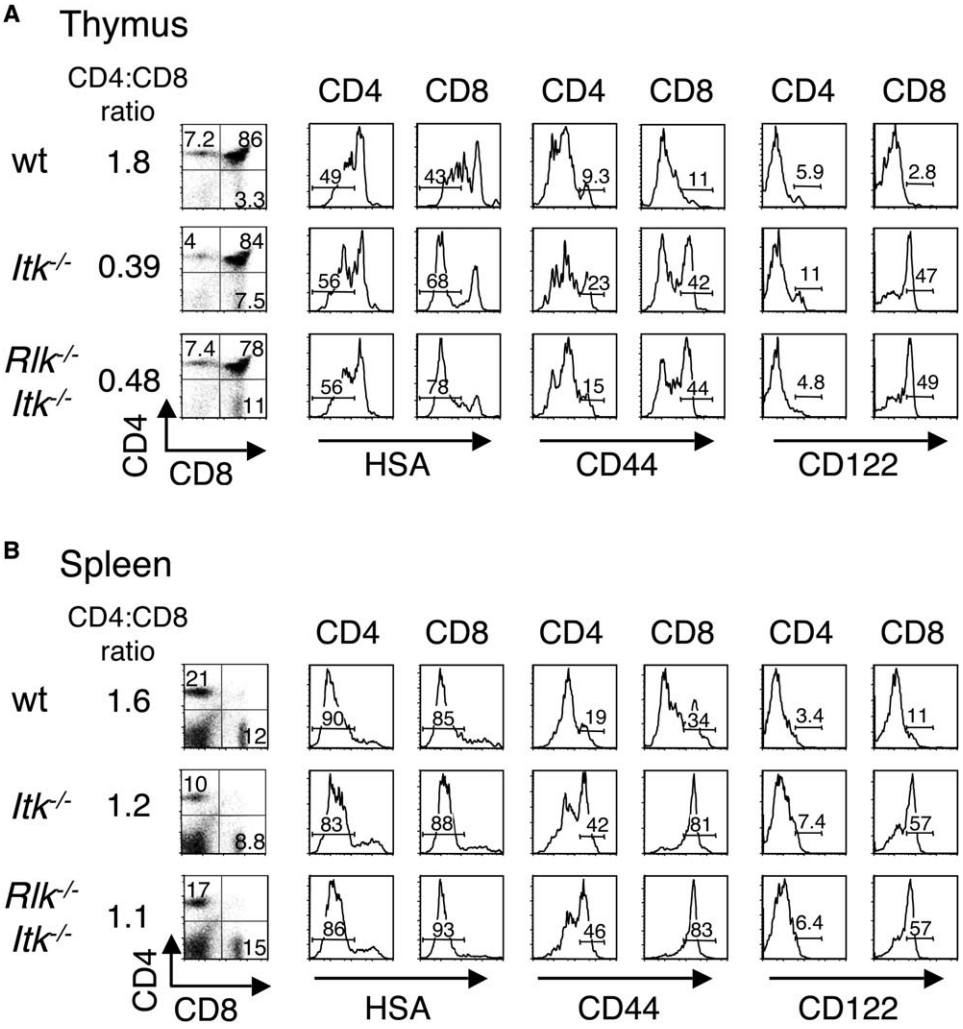

Figure 1. *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ Mice Show Altered CD4:8 Ratios and Increased Expression of Memory Markers in CD4 and CD8 Populations

(A) Profiles of thymocytes harvested from age-matched 8-week-old mice from the indicated lines: wild-type (wt), *Itk*$^{-/-}$, and *Rlk*$^{-/-}$*Itk*$^{-/-}$. Far left, thymocytes stained for CD4 and CD8. The percentages of total cells represented by each T cell subset are given in the respective quadrant. The ratio of CD4:CD8 cells is indicated. Histograms of cells stained for HSA, CD44, and CD122 are shown on the right for CD4 SP and CD8 SP cells. The percentages of gated cells are indicated.

(B) Profiles of splenocytes from the same mice. These data are representative of over eight experiments examining two to three mice each.

of CD44^{hi} cells dropped over time in wt mice but remained high in *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice (Figure S1, Figure 1, and data not shown).

Increased Development of CD8$^+$ T Cells Occurs in the Thymus

Although these developmental studies suggested that the increased numbers of CD8$^+$ T cells in *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice were intrathymically derived, it was still possible that recirculation occurred during the first week of life. To clarify the origin of the increased numbers of CD8$^+$ T cells in these mice, we examined fetal thymic organ cultures. Organ cultures of E15.5 fetal thymi initially have mostly double-negative cells that appeared indistinguishable between cultures from wt and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice (Figures 3A and 3B). Within 2 days of culture, cells from both genotypes had developed into DP cell populations and, by day 6, both wt and *Rlk*$^{-/-}$*Itk*$^{-/-}$ organ cultures had SP cells. However, cultures of *Rlk*$^{-/-}$*Itk*$^{-/-}$ thymi showed increased development of CD8 SP cells that were mature as evidenced by high TCR and low HSA expression (Figure 3B and data not shown). Furthermore, by day 8 of culture, these CD8$^+$ cells also showed increased expression of the memory marker CD122 (Figure 3C). Thus, increased numbers of altered CD8$^+$ T cells develop intrathymically in *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice.

Itk$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ CD8$^+$ Cells Develop in B2m-Deficient Recipients

To better understand the requirements for selection and development of the altered CD8$^+$ cells in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice, we created bone marrow chimeras with wt, MHC class I, and MHC class II-deficient hosts. Transfer of bone marrow from all genotypes into irradiated wt recipients led to similar patterns of T cell development as seen in the original donor animals, demonstrating that the decreased CD4:8 ratios and memory

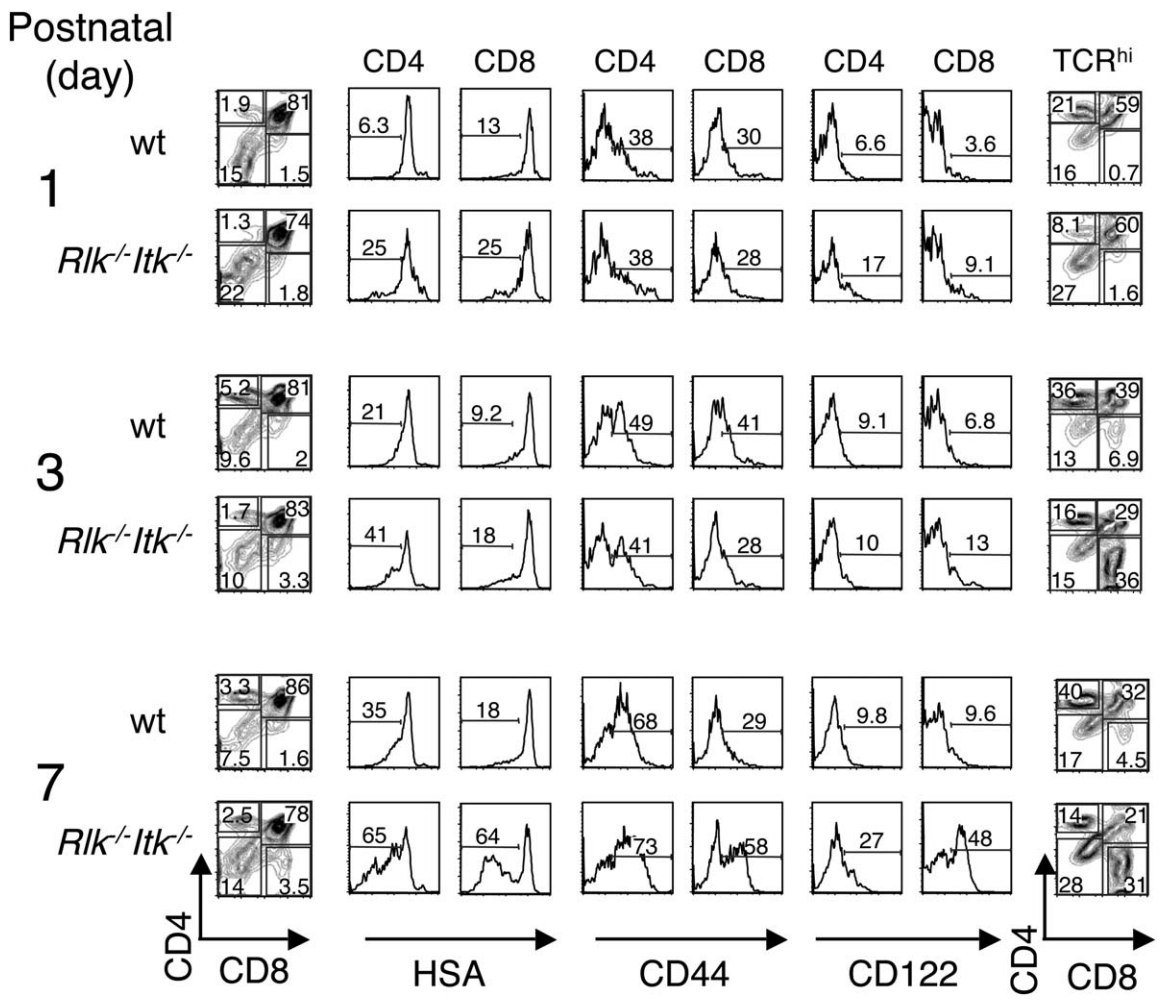

Figure 2. Increased Development of CD8+ T Cells Occurs in the First Week of Life in *Rlk*$^{-/-}$*Itk*$^{-/-}$ Mice

Profiles of thymocytes harvested from age-matched progeny from wt and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice at day 1 (top panels), day 3 (middle panels), and day 7 (bottom panels). Far left, CD4 versus CD8 profiles of thymocytes. Histograms of cells stained for HSA, CD44, and CD122 are shown in the center for CD4 and CD8 SP cells. TCR^{hi}-gated contour plots of anti-CD4 and anti-CD8 T stained thymocytes are shown on the far right. Data are representative of duplicate mice in each of two independent experiments, examining similar days.

cell phenotypes in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice were intrinsic to hematopoietically derived cells (Figure 4A).

Based on a quantitative signaling model of CD4:8 lineage commitment, the increased CD8+ T cell numbers in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice could arise from MHC class II-specific DP precursor cells that are redirected from the CD4 to the CD8 lineage due to impaired TCR signaling. To test this hypothesis, we transferred bone marrow cells from wt, *Itk*$^{-/-}$, and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice into irradiated Beta-2 microglobulin (B2m)-deficient mice. *B2m*$^{-/-}$ mice fail to develop mature CD8+ cells due to the lack of significant MHC class I expression on the selecting thymic epithelium. Accordingly, transfer of wt bone marrow into *B2m*$^{-/-}$ recipients gave rise to very low numbers of mature CD8+ T cells compared to transfers into C57BL/6 recipients (Figures 4A and 5B). Remarkably, transfer of bone marrow from *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ into *B2m*$^{-/-}$ mice gave rise to CD8 SP cells. The CD8+ cells were TCR^{hi}, arguing that they were mature SP cells (Figure 4B). Thus, *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ CD8 SP cells can arise in the absence of selecting MHC class I on the radioresistant thymic epithelium.

Itk$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ CD8+ T Cells Develop Independent of MHC on the Thymic Stroma

Our initial interpretation of the *B2m*$^{-/-}$ transfer data was that the CD8 SP cells arising in these bone marrow transfers were MHC class II-restricted cells that were diverted to the CD8 lineage. For this interpretation to be correct, however, development of these CD8 SP cells should be MHC class II dependent. To address this question, bone marrow transfers were performed into irradiated *B2m*$^{-/-}$*H2-Ab1*$^{-/-}$ (MHC-deficient) hosts. As expected, transfer of wt bone marrow into MHC-deficient hosts failed to yield substantial populations of mature CD4 and CD8 SP cells (Figure 5A). (The few CD4 SP cells that develop in these hosts have been previously shown to be CD4 intermediate cells that fail to develop into fully mature CD4 SP cells [Cosgrove et al., 1991]). Surprisingly, transfer of bone marrow from *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice into irradiated MHC-deficient recipient mice gave rise to CD8+ cells, as well as a more minor population of CD4+ cells. The CD8 SP cells were not observed until late times after transfer, arguing that these were not contaminating mature T cells in the bone

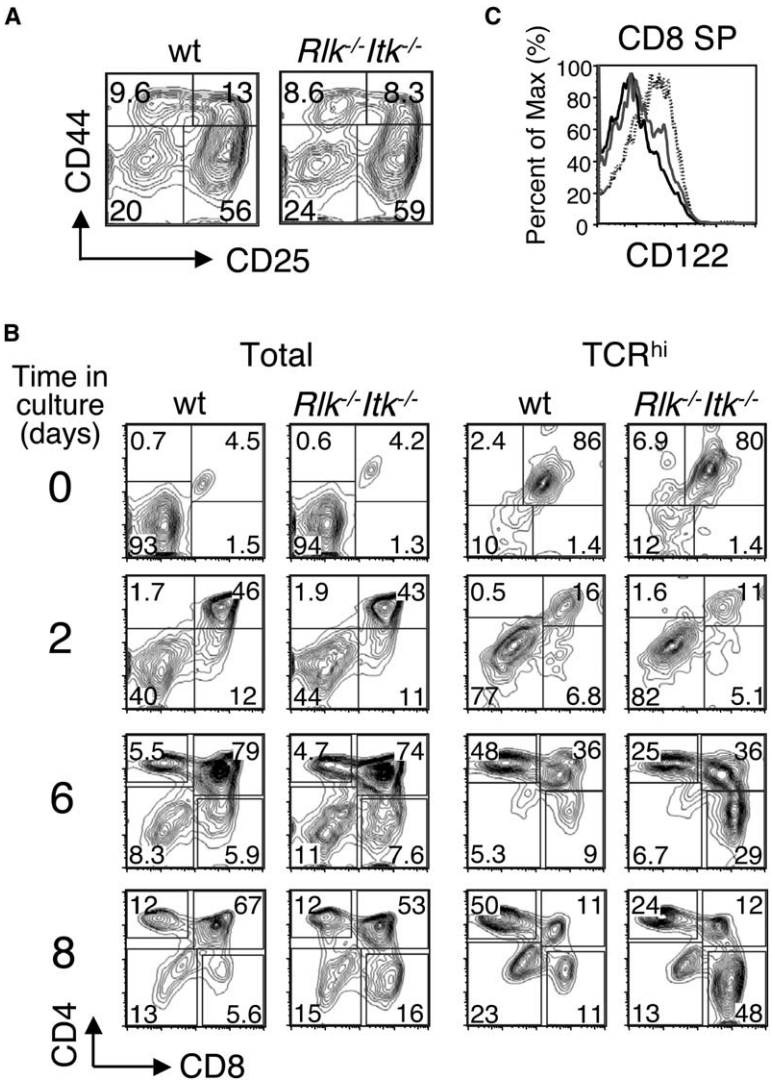

Figure 3. Fetal Thymic Organ Culture: Development of CD8+ Cells Expressing "Memory Cell" Markers Occurs Intrathymically

(A) Profiles of triple-negative (TCR, CD4, and CD8) thymocytes stained with anti-CD44 and anti-CD25 from fetal thymic organ cultures (FTOCs) on day 0.

(B) Immunophenotyping of day 0, 2, 6, and 8 of FTOC. Left, profiles of thymocytes stained with anti-CD4 and anti-CD8. Right, TCR^{hi}-gated CD4 and CD8 profiles.

(C) Analysis of memory marker CD122 on CD8 SP cells derived from day 8 FTOC: solid lines are derived from duplicate wt thymic cultures, dotted lines are from $Rlk^{-/-}Itk^{-/-}$ cultures. Data are representative of duplicate thymi analyzed for each time point in two independent experiments.

marrow (data not shown) and were TCR^{hi}, suggesting that they were mature cell populations despite the lack of selecting MHC on thymic stroma. These CD8 SP cells reached 50%–100% of the numbers of CD8 SP T cells that developed in C57BL/6 recipients (Figure 5B). In contrast, CD4 SP cells in MHC-deficient recipient mice that received $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ bone marrow arose in numbers only slightly greater than in animals that had received bone marrow from wt donors. Moreover, the CD8 SP cells derived from $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ bone marrow also showed high CD122 and CD44 expression (Figures 5A and 4C and data not shown). Thus, the altered $CD8^+$ T cells in $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ animals develop independent of any selecting MHC on the thymic stroma.

Development of $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ $CD8^+$ T Cells Does Require Class I Expression

In bone marrow chimeras, a small amount of selecting MHC is present on the developing T cells and other hematopoietically derived cells. The development of CD8 SP cells in MHC-deficient hosts raised the question of whether MHC-TCR interactions are required at all for their development and full maturation. To address this issue, we performed genetic crosses of $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ animals to $B2m^{-/-}$ mice, where virtually no MHC class I molecules are expressed. In contrast to the bone marrow chimeras, where CD8 SP cells developed in the $B2m^{-/-}$ recipients, genetic deficiency of B2m greatly reduced the number of CD8 SP cells generated in $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ animals (Figure 5C). Although a small number of CD8 SP cells were observed in the thymus, analyses of TCR expression revealed only minimal numbers of mature TCR^{hi} CD8 SP cells, consistent with a requirement for class I for selection and/or maturation of these cells. Thus, most of the unusual CD8 SP cell populations in $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ do require MHC class I for their development, but unlike wt conventional $CD8^+$ T cells, these cells can develop independently of MHC expression on the thymic epithelium.

$Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ CD8 T Cells Share Phenotypes with Class Ib-Selected Cells

The selection of $CD8^+$ T cells independent of MHC on the thymic stroma is reminiscent of selection of certain MHC

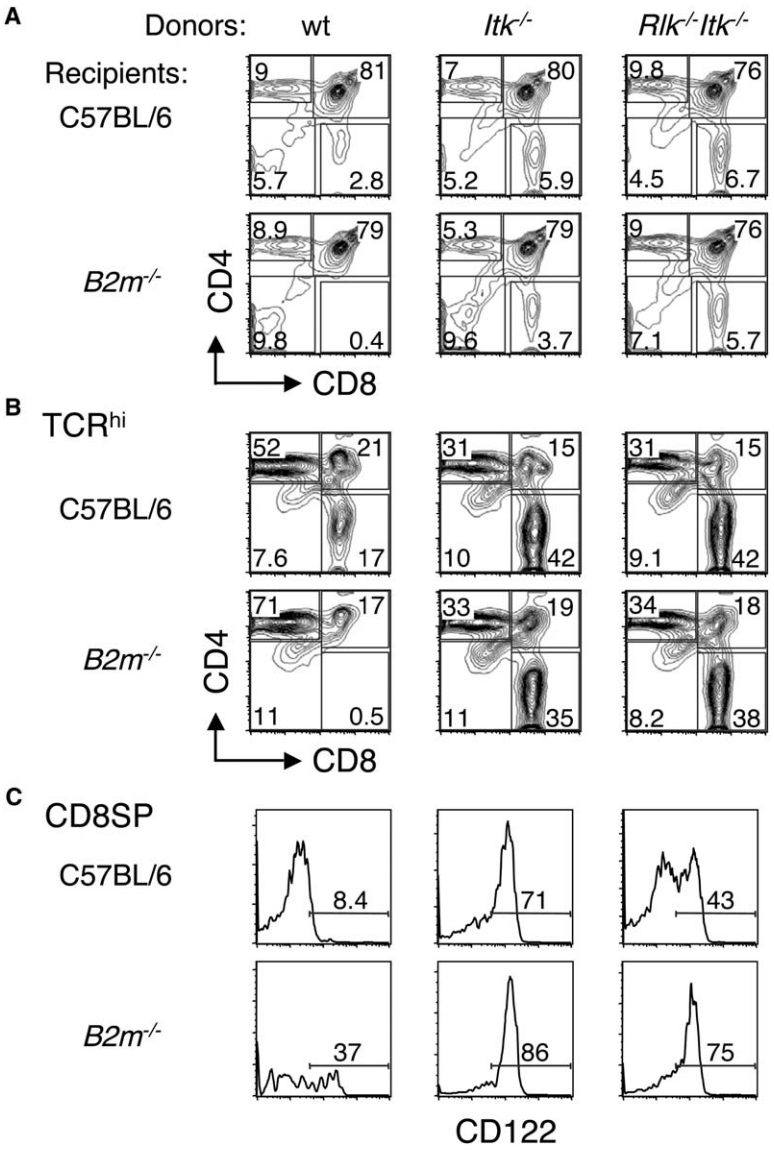

Figure 4. *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ Bone Marrow Transferred into *B2m*$^{-/-}$ Recipients Develop into CD8$^+$ T Cells

(A) Thymocytes stained with anti-CD4 and anti-CD8 from wt (row one) and *B2m*$^{-/-}$ (row two) recipients injected with wt (column one), *Itk*$^{-/-}$ (column two), or *Rlk*$^{-/-}$*Itk*$^{-/-}$ (column three) bone marrow and harvested 8 weeks postinjection.

(B) TCR^{hi}-gated CD4 and CD8 profiles of the same donor-recipient pairs.

(C) CD122 histogram profiles of CD8 SP cells from the same donor-recipient pairs. Data are representative of at least five experiments with triplicate transfers.

class Ib-restricted cells, which can occur on hematopoietic cells within the thymus. These cells include NKT and H2-M3-restricted cells, populations that are also characterized by the expression of memory cell markers and rapid expression of cytokines. CD8$^+$ cells that develop in K^{b}D^b (class Ia)-deficient mice express a number of surface markers that are distinct from classically selected CD8$^+$ T cells, which may result from their selection on hematopoietic cells (Urdahl et al., 2002). Similar to class Ib-restricted cells, mature CD8 SP thymocytes in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice show high CD44 and CD122 expression, slight increases in CD11a, and low expression of β_7 integrin (Figure S2). Peripheral CD8$^+$ cells also resemble cells from K^{b}D^b-deficient mice with increased expression of Ly6C. Moreover, evaluation by intracellular cytokine staining demonstrated that the majority of the CD8 SP cells in these mice rapidly produce IFN-γ in response to stimulation with PMA plus ionomycin, even in the thymus (Figure 6A and Figure S3). Although cytokine expression was most clearly observed in CD8$^+$ cells, a subset of mature CD4$^+$ T cells also rapidly produced cytokines, including IL-4. Thus, CD8$^+$ and a fraction of CD4$^+$ T cells in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice exhibit features that resemble those seen in MHC class Ib-selected innate-type lymphocytes.

To determine whether the altered CD8$^+$ T cells in *Itk*$^{-/-}$ mice are selected by MHC class Ib, we crossed *Itk*$^{-/-}$ mice with *K*$^{b-/-}$*D*$^{b-/-}$ mice. K^{b}D^b-deficient mice select very low numbers of CD8 SP cells in the thymus. However, Itk deficiency led to marked increases in both the percentage and absolute numbers of mature CD8 SP cells in the thymus (Figures 6B and 6C). Although the mature CD8$^+$ T cells in *Itk*$^{-/-}$*K*$^{b-/-}$*D*$^{b-/-}$ mice account for only one-third of the numbers of mature CD8$^+$ cells in class Ia-sufficient Itk-deficient mice, CD8$^+$HSA^{lo} cell numbers in *Itk*$^{-/-}$*K*$^{b-/-}$*D*$^{b-/-}$ mice were increased 26-fold compared to *K*$^{b-/-}$*D*$^{b-/-}$ mice. Thus, mutation of Itk dramatically increases the numbers of cells that can be selected by MHC class Ib; however, a substantial portion of these altered CD8$^+$ T cells still require class Ia for their selection and/or development. Together, our results suggest

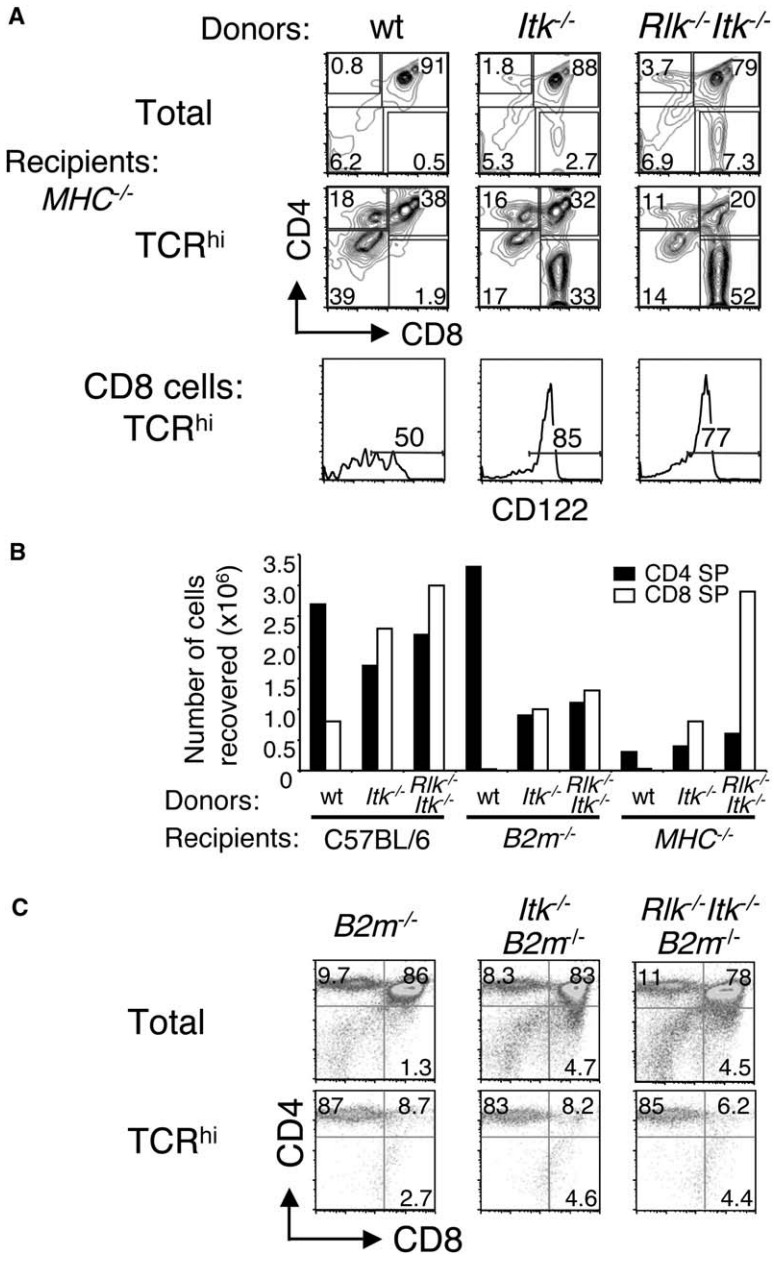

Figure 5. Transfer of Bone Marrow from *Itk*$^{-/-}$ or *Rlk*$^{-/-}$*Itk*$^{-/-}$ Mice into Irradiated MHC-Deficient (*MHC*$^{-/-}$) Host Mice Also Gives Rise to CD8$^+$ Cells

(A) Thymocytes of *MHC*$^{-/-}$ recipients injected with wt (column one), *Itk*$^{-/-}$ (column two), or *Rlk*$^{-/-}$*Itk*$^{-/-}$ (column three) bone marrow and harvested 8 weeks postinjection. Row one, CD4 and CD8 profiles. Row two, TCR^{hi}-gated CD4 and CD8 profiles of the same donor-recipient pairs. Row three, CD122 histogram profiles of CD8 SP cells from the same donor/recipient pairs.

(B) Absolute numbers of CD4 (black bars) and CD8 (white bars) T cells recovered from wt, *B2m*$^{-/-}$, or *MHC*$^{-/-}$ recipients injected with wild-type, *Itk*$^{-/-}$, and *Rlk*$^{-/-}$*Itk*$^{-/-}$ bone marrow. Data are the absolute values from the mice shown in Figures 4 and 5A and are representative of at least four experiments with triplicate transfers.

(C) Genetic crosses to *B2m*$^{-/-}$ mice prevent development of mature CD8 SP cells in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice. Profiles of thymocytes stained with anti-CD4 and anti-CD8 are shown in row one. TCR^{hi}-gated CD4 and CD8 profiles of the same donor-recipient pairs are shown in row two. Data represent analyses of one to two mice per genotype in each of two independent experiments.

that mutations affecting the Tec kinases Itk and Rlk both prevent conventional CD8$^+$ T cell development and permit innate-type cells to be selected by MHC expressed on hematopoietic cells, including both conventional class Ia and nonconventional class Ib.

Signals Required for Conventional versus Altered CD8$^+$ T Cell Development

The development of this large population of altered CD8$^+$ T cells in mice deficient in Itk and Rlk raised the possibility that these cells arise specifically because of their defective signaling. We have previously shown that both *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ thymocytes show impaired activation of Erk1 and Erk2 (Schaeffer et al., 2000), molecules implicated in regulating positive selection, as well as CD4:8 lineage commitment and/or maturation. To evaluate whether altered signaling contributes to these phenotypes, we crossed *Itk*$^{-/-}$ mice to transgenic mice expressing a hypersensitive allele of Erk2, the sevenmaker mutant (*Erk*^{sem}), driven by the CD2 promoter (Sharp et al., 1997). Consistent with the idea that impaired Erk activation in *Itk*$^{-/-}$ mice might contribute to their decreased CD4:8 ratios, *Itk*$^{-/-}$*Erk*^{sem} mice exhibited normalized thymic profiles with increased CD4:8 ratios (Figure 7A). Interestingly, CD8 SP cells in *Itk*$^{-/-}$*Erk*^{sem} mice also appeared phenotypically more similar to wt CD8 SP cells, exhibiting reduced CD122 and CD44 expression and decreased cytokine production compared to CD8 SP cells from *Itk*$^{-/-}$ mice (Figures 7A and 7B). Similarly, when thymic selection was rescued via strong ligands as is seen in *Rlk*$^{-/-}$*Itk*$^{-/-}$ male HY transgenic mice, which we have previously shown switch negative selection to positive selection

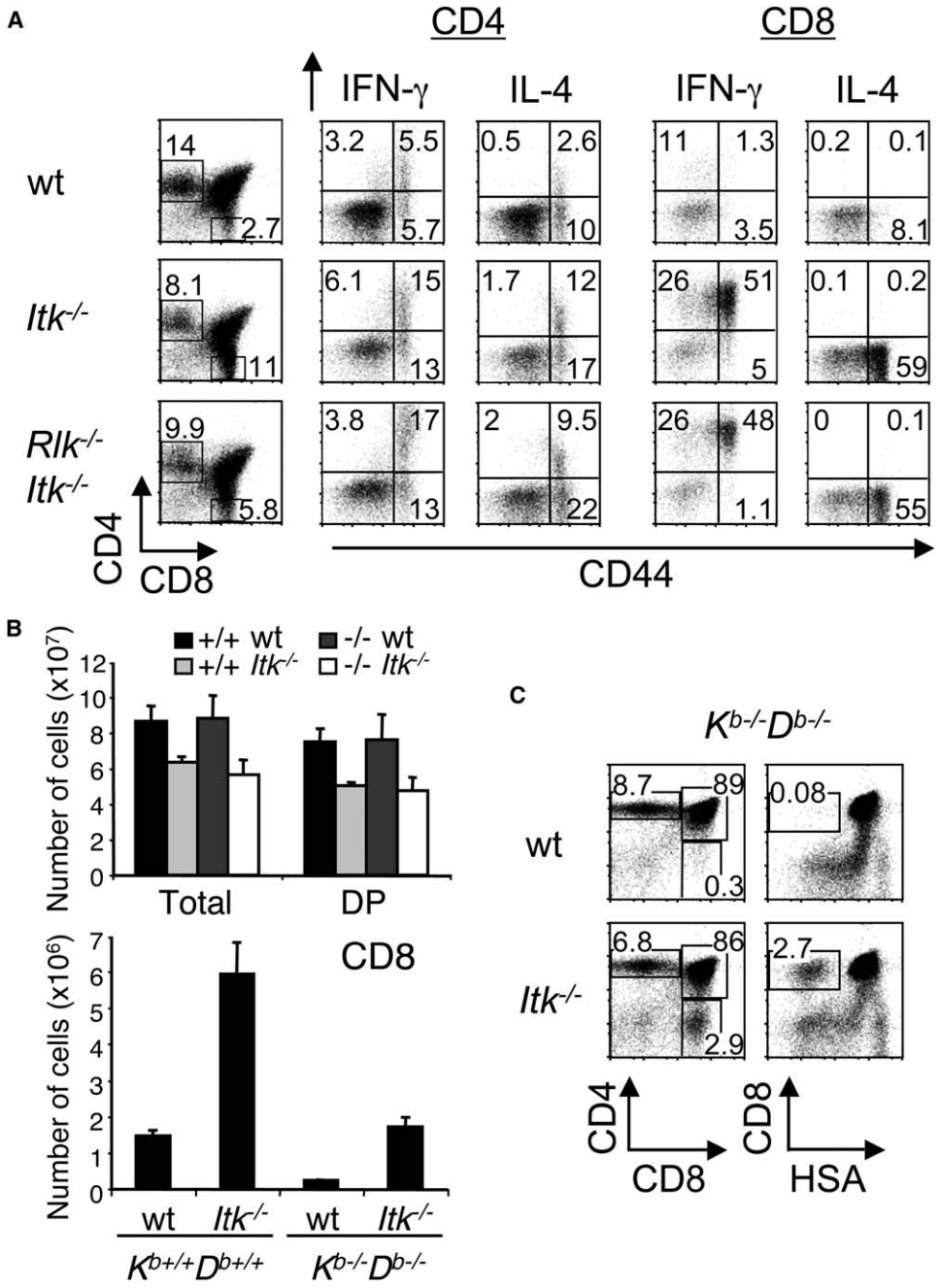

Figure 6. *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ Mature CD8^{+} Thymocytes Resemble MHC Class Ib-Restricted Cells

(A) *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mature CD8^{+} thymocytes produce cytokines. Thymocytes from wt, *Itk*$^{-/-}$, and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice were treated with PMA and ionomycin in the presence of Golgi-plug for 5 hr, and intracellular cytokine analyses were performed by flow cytometry. Cells were costained with anti-CD4, anti-CD8, and anti-CD44. Data are representative of three experiments analyzing two mice per genotype.

(B) Total, DP, and CD8 SP cell numbers of thymocytes in *Itk*$^{-/-}$ mice crossed to $K^{b-/-}D^{b-/-}$ mice (+/+ refers to $K^{b+/+}D^{b+/+}$ and −/− to $K^{b-/-}D^{b-/-}$ mice, respectively). Data represent the average of one to two mice of each genotype in two independent experiments (three to four mice total) ± SEM.

(C) Profiles of thymocytes from $K^{b-/-}D^{b-/-}$ and $K^{b-/-}D^{b-/-}$*Itk*$^{-/-}$ mice stained with anti-CD4, anti-CD8, and anti-HSA.

(Schaeffer et al., 2000), we also observed rescue of these memory cell phenotypes (Figure S4). These results suggest that altered signaling both prevents conventional CD8^{+} T cell development and contributes to the generation of the large population of nonconventional CD8^{+} T cells in *Itk*$^{-/-}$ mice.

Discussion

We present here data demonstrating that Tec family kinases influence the development of distinct CD8^{+} cell lineages in the thymus. Although the CD8^{+} T cells that develop in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice express memory

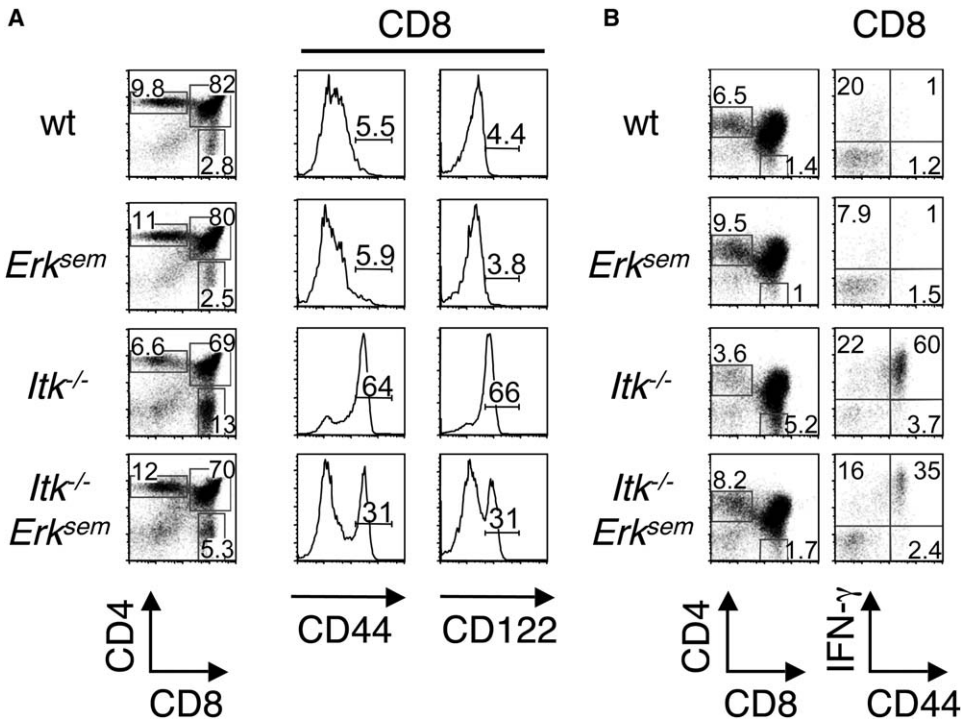

Figure 7. Erk2^{sem} Mutant Partially Rescues Developmental Alterations in *Itk*$^{-/-}$ Mice

(A) Profiles of wt, *Itk*$^{-/-}$, *Erk*^{sem}, and *Itk*$^{-/-}$*Erk*^{sem} thymocytes stained with anti-CD4 and anti-CD8 are shown on the left. Expression of memory cell markers CD122 and CD44 is shown for CD8 SP cells. Data represent similar analyses in three independent experiments.

(B) Reduced IFN-γ expression in stimulated CD44^{hi}CD8^{+} *Itk*$^{-/-}$*Erk*^{sem} thymocytes. Thymocytes were treated as in Figure 6A. Data are representative of three mice examined in two experiments.

cell markers and low HSA, suggesting that they may be mature peripheral cells that had migrated back into the thymus, our data argue that, instead, these altered CD8^{+} cells are intrathymically derived. Indeed, whereas many features of these cells are reminiscent of memory CD8^{+} T cells, there are several distinct characteristics that suggest that they may be more closely related to nonconventional or innate-type T cell lineages. The expression of memory markers, including NK1.1, the ability to express cytokines rapidly, and their dependence on IL-15 (Atherly et al., 2006) are all features shared by memory CD8^{+} T cells and NKT cells. However, the demonstration that these cells can be selected on hematopoietically derived cells rather than the thymic epithelium strongly argues that these cells represent a distinct lineage with characteristics similar to innate-type cells. Thus, although we initially interpreted the increased CD8^{+} T cell numbers as supporting a role for Tec kinases in CD4:8 lineage determination, our data are more consistent with the hypothesis that the CD8^{+} T cells in mice deficient in Itk and Rlk are a lineage distinct from conventional CD8^{+} T cells.

Although thymocytes are usually thought of having three fates, this paradigm does not explain the development of all mature T cell populations. In particular, recent data demonstrate the intrathymic development of several cell lineages, including NKT, CD8αα IELs, and H2-M3-restricted cells that can be selected by agonist peptides or phospholipids associated with nonclassical B2m-associated MHC class Ib molecules and have properties similar to innate cells (Baldwin et al., 2004). These classes of cells are notable for their expression of activation or memory markers and their rapid secretion of cytokines, features that are also shared by virtually all of the CD8^{+} T cells in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice. Moreover, both NKT and H2-M3-restricted cells can be selected by class Ib expressed on hematopoietically derived cells in the thymus, similar to the selection seen in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice. Although the bulk of the CD8 T cells in the *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice do express CD8αβ dimers (data not shown) and are therefore not likely to represent the CD8αα lineage, and express only low NK1.1, the similarities among these different classes of cells is notable. Furthermore, deficiency of Itk markedly increased the number of cells that can be selected on MHC class Ib, arguing that many of these CD8^{+} cells are truly nonconventional innate-type class Ib-restricted lymphocytes. While still only a portion of the CD8^{+} cells in Itk-deficient mice are likely to be selected by class Ib (arguing that these altered cells can also be selected by MHC class Ia), mature T cells in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice, like class Ib-restricted cells, exhibit features at the interface between innate and adaptive immunity. Moreover, virtually all of the CD8^{+} T cells in these mice exhibit these properties, arguing that the Tec kinases are required for normal conventional CD8^{+} T cell development.

The appearance of this large population of CD8 SP cells when *Itk*$^{-/-}$ or *Rlk*$^{-/-}$*Itk*$^{-/-}$ bone marrow is transferred to MHC-deficient hosts is remarkable, demonstrating that these cells develop independent of signals from MHC on the thymic stroma, unlike the majority of

SP cells in wt mice. Although genetic crosses with *B2m*$^{-/-}$ mice argue that selection or development of most of these cells does require MHC class I, our bone marrow transfers show that the selecting class I can be expressed on hematopoietically derived cells. This type of selection is clearly not sufficient for development of a large population of CD8 SP cells after transfer of wt bone marrow cells to *B2m*$^{-/-}$ recipients, raising the possibility that the altered signaling in cells lacking Tec kinases specifically predisposes thymocytes to take this distinct developmental pathway.

Our results suggest that if thymic positive selection signals are normal, thymocytes may preferentially develop into conventional T cells and such innate-type cells may normally be restricted in numbers. However, in the absence of adequate signals, conventional T cell development may not proceed, and under certain conditions, an alternative developmental pathway may be activated by hematopoietic cells that preferentially leads to these innate-type CD8$^+$ cells. Alternatively, these altered lineages may arise from selection on hematopoeitic cells because the impaired signaling in Tec kinase-deficient thymocytes specifically permits selection of innate-type lymphocytes by hematopoietic cells. In particular, the rescue of these phenotypes by the *Erk*^{sem} transgene suggests that signaling defects specifically allow the development of these cells and that the signaling pathways initiated by hematopoietic cells might not require or may even be inhibited by activation of Erk. Although Erk activation defects alone may not account for these phenotypes (Fischer et al., 2005), it is of interest that previous studies of mice expressing a dominant-negative Ras mutant argue that NKT cell development does not require Erk activation (Alberola-Ila et al., 1996). Furthermore, when we rescue selection via a strong ligand such as in male *Rlk*$^{-/-}$*Itk*$^{-/-}$ HY transgenic mice, we no longer see these phenotypes.

It is therefore possible that although the Tec family kinases play positive roles in TCR signals that lead to conventional T cell development, they may have negative roles in other signaling pathways that may permit selection on hematopoietic cells. One possibility is that the reduction of TCR signaling may allow innate-type cells to survive that might normally be deleted by strong TCR signals in conjunction with other pathways initiated by hematopoietic cells, whereas the same reduction of TCR signaling may prevent positive selection of conventional CD8$^+$ cells by the thymic epithelium. Alternatively, prolonged TCR signaling itself may be responsible for either turning off or preventing expression of factors required for pathways involved in the development of this altered CD8 lineage. Such pathways may include those downstream of cytokines, including IL-7 and 15, which have been implicated as second signals for both conventional CD8$^+$ and memory CD8$^+$ T cell development and homeostasis (Ohteki, 2002; Yu et al., 2003). Indeed, TCR signaling has been associated with decreased responsiveness to cytokines (Zhu et al., 2000). Interestingly, IL-15 is also required for the development and maintenance of NKT and CD8$\alpha\alpha$ cells (Ohteki, 2002). A requirement for IL-15 in other lineages that share these phenotypes, such as H2-M3-restricted cells, is not known.

In this regard, it is of note that CD122, the IL-2Rβ subunit, is also a required receptor for IL-15 (Giri et al., 1994). Although CD44^{hi}CD8$^+$ T cells can be divided into CD122$^+$ and CD122$^-$ subpopulations (Judge et al., 2002), *Rlk*$^{-/-}$*Itk*$^{-/-}$ CD8$^+$ T cells are predominantly CD122$^+$ and thus may be particularly sensitive to IL-15. It is therefore relevant that Atherly et al. demonstrate reduced numbers of both CD8 SP thymocytes and mature CD8$^+$ T cells, including all of the CD44^{hi} peripheral CD8$^+$ cells, in *Itk*$^{-/-}$*IL-15*$^{-/-}$ mice (Atherly et al., 2006). Nonetheless, IL-15 may not be the only cause of the increased memory-phenotype cells in the thymus of *Itk*$^{-/-}$ mice—other signals provided by hematopoietic cells may also help initiate or contribute to these phenotypes. However, the critical role of cytokines in CD8 T cell development may help explain why CD8$^+$ T cells are more affected than CD4$^+$ T cells in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice. Increased responsiveness to cytokines may facilitate the establishment of a positive feedback loop affecting both generation and homeostasis of these innate-type, memory-phenotype CD8$^+$ cells in the thymus and periphery. The increased expression of eomesodermin in these cells (Atherly et al., 2006), which can induce CD122 expression (Intlekofer et al., 2005), may well contribute to the sensitivity of these cells to alternative differentiation and maintenance signals.

Nonetheless, although we have concentrated on the CD8$^+$ T cell phenotype, it should be noted that some CD4$^+$ T cells in the thymus and periphery of *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ animals also exhibit memory cell markers and rapid expression of cytokines. Indeed, it is surprising that mature peripheral CD4$^+$ T cells in *Itk*$^{-/-}$ mice show rapid production of both IL-4 and IFN-γ, because in vivo, Itk-deficient mice have defective T_H2 responses (Fowell et al., 1999; Miller et al., 2004; Schaeffer et al., 2001). Thus, both CD8$^+$ and CD4$^+$ T cells that develop under conditions of limited signaling may exhibit properties that distinguish them from conventional T cells. Our results also argue that caution must be taken when examining mature T cell functions from mice with altered signaling and selection in the thymus. It may therefore be of interest to revisit the phenotypes of SP cells in other mouse mutants with impaired TCR signaling. However, we should also note that other factors may specifically contribute to the phenotypes of CD8 and CD4 SP thymocytes in Tec kinase mutant mice, including impaired responses to chemokines and integrins (Finkelstein et al., 2005; Takesono et al., 2004), which might differentially affect CD4$^+$ and CD8$^+$ thymocyte development or exit.

In recent years, there has been a growing appreciation of distinct lineages of thymic-derived cells that possess properties at the boundary of the innate and adaptive immune systems. The development of a large class of cells with features of these lineages may therefore provide information about the signals that regulate the development of these unique and important types of cells. The signaling pathways regulated by the Tec kinases may be critical determinants that differentially regulate the development of conventional versus nonconventional lineages and the balance of adaptive verses innate lymphocyte development. Together with the paper by Berg and colleagues (Atherly et al., 2006), our results highlight the complexity of pathways regulating thymic

development and provide new insights into the consequences of TCR signaling for thymic lineage decisions.

Experimental Procedures

Mouse Strains

Itk$^{-/-}$, *Rlk*$^{-/-}$*Itk*$^{-/-}$ (Schaeffer et al., 1999), *Rlk*$^{-/-}$*Itk*$^{-/-}$HY (Schaeffer et al., 2000), and *Erk*^{sem} transgenics (Sharp et al., 1997) were previously described. *B2m*$^{-/-}$, MHC-deficient (*B2m*$^{-/-}$*H2-Ab1*$^{-/-}$), and $K^{b-/-}D^{b-/-}$ (Perarnau et al., 1999) mice were obtained from the NIAID Taconic Exchange Program. All animals were maintained in SPF conditions. Animals were maintained and experiments performed according to NHGRI animal care and use committee guidelines.

Antibodies and Flow Cytometry

Single cell preparations of thymi and spleens were harvested from adult and neonatal mice of the indicated ages. Red blood cells were lysed in splenocytes with ammonium chloride for ~5 min. Cells were blocked with anti-CD16/32 (2.4G2) and stained with the following antibodies: anti-CD4-PE, -Cy-Chrome, -PerCP-Cy5.5, and -APC (RM4-5); anti-CD8-FITC, -APC, and -Cy-Chrome (53-6.7); anti-CD25-PE (3C7); anti-CD122-PE(TM-β1); anti-CD62L-PE (MEL-14); anti-CD44-FITC (IM7); anti-TCRβ-APC (H57-597); anti-HSA (CD24)-FITC (M1/69); anti-CD11a-FITC (M17/4); anti-β_7 integrin-PE (M293); anti-Ly6C-FITC (AL21); anti-CD8β-PE (53-5.8); anti-NK1.1; and anti-Ly 5.2 (Pharmingen/Becton Dickinson, San Diego, CA). Anti-HY TCR-FITC (T3.70) was from eBioscience (San Diego, CA). Data were collected on a FACSCalibur (Becton-Dickinson, San Diego, CA) and analyzed by using FlowJo (TreeStar, San Carlos, CA). Typically 30,000–50,000 live events were collected per sample (gated by FSC and SSC).

Fetal Thymic Organ Culture

Fetal thymi were harvested from timed pregnant females on day 15.5 pc. Thymic lobes were separated and placed in transwell plates (CoStar) with 1.5 ml DMEM medium (containing 10% fetal calf serum, 10% NCTC 109 hybridoma medium, sodium pyruvate, nonessential amino acids, penicillin/streptomycin, 2-mercaptoethanol, and HEPES) for the indicated time periods.

Bone Marrow Chimeras

Bone marrow chimeras were made according to standard protocol (Coligan et al., 1995). Briefly, bone marrow obtained from the femur and tibia of donor mice was depleted of mature T cells with anti-Thy1.2 (J1J) and anti-Ly1.2 (C3PO) plus low-toxicity rabbit complement (Cederlane Labs, Westbury, NY) for 30 min at 37°C then washed and counted. Recipient mice were injected i.v. with 1–2 × 10^7 cells, 2–10 hr after receiving γ irradiation (1000 rad, Cs source) and were maintained on antibiotic water until sacrifice. Thymocytes, lymph nodes, and splenocytes were harvested and analyzed by flow cytometry either 2 or 7–8 weeks after transfer.

Ex Vivo Stimulation and Intracellular Cytokine Detection

Single cell suspensions of thymocytes or splenocytes (2 × 10^6 cells/ml) from unmanipulated mice were stimulated with PMA (10 ng/ml) and Ionomycin (1 μg/ml) in the presence of GolgiPlug (BD Pharmingen) for 5 hr at 37°C, then washed, stained for CD44, CD8, and CD4, and fixed and permeabilized for intracellular staining with either anti-IFN-γ-PE (XMG1.2) or anti-IL-4-PE (BVD4-1D11) for flow cytometry.

Supplemental Data

Supplemental Data include four figures and three tables and can be found with this article online at http://www.immunity.com/cgi/content/full/25/1/93/DC1/.

Acknowledgments

The authors would like to thank L. Berg, L. Atherly, L. Mijares, R. Handon, R. Germain, and members of the Schwartzberg and Berg laboratories for helpful discussions and communication of results. This work was supported by intramural funding from the National Human Genome Research Institute and the National Institute of Allergy and Infectious Diseases. R.H. was supported in part by the Japanese Society for the Promotion of Science.

Received: November 22, 2005
Revised: April 25, 2006
Accepted: May 5, 2006
Published: July 25, 2006

References

Agus, D.B., Surh, C.D., and Sprent, J. (1991). Reentry of T cells to the adult thymus is restricted to activated T cells. J. Exp. Med. *173*, 1039–1046.

Alberola-Ila, J., Hogquist, K.A., Swan, K.A., Bevan, M.J., and Perlmutter, R.M. (1996). Positive and negative selection invoke distinct signaling pathways. J. Exp. Med. *184*, 9–18.

Atherly, L.O., Lucas, J.A., Felices, M., Yin, C.C., Reiner, S.L., and Berg, L.J. (2006). The Tec kinases Itk and Rlk regulate the development of conventional CD8+ T cells. Immunity *25*, this issue, 79–91.

Baldwin, T.A., Hogquist, K.A., and Jameson, S.C. (2004). The fourth way? Harnessing aggressive tendencies in the thymus. J. Immunol. *173*, 6515–6520.

Bendelac, A. (1995). Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. J. Exp. Med. *182*, 2091–2096.

Bendelac, A., Killeen, N., Littman, D.R., and Schwartz, R.H. (1994). A subset of CD4+ thymocytes selected by MHC class I molecules. Science *263*, 1774–1778.

Bendelac, A., Lantz, O., Quimby, M.E., Yewdell, J.W., Bennink, J.R., and Brutkiewicz, R.R. (1995). CD1 recognition by mouse NK1+ T lymphocytes. Science *268*, 863–865.

Berg, L.J., Finkelstein, L.D., Lucas, J.A., and Schwartzberg, P.L. (2005). Tec family kinases in T lymphocyte development and function. Annu. Rev. Immunol. *23*, 549–600.

Bix, M., Coles, M., and Raulet, D. (1993). Positive selection of Vβ8+ CD4−8− thymocytes by class I molecules expressed by hematopoietic cells. J. Exp. Med. *178*, 901–908.

Bommhardt, U., Basson, M.A., Krummrei, U., and Zamoyska, R. (1999). Activation of the extracellular signal-related kinase/mitogen-activated protein kinase pathway discriminates CD4 versus CD8 lineage commitment in the thymus. J. Immunol. *163*, 715–722.

Brugnera, E., Bhandoola, A., Cibotti, R., Yu, Q., Guinter, T.I., Yamashita, Y., Sharrow, S.O., and Singer, A. (2000). Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. Immunity *13*, 59–71.

Chung, B., Aoukaty, A., Dutz, J., Terhorst, C., and Tan, R. (2005). Signaling lymphocytic activation molecule-associated protein controls NKT cell functions. J. Immunol. *174*, 3153–3157.

Coligan, J., Kruisbeek, A., Margulies, D., Shevach, E., and Strober, W. (1995). Current Protocols in Immunology (New York, NY: John Wiley and Sons).

Cosgrove, D., Gray, D., Dierich, A., Kaufman, J., Lemeur, M., Benoist, C., and Mathis, D. (1991). Mice lacking MHC class II molecules. Cell *66*, 1051–1066.

Das, G., Sheridan, S., and Janeway, C.A., Jr. (2001). The source of early IFN-γ that plays a role in Th1 priming. J. Immunol. *167*, 2004–2010.

Dutton, R.W., Bradley, L.M., and Swain, S.L. (1998). T cell memory. Annu. Rev. Immunol. *16*, 201–223.

Eberl, G., Lowin-Kropf, B., and MacDonald, H.R. (1999). Cutting edge: NKT cell development is selectively impaired in Fyn- deficient mice. J. Immunol. *163*, 4091–4094.

Finkelstein, L., Shimizu, Y., and Schwartzberg, P. (2005). Tec kinases regulate TCR-mediated recruitment of signaling molecules and integrin-dependent cell adhesion. J. Immunol. *175*, 5923–5930.

Fischer, A.M., Katayama, C.D., Pages, G., Pouyssegur, J., and Hedrick, S.M. (2005). The role of erk1 and erk2 in multiple stages of T cell development. Immunity *23*, 431–443.

Fowell, D.J., Shinkai, K., Liao, X.C., Beebe, A.M., Coffman, R.L., Littman, D.R., and Locksley, R.M. (1999). Impaired NFATc translocation

and failure of Th2 development in Itk-deficient CD4$^+$ T cells. Immunity *11*, 399–409.

Gadue, P., Morton, N., and Stein, P.L. (1999). The Src family tyrosine kinase Fyn regulates natural killer T cell development. J. Exp. Med. *190*, 1189–1196.

Gangadharan, D., and Cheroutre, H. (2004). The CD8 isoform CD8$\alpha\alpha$ is not a functional homologue of the TCR co-receptor CD8$\alpha\beta$. Curr. Opin. Immunol. *16*, 264–270.

Gapin, L., Matsuda, J.L., Surh, C.D., and Kronenberg, M. (2001). NKT cells derive from double-positive thymocytes that are positively selected by CD1d. Nat. Immunol. *2*, 971–978.

Germain, R.N. (2002). T-cell development and the CD4–CD8 lineage decision. Nat. Rev. Immunol. *2*, 309–322.

Giri, J.G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L.S., Cosman, D., and Anderson, D. (1994). Utilization of the β and γ chains of the IL-2 receptor by the novel cytokine IL-15. EMBO J. *13*, 2822–2830.

Hernandez-Hoyos, G., Sohn, S.J., Rothenberg, E.V., and Alberola-Ila, J. (2000). Lck activity controls CD4/CD8 T cell lineage commitment. Immunity *12*, 313–322.

Intlekofer, A.M., Takemoto, N., Wherry, E.J., Longworth, S.A., Northrup, J.T., Palanivel, V.R., Mullen, A.C., Gasink, C.R., Kaech, S.M., Miller, J.D., et al. (2005). Effector and memory CD8$^+$ T cell fate coupled by T-bet and eomesodermin. Nat. Immunol. *6*, 1236–1244.

Judge, A.D., Zhang, X., Fujii, H., Surh, C.D., and Sprent, J. (2002). Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8$^+$ T cells. J. Exp. Med. *196*, 935–946.

Kennedy, M.K., Glaccum, M., Brown, S.N., Butz, E.A., Viney, J.L., Embers, M., Matsuki, N., Charrier, K., Sedger, L., Willis, C.R., et al. (2000). Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J. Exp. Med. *191*, 771–780.

Kerksiek, K.M., Busch, D.H., Pilip, I.M., Allen, S.E., and Pamer, E.G. (1999). H2–M3-restricted T cells in bacterial infection: rapid primary but diminished memory responses. J. Exp. Med. *190*, 195–204.

Legname, G., Seddon, B., Lovatt, M., Tomlinson, P., Sarner, N., Tolaini, M., Williams, K., Norton, T., Kioussis, D., and Zamoyska, R. (2000). Inducible expression of a p56^{Lck} transgene reveals a central role for Lck in the differentiation of CD4 SP thymocytes. Immunity *12*, 537–546.

Lindahl, K.F., Byers, D.E., Dabhi, V.M., Hovik, R., Jones, E.P., Smith, G.P., Wang, C.R., Xiao, H., and Yoshino, M. (1997). H2-M3, a full-service class Ib histocompatibility antigen. Annu. Rev. Immunol. *15*, 851–879.

Liu, K.Q., Bunnell, S.C., Gurniak, C.B., and Berg, L.J. (1998). T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. J. Exp. Med. *187*, 1721–1727.

Loveland, B., Wang, C.R., Yonekawa, H., Hermel, E., and Lindahl, K.F. (1990). Maternally transmitted histocompatibility antigen of mice: a hydrophobic peptide of a mitochondrially encoded protein. Cell *60*, 971–980.

Lucas, J.A., Atherly, L.O., and Berg, L.J. (2002). The absence of Itk inhibits positive selection without changing lineage commitment. J. Immunol. *168*, 6142–6151.

Miller, A.T., Wilcox, H.M., Lai, Z., and Berg, L.J. (2004). Signaling through Itk promotes T helper 2 differentiation via negative regulation of T-bet. Immunity *21*, 67–80.

Min, B., McHugh, R., Sempowski, G.D., Mackall, C., Foucras, G., and Paul, W.E. (2003). Neonates support lymphopenia-induced proliferation. Immunity *18*, 131–140.

Nichols, K.E., Hom, J., Gong, S.Y., Ganguly, A., Ma, C.S., Cannons, J.L., Tangye, S.G., Schwartzberg, P.L., Koretzky, G.A., and Stein, P.L. (2005). Regulation of NKT cell development by SAP, the protein defective in XLP. Nat. Med. *11*, 340–345.

Ohteki, T. (2002). Critical role for IL-15 in innate immunity. Curr. Mol. Med. *2*, 371–380.

Ohteki, T., and MacDonald, H.R. (1994). Major histocompatibility complex class I related molecules control the development of CD4$^+$8$^-$ and CD4$^-$8$^-$ subsets of natural killer 1.1$^+$ T cell receptor-α/β^+ cells in the liver of mice. J. Exp. Med. *180*, 699–704.

Pasquier, B., Yin, L., Fondaneche, M.C., Relouzat, F., Bloch-Queyrat, C., Lambert, N., Fischer, A., de Saint-Basile, G., and Latour, S. (2005). Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. J. Exp. Med. *201*, 695–701.

Perarnau, B., Saron, M.F., San Martin, B.R., Bervas, N., Ong, H., Soloski, M.J., Smith, A.G., Ure, J.M., Gairin, J.E., and Lemonnier, F.A. (1999). Single *H2K^b*, *H2D^b* and double *H2K^{b}D^b* knockout mice: peripheral CD8$^+$ T cell repertoire and anti-lymphocytic choriomeningitis virus cytolytic responses. Eur. J. Immunol. *29*, 1243–1252.

Reinhardt, R.L., Khoruts, A., Merica, R., Zell, T., and Jenkins, M.K. (2001). Visualizing the generation of memory CD4 T cells in the whole body. Nature *410*, 101–105.

Schaeffer, E., Yap, G., Lewis, C.M., Czar, M.J., McVicar, D.W., Sher, A., and Schwartzberg, P. (2001). Mutation of Tec family kinases alters T helper cell differentiation. Nat. Immunol. *2*, 1183–1188.

Schaeffer, E.M., Debnath, J., Yap, G., McVicar, D., Liao, X.C., Littman, D.R., Sher, A., Varmus, H.E., Lenardo, M.J., and Schwartzberg, P.L. (1999). Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. Science *284*, 638–641.

Schaeffer, E.M., Broussard, C., Debnath, J., Anderson, S., McVicar, D.W., and Schwartzberg, P.L. (2000). Tec family kinases modulate thresholds for thymocyte development and selection. J. Exp. Med. *192*, 987–1000.

Sharp, L.L., Schwarz, D.A., Bott, C.M., Marshall, C.J., and Hedrick, S.M. (1997). The influence of the MAPK pathway on T cell lineage commitment. Immunity *7*, 609–618.

Singer, A., and Bosselut, R. (2004). CD4/CD8 coreceptors in thymocyte development, selection, and lineage commitment: analysis of the CD4/CD8 lineage decision. Adv. Immunol. *83*, 91–131.

Stanic, A.K., Bezbradica, J.S., Park, J.-J., Kaer, L.V., Boothby, M.R., and Joyce, S. (2004). Cutting edge: the ontogeny and function of Vα14Jα18 natural T lymphocytes require signal processing by protein kinase C θ and NF-κB. J. Immunol. *172*, 4667–4671.

Starr, T.K., Jameson, S.C., and Hogquist, K.A. (2003). Positive and negative selection of T cells. Annu. Rev. Immunol. *21*, 139–176.

Takesono, A., Horai, R., Mandai, M., Dombroski, D., and Schwartzberg, P.L. (2004). Requirement for tec kinases in chemokine-induced migration and activation of cdc42 and rac. Curr. Biol. *14*, 917–922.

Urdahl, K.B., Sun, J.C., and Bevan, M.J. (2002). Positive selection of MHC class Ib-restricted CD8$^+$ T cells on hematopoietic cells. Nat. Immunol. *3*, 772–779.

Wilkinson, B., and Kaye, J. (2001). Requirement for sustained MAPK signaling in both CD4 and CD8 lineage commitment: a threshold model. Cell. Immunol. *211*, 86–95.

Yamagata, T., Mathis, D., and Benoist, C. (2004). Self-reactivity in thymic double-positive cells commits cells to a CD8$\alpha\alpha$ lineage with characteristics of innate immune cells. Nat. Immunol. *5*, 597–605.

Yoshimoto, T., and Paul, W.E. (1994). CD4^{pos}, NK1.1^{pos} T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. J. Exp. Med. *179*, 1285–1295.

Yu, Q., Erman, B., Bhandoola, A., Sharrow, S.O., and Singer, A. (2003). In vitro evidence that cytokine receptor signals are required for differentiation of double positive thymocytes into functionally mature CD8$^+$ T cells. J. Exp. Med. *197*, 475–487.

Zhu, J., Huang, H., Guo, L., Stonehouse, T., Watson, C.J., Hu-Li, J., and Paul, W.E. (2000). Transient inhibition of interleukin 4 signaling by T cell receptor ligation. J. Exp. Med. *192*, 1125–1134.