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Requirements for Selection of Conventional and Innate T Lymphocyte Lineages

Reiko Horai,[1,2] Kristen L. Mueller,[1] Robin A. Handon,[1] Jennifer L. Cannons,[1] Stacie M. Anderson,[1] Martha R. Kirby,[1] and Pamela L. Schwartzberg[1,*]
[1]National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA
[2]Present address: National Eye Institute, National Institutes of Health, Bethesda, MD 20892, USA.
*Correspondence: pams@mail.nih.gov
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SUMMARY

Mice deficient in the Tec kinase Itk develop a large population of CD8$^+$ T cells with properties, including expression of memory markers, rapid production of cytokines, and dependence on Interleukin-15, resembling NKT and other innate T cell lineages. Like NKT cells, these CD8$^+$ T cells can be selected on hematopoietic cells. We demonstrate that these CD8$^+$ T cell phenotypes resulted from selection on hematopoietic cells—forcing selection on the thymic stroma reduced the number and innate phenotypes of mature Itk-deficient CD8$^+$ T cells. We further show that, similar to NKT cells, selection of innate-type CD8$^+$ T cells in *Itk*$^{-/-}$ mice required the adaptor SAP. Acquisition of their innate characteristics, however, required CD28. Our results suggest that SAP and Itk reciprocally regulate selection of innate and conventional CD8$^+$ T cells on hematopoietic cells and thymic epithelium, respectively, whereas CD28 regulates development of innate phenotypes resulting from selection on hematopoietic cells.

INTRODUCTION

Although lymphocytes are generally thought to provide the adaptive arm of the immune system, there is a growing appreciation that certain T cell lineages exhibit properties of innate cells and contribute to immediate, early responses to pathogens. Among the earliest lymphocyte responses are those of NKT cells, H2-M3-restricted cells, and other T lymphocytes that are selected by MHC class Ib molecules and that possess properties of innate immune cells (Behar and Porcelli, 2007; Kerksiek et al., 1999; Seaman et al., 2000; Urdahl et al., 2002). These features include expression of memory markers, rapid production of cytokines (within hours of stimulation), and dependence on Interleukin-15 (IL-15) for their homeostasis or expansion (Das et al., 2001; Ohteki, 2002; Urdahl et al., 2002; Yoshimoto and Paul, 1994). Unlike memory T cells, which exhibit these properties after initial activation in the periphery, innate lymphocyte lineages develop these properties within the thymus (reviewed in Baldwin et al. [2004] and Berg [2007]).

The development of T cells having these distinct innate properties within the thymus suggests that the requirements for their selection and differentiation might differ from those required for conventional T cell populations. In particular, NKT cells and other MHC class Ib-restricted cells that arise in H-2Kb-H-2Db (KbDb)-deficient animals can be selected on hematopoietic cells within the thymus, unlike conventional T cells that are selected by recognition of peptide-MHC molecule ligands on the thymic stroma (Bendelac, 1995; Bix et al., 1993; Ohteki and MacDonald, 1994; Urdahl et al., 2002). Moreover, a number of signaling molecules that are not required for conventional T cell development have been implicated in NKT cell development. These include the adaptor molecule SAP (SH2D1A), the tyrosine kinase Fyn, protein kinase C-theta (PKC-θ), and members of the nuclear factor of kappa B (NF-κB) transcription factors (Chung et al., 2005; Eberl et al., 1999; Gadue et al., 1999; Nichols et al., 2005; Pasquier et al., 2005; Schmidt-Supprian et al., 2004; Sivakumar et al., 2003; Stanic et al., 2004). Intriguingly, all of these molecules have been implicated in signaling pathways downstream from SLAM family receptors, a class of cell-surface molecules with complex roles in multiple hematopoietic cell lineages (Cannons et al., 2004; Ma et al., 2007). Nonetheless, Fyn, PKC-θ, and NF-κB are also activated in mature T cells by T cell receptor (TCR) engagement, thereby limiting our understanding of the specific roles they play in development of innate lymphocytes.

Itk is a Tec family tyrosine kinase that contributes to TCR-induced phospholipase C-γ phosphorylation, Ca^{2+} mobilization, and ERK activation (Berg et al., 2005). Itk also participates in TCR- and chemokine-induced actin reorganization and "inside-out signaling" to integrins (Gomez-Rodriguez et al., 2007). *Itk*$^{-/-}$ mice show impaired positive selection of both MHC class I- and class II-specific TCR transgenes (Liao and Littman, 1995; Lucas et al., 2002; Schaeffer et al., 2000). Surprisingly, however, *Itk*$^{-/-}$ mice with polyclonal repertoires only show reduced numbers of CD4 single positive (SP) thymocytes, whereas CD8 SP thymocytes are relatively normal in number. Further analyses of CD8 SP thymocytes in these animals demonstrated that most of these cells exhibit properties of innate T lymphocyte lineages; these CD8$^+$ T cells express memory markers including CD44 and CD122,

rapidly produce cytokines ex vivo, and are IL-15 dependent (Atherly et al., 2006; Berg, 2007; Broussard et al., 2006; Dubois et al., 2006). Fetal thymic organ cultures demonstrated that these cells develop within the thymus and are not the result of activation in the periphery (Broussard et al., 2006). Strikingly, bone-marrow transfers into $B2m^{-/-}$ and $B2m^{-/-}H2\text{-}Ab1^{-/-}$ mice revealed that the $CD8^+$ T cells in $Itk^{-/-}$ mice, like NKT cells and $CD8^+$ T cells that are selected by MHC class Ib molecules, can be selected on hematopoietic cells, independent of the presence of selecting MHC class I molecules on the thymic stroma (Broussard et al., 2006). Thus, the $CD8^+$ T cells that develop in $Itk^{-/-}$ mice resemble innate lymphocyte lineages. Moreover, because the majority of $CD8^+$ T cells in $Itk^{-/-}$ mice exhibit these characteristics, Itk appears to differentially affect the development of conventional and innate-type $CD8^+$ T lineages.

The large number of "innate-like" $CD8^+$ T cells that develop in $Itk^{-/-}$ mice provides an opportunity to analyze the requirements for the development of innate T cell phenotypes. We demonstrate here that the innate characteristics of $CD8^+$ T cells in $Itk^{-/-}$ mice resulted from selection on hematopoietic cells—forcing selection on MHC expressed by the thymic stroma prevented these innate $CD8^+$ T cell phenotypes. We further found that costimulatory signals from hematopoietic cells are required for the development of the innate $CD8^+$ T cell population in $Itk^{-/-}$ mice. Selection of the innate-type $CD8^+$ T cells on hematopoietic cells in $Itk^{-/-}$ mice was dependent on the adaptor molecule SAP, which is required for signaling from SLAM family receptors on T lymphocytes. Development of the innate characteristics of $CD8^+$ T cells in $Itk^{-/-}$ mice also required CD28 costimulation. However, CD28 deficiency did not prevent selection on hematopoietic cells but rather prevented the acquisition of the innate phenotypes. Together, these data suggest that Itk, SAP, and CD28 play distinct roles in innate T cell development, in which Itk and SAP-mediated pathways differentially regulate selection of conventional T cells on the thymic stroma versus nonconventional, innate $CD8^+$ T cell lineages on hematopoietic cells, respectively, and CD28 influences the acquisition of innate-like phenotypes.

RESULTS

Itk Deficiency Increases Selection of MHC Class Ib-Restricted Cells on Hematopoietic Cells

We have previously demonstrated that development of $CD8^+$ T cells in $Itk^{-/-}$ mice requires MHC class I, but like MHC class Ib-restricted cells, these cells can be selected on hematopoietic cells: $CD8^+$ T cells fail to develop in $Itk^{-/-}B2m^{-/-}$ mice, and yet they develop when $Itk^{-/-}$ bone marrow is transferred into $B2m^{-/-}$ mice in which MHC class I expression is restricted to the transferred hematopoietic cells (Broussard et al., 2006). We have further shown that Itk deficiency increases the number of $CD8^+$ T cells developing in $H\text{-}2Kb^{-/-}H\text{-}2Db^{-/-}$ ($Kb^{-/-}Db^{-/-}$) mice, suggesting that Itk deficiency increases selection of MHC class Ib-restricted cells (Broussard et al., 2006). Nonetheless, further examination of these cells demonstrated that a larger proportion of the mature (HSA^{lo}) CD8 SP and peripheral $CD8^+$ T cells in $Itk^{-/-}$ and $Itk^{-/-}H\text{-}2Kb^{-/-}H\text{-}2Db^{-/-}$ mice exhibited memory markers ($CD122^{hi}$ $CD44^{hi}$, β_7 $integrin^{lo}$) than in $H\text{-}2Kb^{-/-}H\text{-}2Db^{-/-}$ mice, which were more variable in their phenotype (Figure 1A and Figure S1 in the Supplemental Data available online). Moreover, the increased percentages and number of mature CD8 SP cells in $Itk^{-/-}H\text{-}2Kb^{-/-}H\text{-}2Db^{-/-}$ mice only partially accounted for the number of mature CD8 SP cells in $Itk^{-/-}$ mice, suggesting that many of the nonconventional $CD8^+$ T cells in $Itk^{-/-}$ mice are selected by MHC class Ia molecules (Figure 1A and Broussard et al. [2006]). These results suggested that selection on MHC class Ib alone does not account for the altered development of $CD8^+$ T cells in $Itk^{-/-}$ mice—rather, it is the loss of Itk that predisposes $CD8^+$ T cells to have these phenotypes, irrespective of the selecting MHC ligand.

To further evaluate how Itk affects selection of MHC class Ib-restricted cells, we performed bone-marrow transfers into $B2m^{-/-}$ mice. Bone-marrow transfers from $Itk^{-/-}H\text{-}2Kb^{-/-}H\text{-}2Db^{-/-}$ mice into $B2m^{-/-}$ recipients gave rise to more CD8 SP cells than $H\text{-}2Kb^{-/-}H\text{-}2Db^{-/-} \rightarrow B2m^{-/-}$ recipients (Figure 1B), demonstrating that Itk deficiency specifically increased selection of MHC class Ib-restricted cells on hematopoietic cells. These observations raise the possibility that Itk deficiency increases the numbers of innate-like cells by promoting selection on hematopoietic cells.

Development of Innate-like $CD8^+$ T Cells Requires Selection on Hematopoietic Cells

Although adoptive-transfer experiments show that the innate-like $CD8^+$ T cells in $Itk^{-/-}$ mice can be selected on hematopoietic cells (Broussard et al., 2006), it remained unclear whether these $CD8^+$ T cells are only selected on hematopoietic cells or can also be selected on the thymic stroma. Furthermore, it was unclear how these potential selection pathways might affect the development of these cells.

To address these questions, we generated bone-marrow chimeras that expressed MHC class I only on thymic stromal cells by transferring bone-marrow cells from $Itk^{-/-}B2m^{-/-}$ or $B2m^{-/-}$ controls into wild-type (WT) mice. In this situation, MHC class I is only expressed on the radioresistant thymic stroma, not on hematopoietic cells. Analyses of these chimeras revealed that $CD8^+$ T cells could develop in mice that had received bone marrow from either $B2m^{-/-}$ or $Itk^{-/-}B2m^{-/-}$ mice (Figures 2A and 2B). However, although intact $Itk^{-/-}$ mice or chimeras in which $Itk^{-/-}$ bone marrow was transferred into WT mice exhibited elevated numbers of CD8 SP cells compared to WT mice (see Figure 1 and (Broussard et al. [2006]), fewer mature ($HSA^{lo}TCR^{hi}$) CD8 SP cells arose in the $Itk^{-/-}B2m^{-/-} \rightarrow$ WT bone-marrow chimeras compared to the $B2m^{-/-} \rightarrow$ WT chimeras (Figures 2A and 2B). Thus, Itk-deficient $CD8^+$ T cells can be selected on the thymic stroma, although their selection is less efficient. Notably, the Itk-deficient CD8 SP cells that developed via recognition of MHC class I molecules on

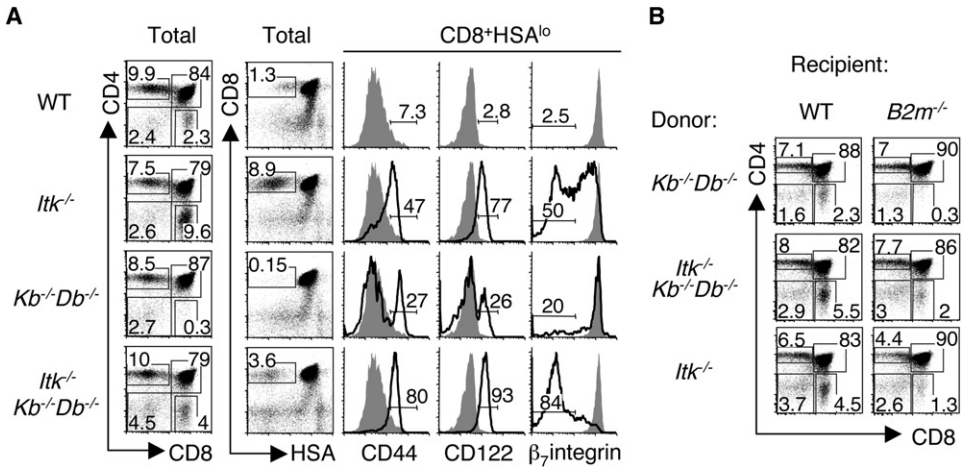

Figure 1. Itk Deficiency Increases Selection on Hematopoietic Cells

(A) Thymocyte profiles of wild-type (WT), *Itk*$^{-/-}$, *H-2Kb*$^{-/-}$*H-2Db*$^{-/-}$ (*Kb*$^{-/-}$*Db*$^{-/-}$), and *Itk*$^{-/-}$*H-2Kb*$^{-/-}$*H-2Db*$^{-/-}$ (*Itk*$^{-/-}$*Kb*$^{-/-}$*Db*$^{-/-}$) mice demonstrating increased expression of CD44 and CD122 and decreased expression of β_7 integrin in *Itk*$^{-/-}$, *H-2Kb*$^{-/-}$*H-2Db*$^{-/-}$, and *Itk*$^{-/-}$*H-2Kb*$^{-/-}$*H-2Db*$^{-/-}$ CD8 SP cells. Data are representative of two experiments that used one to two mice per genotype. Numbers represent the percentage of cell in each gate. WT profiles are shown in gray.

(B) Bone-marrow transfer of *H-2Kb*$^{-/-}$*H-2Db*$^{-/-}$, *Itk*$^{-/-}$*H-2Kb*$^{-/-}$*H-2Db*$^{-/-}$, and *Itk*$^{-/-}$ donor cells (2×10^7 cells) into WT or *B2m*$^{-/-}$ recipients. A representative transfer of WT bone marrow into *B2m*$^{-/-}$ recipients is shown in Figure 4. Results are representative of two transfer experiments that used four to five donor mice and two to five recipients per transfer for each genotype.

radioresistant stromal cells had reduced surface expression of CD44 and CD122, similar to conventional and not innate CD8$^+$ T cells (Figure 2A). These results argue that Itk is required for efficient selection on the thymic stroma and, moreover, that selection on hematopoietic cells is required for development of the innate-type CD8$^+$ T cells in Itk-deficient mice.

SAP Deficiency Prevents Selection of Innate-like CD8$^+$ T Cells in *Itk*$^{-/-}$ Mice

To further understand the requirements for development of the innate-like CD8$^+$ T cells in *Itk*$^{-/-}$ mice, we considered what signals provided by hematopoietic cells might affect development of innate cell lineages. Hematopoietic cells express a variety of costimulatory molecules known to

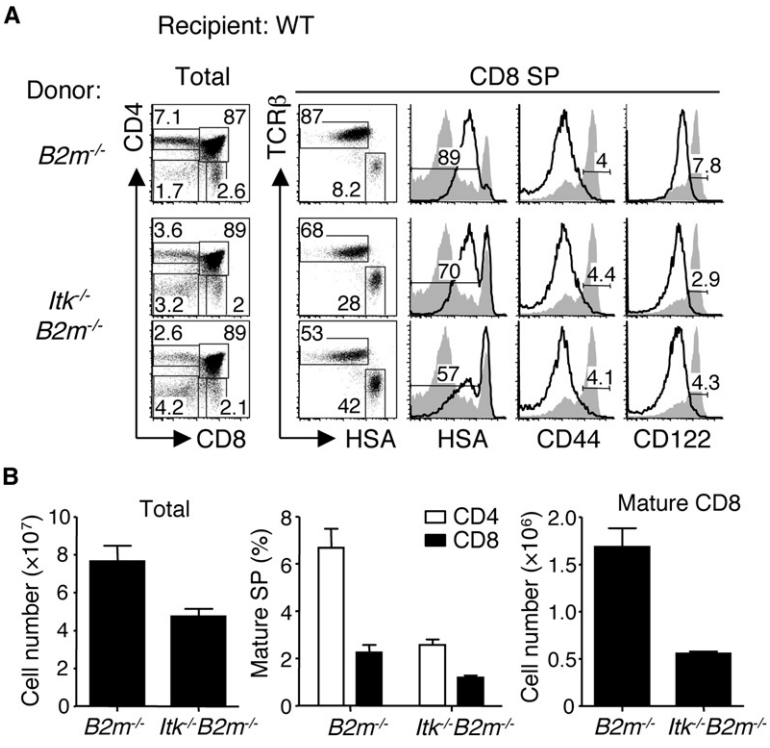

Figure 2. Selection on Thymic Stroma Prevents Development of Innate Phenotypes of *Itk*$^{-/-}$ CD8$^+$ T Cells

(A) Thymic profiles of WT chimeric mice that received 1.8×10^7 *B2m*$^{-/-}$ or *Itk*$^{-/-}$*B2m*$^{-/-}$ bone-marrow cells. Experiments were repeated twice with three to four donor mice and four recipient mice per genotype per experiment. Bold line histograms indicate the CD8 profiles of chimeric mice, and filled gray histograms indicate the CD8 profiles of thymocytes from *Itk*$^{-/-}$ mice stained at the same time to indicate differences in CD44 and CD122 levels (WT, data not shown). Two examples of *Itk*$^{-/-}$*B2m*$^{-/-}$ → WT chimeras are shown.

(B) Cellularity and percentages of total thymocytes, CD4 and CD8 SP cells, and mature CD8 SP cells from chimeras receiving the indicated bone marrow. Mature CD8$^+$ T cell numbers were calculated by gating on CD8 SP and HSA^{lo}TCRβ^{hi} populations as indicated in (A). Averages ± SEM of four recipients per genotype are shown.

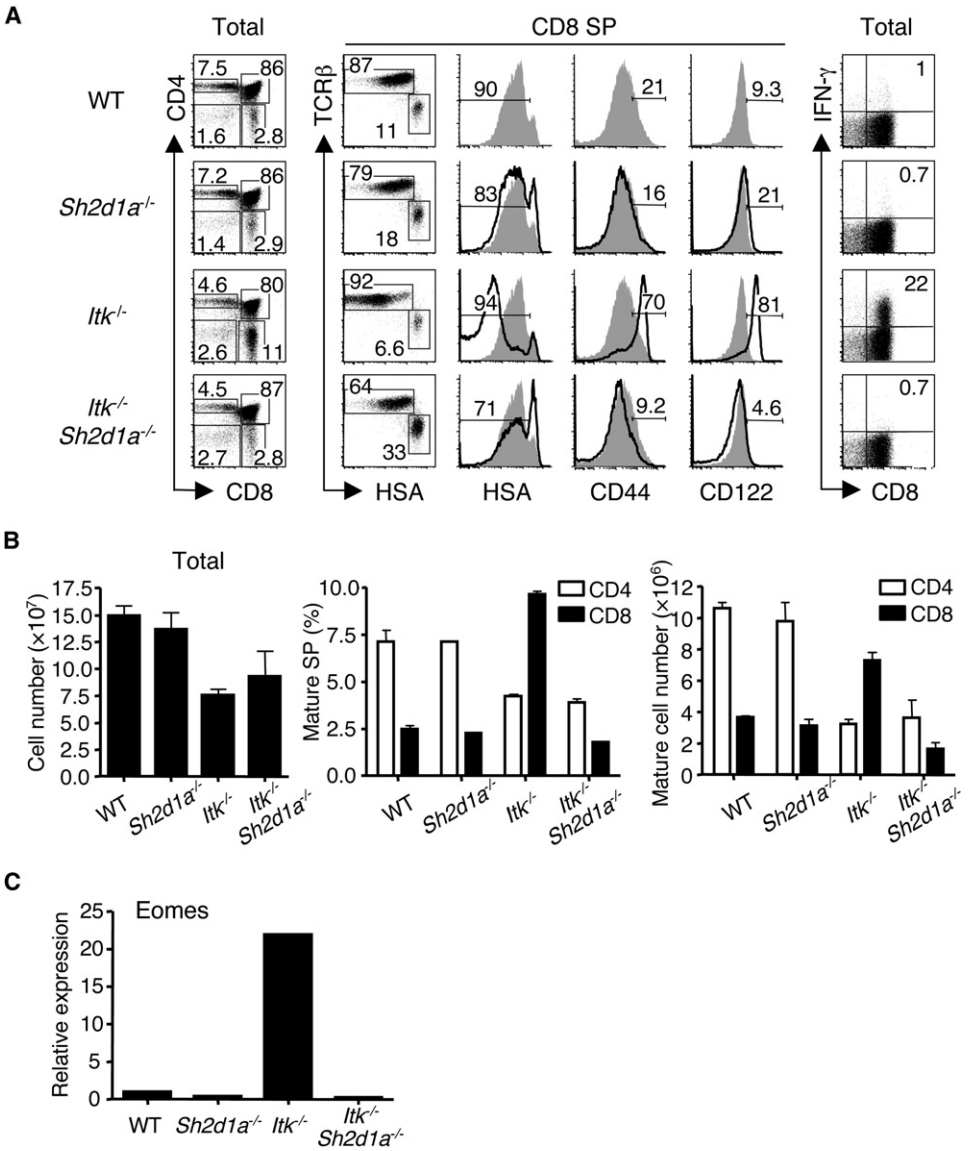

Figure 3. SAP Is Required for the Development of Innate-like CD8$^+$ T Cells in *Itk*$^{-/-}$ Mice

(A) Thymocyte profiles of WT, *Sh2d1a*$^{-/-}$, *Itk*$^{-/-}$, and *Itk*$^{-/-}$*Sh2d1a*$^{-/-}$ mice. In the second column, gating distinguishes mature (HSA^{lo}TCRβ^{hi}) and immature (HSA^{hi}TCRβ^{lo}) CD8 SP subsets. Numbers represent the percentage of cells in each gate. WT profiles are shown in gray. Right-hand column shows intracellular staining for IFN-γ produced from thymocytes after stimulation ex vivo for 4 hr with PMA and Ionomycin.

(B) Thymic cellularity of WT, *Sh2d1a*$^{-/-}$, *Itk*$^{-/-}$, and *Itk*$^{-/-}$*Sh2d1a*$^{-/-}$ mice. We calculated mature SP cells by gating on HSA^{lo}TCRβ^{hi} populations as in (A). Graphs represent averages ± SEM. For (A) and (B), one representative experiment of three independent experiments that used three mice per genotype is shown.

(C) Eomes mRNA expression in WT, *Sh2d1a*$^{-/-}$, *Itk*$^{-/-}$, and *Itk*$^{-/-}$*Sh2d1a*$^{-/-}$ sorted CD8 SP thymocyte as examined by real-time PCR. One representative experiment of two independent experiments that used two to three pooled mice per genotype is shown.

contribute to T cell activation. The SLAM family of receptors is one such family of immunomodulatory receptors expressed on hematopoietic cells that mediate cellular signals through homotypic interactions (Ma et al., 2007). SAP, a small SH2-domain-containing adaptor that mediates signaling through SLAM family receptors, is strictly required for development of NKT cells, which are also selected on hematopoietic cells (Chung et al., 2005; Nichols et al., 2005; Pasquier et al., 2005). We therefore asked whether SAP is required for the development of the innate-like CD8$^+$ T cells in *Itk*$^{-/-}$ mice.

To address this question, we interbred Itk-deficient and SAP-deficient (*Sh2d1a*$^{-/-}$) mice. SAP deficiency decreased both the percentage and number of CD8 SP thymocytes in *Itk*$^{-/-}$ mice and completely prevented the innate-like phenotypes of the CD8 SP cells as determined by expression of CD44 and CD122 (Figures 3A and 3B). The percentage of CD8 SP cells producing IFN-γ upon

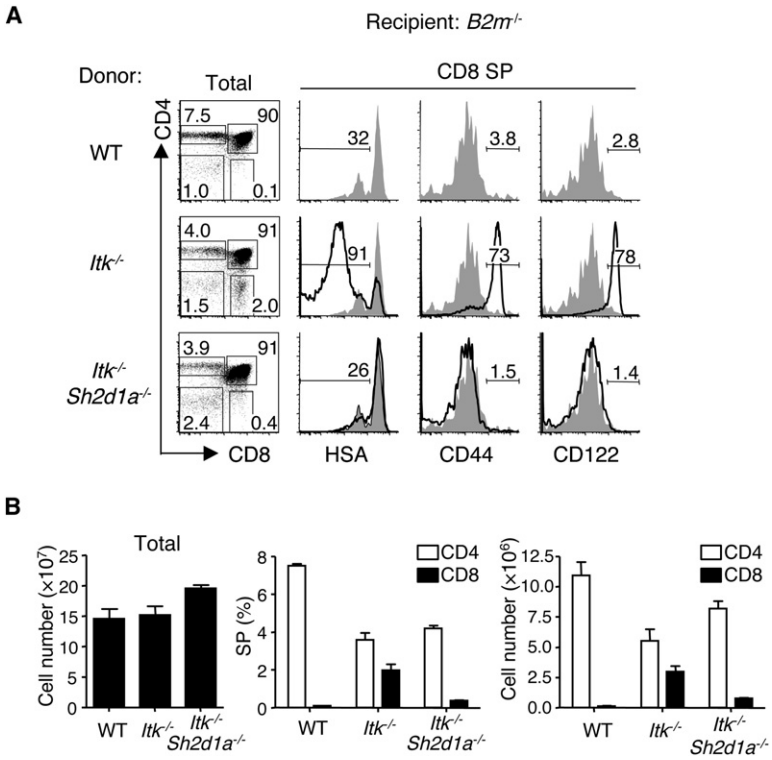

Figure 4. SAP Is Required for Thymic Selection on Hematopoietic Cells

WT, $Itk^{-/-}$ or $Itk^{-/-}Sh2d1a^{-/-}$ bone marrow (2×10^7 cells) was transferred into $B2m^{-/-}$ recipients and mice were analyzed 7 weeks after transfer. Data are representative of two experiments examining a minimum of three recipient mice per donor genotype. In (A), representative thymocyte profiles for each genotype are shown. Profiles of WT→$B2m^{-/-}$ chimeras are shown in gray. (B) shows the cellularity of thymocyte populations of WT, $Itk^{-/-}$, and $Itk^{-/-}Sh2d1a^{-/-}$ bone-marrow chimeras as in (A). Graphs represent averages ± SEM.

ex vivo stimulation was also dramatically reduced in $Itk^{-/-}Sh2d1a^{-/-}$ CD8 SP cells compared with $Itk^{-/-}$ CD8 SP cells (Figure 3A).

Development of the innate cell properties in $CD8^+$ T cells in Itk-deficient mice has been reported to correlate with expression of the T box transcription factor Eomes (Atherly et al., 2006), which induces expression of memory cell markers including CD122 and CD44 (Intlekofer et al., 2005). CD8 SP cells in Itk-deficient mice show high expression of Eomes mRNA (Atherly et al., 2006). To evaluate whether SAP deficiency affects expression of Eomes, we sorted thymic cell populations from $Itk^{-/-}$, $Itk^{-/-}Sh2d1a^{-/-}$, and control mice. SAP deficiency strongly reduced expression of Eomes in $Itk^{-/-}$ CD8 SP cells (Figure 3C), suggesting that SAP-mediated signals participate in pathways leading to the induction of Eomes mRNA. Together, these data suggest that SAP-mediated signaling is required for the development of the innate-like $CD8^+$ T cells in $Itk^{-/-}$ mice.

SAP Is Required for Selection on Hematopoietic Cells

SAP deficiency could prevent development of the innate-type $CD8^+$ T cells in Itk-deficient mice either by affecting selection on hematopoietic cells or by preventing the development of the innate characteristics of cells selected on hematopoietic cells. Analyses of thymic cell populations in $Itk^{-/-}Sh2d1a^{-/-}$ mice revealed decreased percentages of CD8 SP cells in the thymus compared to those in $Itk^{-/-}$ mice (Figures 3A and 3B), similar to what we observed in the $Itk^{-/-}B2m^{-/-}$ → WT bone-marrow chimeras (Figure 2). Moreover, CD8 SP cells in $Itk^{-/-}Sh2d1a^{-/-}$ mice included more immature ($HSA^{hi}TCR^{lo}$) cells (Figure 3A) resulting in lower percentages and numbers of mature CD8 SP cells compared to those in WT mice (Figure 3B). Thus, the thymic profiles in $Itk^{-/-}Sh2d1a^{-/-}$ mice resemble those of chimeras in which selection was fixed on the thymic stroma. These results suggested that SAP deficiency might prevent the selection of the innate $CD8^+$ T cell population on hematopoietic cells in $Itk^{-/-}$ mice.

To address specifically whether SAP deficiency affected selection of $Itk^{-/-}$ $CD8^+$ T cells on hematopoietic cells, we performed transfers of WT, $Itk^{-/-}$, and $Itk^{-/-}Sh2d1a^{-/-}$ bone marrow into $B2m^{-/-}$ mice. Although transfers of $Itk^{-/-}$ bone marrow into $B2m^{-/-}$ mice permitted development of a large population of CD8 SP cells, transfer of $Itk^{-/-}Sh2d1a^{-/-}$ bone marrow gave rise to only low percentages and numbers of CD8 SP cells that did not appear to be mature on the basis of TCR and HSA expression (Figures 4A and 4B and data not shown). Thus, in the absence of SAP, $Itk^{-/-}$ cells cannot be selected efficiently on hematopoietic cells. These data suggest that SAP is required for the selection of the innate-like $CD8^+$ T cells on hematopoietic cells in Itk-deficient mice.

CD28 Deficiency Also Prevents Development of Innate Cell Lineages in $Itk^{-/-}$ Mice

One of the other key costimulatory molecules involved in T cell function is CD28, which is critical for full activation of mature T cells. Nonetheless, the role of CD28 in the thymus is relatively poorly understood. CD28's interactions

with B7-1 (CD80) and B7-2 (CD86) are required for development of $CD4^+CD25^+FoxP3^+$ T cells, an important regulatory T cell lineage (Liston and Rudensky, 2007; Salomon et al., 2000; Tai et al., 2005; Tang et al., 2003). Other evidence suggests that signals from hematopoietic cells via CD28 provide distinct signals that might be associated with cell deletion and negative selection (Amsen and Kruisbeek, 1996; Buhlmann et al., 2003; Kishimoto et al., 1996; Lucas and Germain, 2000; Punt et al., 1994; Teh and Teh, 2001). However, yet other data demonstrate that CD28 influences the efficiency of positive selection in the HY TCR transgenic system (Vacchio et al., 2005).

To determine whether CD28 contributes to the development of innate $CD8^+$ T cells in Itk-deficient mice, we interbred *Itk*$^{-/-}$ and *Cd28*$^{-/-}$ mice. Similar to *Itk*$^{-/-}$*Sh2d1a*$^{-/-}$ mice, CD28 deficiency decreased the development of nonconventional $CD8^+$ T cells in *Itk*$^{-/-}$*Cd28*$^{-/-}$ mice: *Itk*$^{-/-}$*Cd28*$^{-/-}$ CD8 SP cells exhibited reduced CD44 and CD122 expression and decreased IFN-γ production, although these phenotypes were more variable than in *Itk*$^{-/-}$*Sh2d1a*$^{-/-}$ mice (Figure 5A). Similarly, *Itk*$^{-/-}$*Cd28*$^{-/-}$ CD8 SP cells had decreased expression of Eomes (Figure 5C). However, CD28 deficiency did not decrease either the percentages or numbers of thymic CD8 SP cells (Figures 5A and 5B), in contrast to the phenotypes observed in *Itk*$^{-/-}$*Sh2d1a*$^{-/-}$ mice. Instead, an increase in CD4 SP cells was observed. Thus, CD28 deficiency appeared to improve selection of both CD4 and CD8 conventional SP cells and also prevent the appearance of $CD8^+$ T cells with innate characteristics.

To address whether the effects of CD28 were mediated by interactions with CD80 and CD86 expressed on the thymic stroma or on hematopoietic cells, we used *Cd80*$^{-/-}$*Cd86*$^{-/-}$ mice as recipient mice for bone-marrow transfers from *Itk*$^{-/-}$ or WT donor mice. Transfer of *Itk*$^{-/-}$ bone-marrow cells into *Cd80*$^{-/-}$*Cd86*$^{-/-}$ mice still gave rise to CD8 SP cells with innate phenotypes, as evidenced by high expression of CD44 and CD122 as well as low expression of HSA (Figure 6A). These CD8 SP cell populations were similar to those seen in *Itk*$^{-/-}$ mice or *Itk*$^{-/-}$ → WT bone-marrow chimeras (data not shown). Similar results were obtained with bone-marrow transfers into *Cd28*$^{-/-}$ hosts (data not shown). These results suggest that CD28 does not require CD80 or CD86 expressed on the thymic stroma to provide signals for the generation of innate-type lymphocytes.

To further evaluate how CD28 costimulation affects development of innate cell lineages, we performed bone-marrow transfers into *B2m*$^{-/-}$ recipients. Bone-marrow-transfer experiments that used *Itk*$^{-/-}$*Cd28*$^{-/-}$ donors into *B2m*$^{-/-}$ recipients revealed that CD28 deficiency on hematopoietic cells still permitted development of CD8 SP cells at numbers similar to that seen in *Itk*$^{-/-}$ → *B2m*$^{-/-}$ chimeras (Figures 6B and 6C). These findings contrast with those in *Itk*$^{-/-}$*Sh2d1a*$^{-/-}$ → *B2m*$^{-/-}$ bone-marrow transfers, in which very few CD8 SP cells developed (Figure 4). Nonetheless, the CD8 SP cells that developed in *Itk*$^{-/-}$*Cd28*$^{-/-}$ → *B2m*$^{-/-}$ bone-marrow transfers were $CD44^{lo}CD122^{lo}$, i.e., they did not resemble innate cells, despite their selection on hematopoietic cells (Figure 6B). Thus, CD28 deficiency on hematopoietic cells was sufficient to prevent the innate phenotypes of the $CD8^+$ T cells in *Itk*$^{-/-}$ mice.

Together, these results indicate that CD28-B7 signals are not required for selection of $CD8^+$ T cells on hematopoietic cells in *Itk*$^{-/-}$ mice but are important for the development of an innate cell program in cells selected on hematopoietic cells (Figure 6B). Thus, Itk, SAP, and CD28 influence the development of innate cells by distinct mechanisms: SAP and Itk reciprocally regulate selection of innate $CD8^+$ T cells on hematopoietic cells versus the thymic stroma, whereas costimulation through CD28 promotes the acquisition of innate-type phenotypes in hematopoietically selected cells.

DISCUSSION

We present here experiments examining the requirements for selection of innate-like $CD8^+$ T cell populations in mice deficient in Itk. We found that development of these cells required selection on hematopoietic cells and was dependent on the adaptor molecule SAP, thereby implicating homotypic interactions between SLAM family members in this process. Moreover, we found that CD28 was required for full development of innate cell characteristics upon selection on hematopoietic cells; such a finding raises the possibility that CD28 costimulation plays a role in the maturation of other innate cell lineages.

Our data clearly demonstrate that selection on hematopoietic cells plays a fundamental role in determining the phenotypic characteristics of these innate-type cells, a finding previously suggested by examination of the few $CD8^+$ T cells selected in K^bD^b-deficient mice (Urdahl et al., 2002) and the $CD4^+$ T cells selected in CIITA transgenic mice (Li et al., 2007). Our findings further argue that signaling pathways dependent on SAP are a critical part of this selection pathway directed by hematopoietic cells. It should be noted that not all innate lymphocyte lineages might follow these rules—the mechanisms driving selection of CD8$\alpha\alpha$ lineages, which also exhibit characteristics of innate cells, remain unclear (Lambolez et al., 2007). Moreover, how these lineages relate to other regulatory T cell lineages in the thymus remains an open question.

Our results suggest that Itk might be one of the few molecules that specifically affects selection of conventional versus innate-type lineages. Of note, other molecules implicated in TCR signaling, including Lck, ZAP-70, LAT, and SLP-76 affect development of all T lymphocyte lineages, perhaps because of their more profound effects on pre-TCR and TCR signaling (Starr et al., 2003). Yet other molecules, including SAP, Fyn, PKC-θ, NF-κB1, Eomes, and T-bet help define pathways regulating nonconventional, innate-like lineages such as NKT cells (Chung et al., 2005; Eberl et al., 1999; Gadue et al., 1999; Godfrey and Berzins, 2007; Intlekofer et al., 2005; Nichols et al., 2005; Pasquier et al., 2005; Schmidt-Supprian et al., 2004; Sivakumar et al., 2003; Stanic et al., 2004). In contrast, Itk appears to be required specifically for efficient

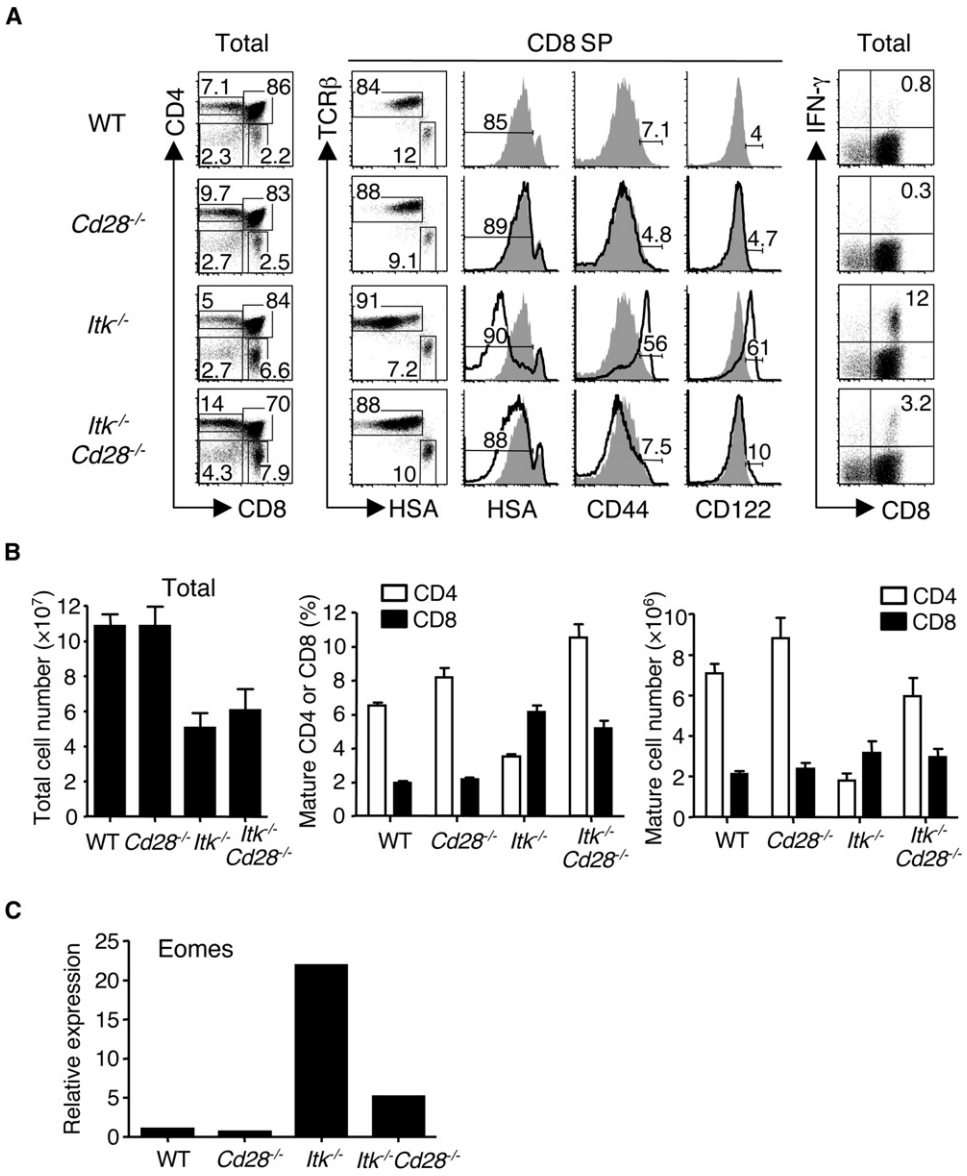

Figure 5. CD28 Deficiency Prevents Innate CD8$^+$ T Cell Phenotypes in *Itk*$^{-/-}$ Mice

(A) Thymic profiles of WT, *Cd28*$^{-/-}$, *Itk*$^{-/-}$, and *Itk*$^{-/-}$*Cd28*$^{-/-}$ mice. Gating in the second column distinguishes mature (HSA^{lo}TCRβ^{hi}) and immature (HSA^{hi}TCRβ^{lo}) CD8 SP subsets. WT profiles are shown in gray. IFN-γ production from thymocytes stimulated for 4 hr ex vivo with PMA and ionomycin is shown in the right column. Data are one representative of five independent experiments that used one to three mice for each genotype.

(B) Cellularity of total thymocytes and mature CD4 and CD8 SP cells. We calculated mature SP cells by gating on HSA^{lo}TCRβ^{hi} populations as in (A). Averages ± SEM are shown. WT and *Cd28*$^{-/-}$ mice, n = 5. *Itk*$^{-/-}$ and *Itk*$^{-/-}$*Cd28*$^{-/-}$ mice, n = 6.

(C) Eomes expression in CD8 SP thymocytes. Data are from one representative of three independent sorting experiments that used two to three mice pooled per genotype.

differentiation of conventional CD8$^+$ T cell lineages (Atherly et al., 2006; Berg, 2007; Broussard et al., 2006).

In particular, our data suggest that Itk deficiency specifically prevents efficient positive selection of mature CD8 SP cells on the thymic stroma. Indeed, when selection is forced to occur on the thymic stroma, as in *Itk*$^{-/-}$*B2m*$^{-/-}$ bone-marrow transfers into WT mice, fewer mature CD8$^+$ T cells develop. In *Itk*$^{-/-}$ mice, perhaps because selection on hematopoietic cells can still occur, development of non-conventional cells becomes a major route of CD8$^+$ T cell development. The large numbers of innate-type CD8$^+$ T cells that develop in *Itk*$^{-/-}$ mice could result from expansion secondary to the lower numbers of mature conventional SP cells. However, the observation that large numbers of CD8 SP cells develop when *Itk*$^{-/-}$ but not WT bone marrow is transferred into *B2m*$^{-/-}$ and *B2m*$^{-/-}$*H2-Ab1*$^{-/-}$ mice (Broussard et al., 2006) raise the possibility that Itk functions as a negative regulator of signaling

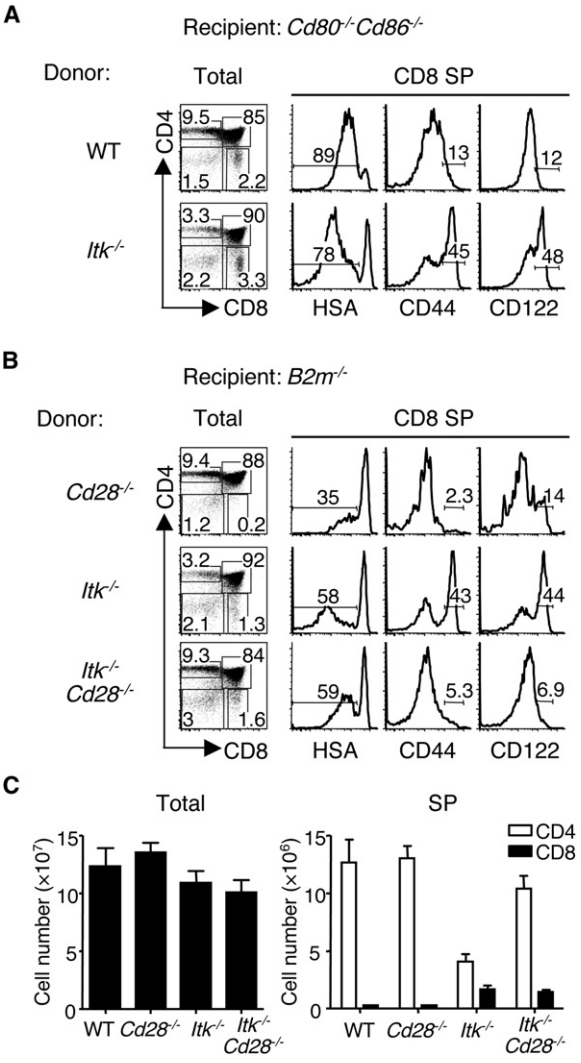

Figure 6. CD28-B7 Costimulation on Hematopoeitic Cells Is Required for Development of Innate CD8$^+$ T Cell Phenotypes

(A) Bone-marrow transfer of WT and *Itk*$^{-/-}$ cells (1.6 × 10^7 cells) into three *Cd80*$^{-/-}$*Cd86*$^{-/-}$ recipients. Data are representative of one experiment with *Itk*$^{-/-}$ cells and one experiment with *Rlk*$^{-/-}$*Itk*$^{-/-}$ cells transferred into *Cd80*$^{-/-}$*Cd86*$^{-/-}$ mice. In these experiments, *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ cells behaved similarly.

(B) Bone-marrow transfer of *Cd28*$^{-/-}$, *Itk*$^{-/-}$, and *Itk*$^{-/-}$*Cd28*$^{-/-}$ cells (1.5 × 10^7 cells) into *B2m*$^{-/-}$ recipients. Data are representative of one of two experiments that used three to four recipients per genotype.

(C) Cellularity data of bone-marrow transfers as in (B). Data are averages ± SEM of three recipients each for WT and *Cd28*$^{-/-}$ donors, six recipients for *Itk*$^{-/-}$ donors, and seven recipients for *Itk*$^{-/-}$*Cd28*$^{-/-}$ donors.

pathways that are required for selection of innate CD8$^+$ T cells on hematopoietic cells. The differential effects of Itk on the selection of conventional CD8$^+$ T cells on the thymic stroma versus innate CD8$^+$ T cells selected on hematopoietic cells further suggest that Itk-dependent pathways might serve as a rheostat for determining the balance of adaptive versus innate T cell lineages. Thus, Itk-dependent pathways might normally prevent efficient selection of T cells on hematopoietic cells, thereby guaranteeing that the majority of mature T cells represent the adaptive arm of the immune system. Why Itk deficiency specifically affects selection on thymic stroma remains an important question and might reflect the relative importance of TCR versus costimulatory signals for the development of these distinct T cell lineages; development of innate CD8$^+$ T cells in *Itk*$^{-/-}$ mice can be prevented by either increasing TCR signal strength or by reducing costimulatory signals (Atherly et al., 2006; Broussard et al., 2006, and this paper).

One question that arises from this work is why CD8$^+$ T cells are the primary cells affected in *Itk*$^{-/-}$ mice. Analyses of TCR transgenic mice demonstrate that Itk is required for efficient positive selection of both CD4$^+$ and CD8$^+$ T cells, yet only a large population of innate CD8$^+$ T cells develop. These observations could imply that Itk is specifically required for the development of conventional CD8$^+$ T cells (Berg, 2007). However, the specific development of innate CD8$^+$ (but not CD4$^+$) T cells could also result from the lack of appreciable MHC class II expression on murine double positive (DP) thymocytes. Although we do not know which hematopoietic cells are responsible for selection of the innate-type CD8$^+$ T cells in *Itk*$^{-/-}$ mice, DP thymocytes are the major hematopoietic cell population in the thymus and have been shown to mediate selection of NKT cells. If MHC class II molecules were expressed on thymocytes, as in CIITA transgenic mice (Choi et al., 2005; Li et al., 2005), a larger population of CD4$^+$ T cells with innate cell phenotypes might be observed.

Our results also highlight the role of SAP and SLAM family members in the development of innate cell lineages. The SLAM family receptors are emerging as important immunoregulatory receptors that mediate interactions between hematopoeitic cells to regulate T helper cell polarization, humoral immunity, and the development of autoantibodies, host responses to pathogens, and NKT cell development (Griewank et al., 2007 [this issue of *Immunity*]; Ma et al., 2007). Mutations affecting SAP lead to profound immune dysfunction in X linked lymphoproliferative disease (XLD). Our results provide evidence that signaling pathways involving SAP might be required for multiple innate cells selected on hematopoietic cells. Thus, Itk and SAP play complementary roles as determinants of the balance of conventional and innate T cell lineages, respectively. An important question that remains is which SLAM family members participate in hematopoietic selection and whether they are the same for all innate T cell lineages. Furthermore, whether SAP and SLAM family receptors are required for later stages of development of innate T cell lineages remains unknown.

Nonetheless, SAP-associated receptors are not the only costimulatory pathways affecting development of these innate cell lineages. A growing body of data indicates that CD28 participates in the thymic development of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells (Liston and Rudensky, 2007; Salomon et al., 2000; Tai et al., 2005; Tang et al., 2003). It is therefore of interest that CD8$^+$CD122$^+$ cells have also been described to have regulatory T cell function (Rifa'i et al., 2004), suggesting parallels between

these two lineages. It is also of interest that CD28 and B7's might not be required for selection of $CD4^+CD25^+$ cells but rather for their maturation and acquisition of FoxP3 expression and regulatory function (Liston and Rudensky, 2007). Similarly, our data demonstrate that CD28 is not required for selection of $CD8^+$ T cells on hematopoietic cells but rather for the full acquisition of the innate cell program, including the induction of high levels of Eomes mRNA. Indeed, our data raise the possibility that maturation of other innate cell lineages might also be partially dependent on CD28. The role of CD28 might be more complex, however, because CD28 deficiency appears to increase the selection of both CD4 and CD8 SP cells in $Itk^{-/-}$ mice, perhaps reflecting its possible role in negative selection. Indeed, one potential reason for why $Itk^{-/-}$ mice develop this large population of innate $CD8^+$ T cells is that cells that would normally undergo negative selection from agonist peptides in the thymus might not be efficiently deleted (Schaeffer et al., 2000). It should also be noted that neither SAP nor CD28 deficiency increased the low numbers of total thymocytes in $Itk^{-/-}$ mice, which are likely to result from Itk's effects on pre-TCR signaling (Lucas et al., 2003).

Together, our data help define pathways that are differentially required for T cell selection on thymic stroma versus hematopoietic cells and the generation of conventional and innate T cell lineages that are required for proper immune homeostasis and responses to infections. In particular, our results suggest that costimulation is critical for the development of innate T cell phenotypes in the thymus: Whereas SAP and Itk reciprocally regulate selection of innate $CD8^+$ T cells on hematopoietic cells, costimulation through CD28 influences the maturation and acquisition of innate-type phenotypes on cells selected on hematopoietic cells. The shared requirement for SAP-mediated signaling for development of these innate-type $CD8^+$ T cells and CD1d-restricted NKT cells suggests these findings might help define common requirements for the development of multiple innate T lymphocyte lineages and raises the possibility that absence of these lineages contributes to phenotypes associated with XLP.

EXPERIMENTAL PROCEDURES

Mice

$Itk^{-/-}$ and $Sh2d1a^{-/-}$ mice on the C57BL/6 background are previously described (Liao and Littman, 1995; Czar et al., 2001). $H\text{-}2Kb^{-/-}H\text{-}2Db^{-/-}$ ($Kb^{-/-}Db^{-/-}$) (Perarnau et al., 1999) and $B2m^{-/-}$ (Zijlstra et al., 1989) mice were obtained from Taconic, and C57BL/6, $Cd28^{-/-}$ (Shahinian et al., 1993) and $Cd80^{-/-}Cd86^{-/-}$ (Borriello et al., 1997) mice were obtained from Jackson Laboratory. All animals were bred and maintained under specific pathogen-free conditions in the National Human Genome Research Institute (NHGRI) Animal Facility, and experiments were performed according to NHGRI animal care and use committee guidelines.

Thymocyte Preparation and Sorting

Single-cell suspensions of thymocytes were prepared from age-matched mice between 6 and 10 weeks old. For total thymocyte sorting, cells were stained with PerCP-Cy5.5-anti-CD4 (RM4-5) and APC-anti-CD8 (53-6.7) antibodies (BD Biosciences, San Diego, CA) and sorted for $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ populations.

Flow Cytometry

Antibodies used for the staining are as follows: FITC-TCRβ (H57-597), PE-β_7 integrin (M293), PE-CD62L (MEL-14), PE-Cy5-HSA/CD24, Biotin-CD122 (TM-β1), Streptavidin PE-Cy7, APC-CD44 (IM7), APC-Alexa Fluoro 750-CD8, and Pacific Blue-CD4. Cells were acquired by FACSCalibur or LSRII (BD Biosciences), and data were analyzed with Flowjo software (Tree Star, Ashland, OR).

Ex Vivo Stimulation and Intracellular Cytokine Staining

Thymocytes and splenocytes were stimulated ex vivo with PMA and Ionomycin in the presence of Brefeldin A (BD Biosciences) as previously described (Broussard et al., 2006). After 4 hr, cells were harvested and stained with FITC-anti-CD44 (IM7), Pacific Blue-anti-CD4, and PerCP-Cy5.5-anti-CD8 antibodies. Cells were fixed with 4% PFA and permeabilized with PBS containing 0.1% BSA and 0.05% Triton X-100. Intracellular cytokines were stained with APC-anti-IFN-γ (XMG1.2) antibody.

Bone-Marrow Cell Transfers

Bone-marrow cells were prepared from femur and tibiae as previously described (Broussard et al., 2006). Recipient mice were sublethally irradiated at 950–1000 rad, depending on the age and body size, and 1.5 to 2 × 10^7 cells were transferred intravenously. Thymocytes and splenocytes for flow-cytometric analyses were harvested 7 weeks after the transfer, and thymocytes for real time PCR analyses were harvested 5 weeks after the transfer.

RNA Isolation and Real-Time PCR

Total and sorted thymocytes were lysed in TRIzol (Invitrogen), and total RNA was extracted with RNeasy Mini Kit (QIAGEN). cDNA was synthesized from total RNA with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR was performed with a 7900 sequence detection system (Applied Biosystems). TaqMan Endogenous Control for Eukaryotic 18S rRNA (VIC-MGB probe), and the primer and probe sets for murine Eomes (Mm01351988_m1, FAM-MGB probe) were from Applied Biosystems. Results were normalized to 18S rRNA and expressed relative to WT levels (WT = 1).

Supplemental Data

One figure is available at http://www.immunity.com/cgi/content/full/27/5/775/DC1/.

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