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Development of Innate CD4⁺ and CD8⁺ T Cells in Itk-Deficient Mice Is Regulated by Distinct Pathways

Amanda L. Prince,* Zachary Kraus,† Shannon A. Carty,‡ Caleb Ng,‡ Catherine C. Yin,* Martha S. Jordan,‡ Pamela L. Schwartzberg,‡ and Leslie J. Berg*

T cell development in the thymus produces multiple lineages of cells, including innate T cells such as γδ TCR⁺ cells, invariant NKT cells, mucosal-associated invariant T cells, and H2-M3-specific cells. Although innate cells are generally a minor subset of thymic development produces a wide range of T cell subsets with varying functions in immune responses. In addition to conventional naive CD4⁺ and CD8⁺ T cells, which require prolonged activation and differentiation to acquire protective effector functions, several subsets of T cells with innate effector functions are now known to develop in the thymus (1, 2). This latter group includes several distinct categories of γδ T cells, CD1d-specific invariant NKT (iNKT) cells, MR1-specific mucosal-associated invariant T (MAIT) cells, H2-M3-specific CD8⁺ T cells, and Foxp3⁺ regulatory T cells, among others (1, 2). Although a thorough understanding of the signals giving rise to each of these T cell lineages has not yet been achieved, recent studies indicate a role for the strength of TCR signaling, extrinsic signals provided by cytokines, as well as components of intrinsic developmental programming in this process (3–8).

One important clue to dissecting the signals regulating T cell lineage development has come from studies of genetically altered mice. In the absence of the Tec kinase Itk, as well as in DBA/2 mice lacking the transcription factors Krüppel-like factor 2, inhibitor of DNA-binding 3, and CREB-binding protein, conventional CD8⁺ T cells developing in the thymus are converted into innate/memory-like CD8⁺ T cells expressing high levels of the effector-promoting transcription factor eomesodermin (Eomes) (6, 8–10). Although the involvement of Itk indicates a role for TCR signaling in this process, further evidence supporting this conclusion comes from studies demonstrating an identical phenotype in mice expressing a mutant form of the adapter protein SLP-76 (11, 12), which lacks the ability to recruit Itk in response to TCR stimulation (7). Taken together, these data indicate that intact TCR signaling pathways are critical for the normal development of conventional CD8⁺ T cells. Interestingly, impaired TCR signaling is not the only requirement for the development of innate/memory CD8⁺ T cells expressing high levels of Eomes. Studies by Hogquist and colleagues (8) first demonstrated a requirement for exogenous IL-4 to induce Eomes expression in CD8⁺ CD69⁺ T cells (3, 6, 7). Thus, in the absence of the IL-4Rα (CD124), Eomes is no longer expressed in Itk⁻/⁻ CD8⁺ T cells (8). Further evidence indicates that a subset of T cells expressing the transcription factor promyelocytic leukemia zinc finger (PLZF) are involved in this pathway and are the likely source of the excess IL-4 (6–8).

Data from our laboratory and others have shown that in the absence of Itk, there is an increase in PLZF⁺ γδ T cells expressing the Vγ1.1 Vδ6.3 TCR, a subset known as γδ NKT cells (11, 12). In general, it is thought that these γδ NKT cells are responsible for the excess IL-4 in the absence of Itk, and thus for the development of Eomes⁺ innate/memory CD8⁺ T cells (13). Although it has been demonstrated that γδ NKT cells are responsible for the hyper-IgE syndrome seen in Itk⁻/⁻ mice (11, 12), it is not currently known whether γδ NKT cells are required to induce Eomes expression in CD8⁺ T cells. Multiple cell types (e.g., αβ iNKT cells, MAIT cells)
are capable of IL-4 production. Therefore, even though γδ NKT cells are the most likely candidate for the excess IL-4 acting on CD8+ thymocytes, it remains possible that other cell types are contributing to this process.

Additionally, the effect of thymic IL-4 on the development of conventional CD4+ αβ T cells has not been addressed. Whereas numerous studies have documented that itk−/− mice have an increased frequency of activated CD4+ αβ T cells, it is not known whether these cells are expressing Eomes similarly to itk−/− CD8+ αβ T cells (14). In this study, we examined itk−/− CD4+ αβ thymocytes and found a substantial subset of conventional T cells that are upregulating Eomes in response to the environment. Using these innate/memory Eomes+ CD4+ and CD8+ thymocytes to track the environmental contribution to innate T cell development in itk−/− mice, we demonstrate that this phenotype is not induced by γδ T cells.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 mice were purchased from either Taconic Farms (Hudson, NY), The Jackson Laboratory (Bar Harbor, ME), or Charles River Laboratories International (Wilmington, MA). The itk−/− mice were previously described (15–17) and housed at the University of Massachusetts Medical School in accordance with the Institutional Animal Care and Use Committee and in a specific pathogen-free environment. IL-4 reporter (Ab) mice were a gift from the laboratory of Raymond Welsh and were crossed to mice to generate Y145F/itk−/− mice (21). When conventional, mature (TCRββHSAβ) CD1d tetramer CD4SP thymocytes were analyzed, we found an increased population of itk−/− cells expressing Eomes compared with WT CD4SP thymocytes (Fig. 1C). Similar to the CD8SP thymocytes, Eomes+ itk−/− CD4SP thymocytes also expressed increased levels of IL-4Rx (CD124) and have a memory-like phenotype characterized by high expression of CD44, CD62L, IL-2Rβ (CD122), and CXCR3 (Fig. 1B, 1D).

To determine whether itk−/− CD4+ αβ T cells also underwent altered development, we characterized CD4+CD8− (CD4SP) thymocytes from itk−/− mice. For this analysis, we excluded invariant αβ NKT cells, which are known to have a memory-like phenotype (21). When conventional, mature (TCRββHSAα) CD1d tetramer CD4SP thymocytes were analyzed, we found an increased population of itk−/− cells expressing Eomes compared with WT CD4SP thymocytes (Fig. 1C). Similar to the CD8SP thymocytes, Eomes+ itk−/− CD4SP thymocytes also expressed increased levels of CD44, CD62L, IL-2Rβ (CD122), IL-4Rx (CD124), and CXCR3 (Fig. 1C, 1E). In addition to the upregulated expression of Eomes, CD44, CD62L, IL-2Rβ, IL-4Rx, and CXCR3, itk−/− Eomes+ CD8SP thymocytes produce IFN-γ upon ex vivo stimulation (15, 20). This effector function is shared with itk−/− CD4SP thymocytes (Fig. 2). Taken together, these data indicate that innate-like CD4+ αβ T cells expressing Eomes develop in the absence of Itk.

IL-15 regulates the expansion of Eomes+ T cells

Previously, we and others demonstrated the importance of IL-15 for the development and/or maintenance of itk−/− CD44hi peripheral CD8+ T cells (15, 22). Additionally, we have observed a slight reduction in the overall thymic cellularity of itk−/− il15−/− mice (Supplemental Fig. 1A). Furthermore, as shown in Supplemental Fig. 1B, itk−/− Eomes+ CD8SP thymocytes are substantially reduced in number in the absence of IL-15. However, the presence of a significant residual population of itk−/− Eomes+ CD8SP cells, even in the absence of IL-15, indicates that IL-15 is critical for the maintenance and/or expansion of these cells, rather than for their development per se. In contrast, the frequency and number of itk−/− Eomes+ CD4SP thymocytes increased dramatically in the absence of IL-15 (Supplemental Fig. 1C). This increase is likely due to the reduced number of Eomes+ itk−/− CD8SP cells that would compete with the CD4+ T cells for survival cytokines, such as IL-7 or IL-4. These data indicate that CD4+ Eomes+ T cells are independent of IL-15 for their development, maintenance, and expansion. Thus, although both of these subsets of innate T cells in itk−/− mice express Eomes, they are differentially regulated by the cytokine IL-15.

Conventional CD4+ T cells promote the expansion and development of Eomes-expressing αβ T cells

Eomes-expressing itk−/− CD8+ T cells are conventional αβ T cells that are converted to this innate-like phenotype by IL-4 (6, 8). To determine whether Eomes+ CD4+ T cells were also conventional T cells that are converted to an innate-like phenotype, we examined the development of these cells in itk−/− mice lacking all.
MHC class II expression ($H2^{dABA1-Ea}$). Unexpectedly, we first observed that, in the absence of MHC class II, there is an overall reduction in the thymic cellularity of Itk-deficient mice lacking MHC class II (Fig. 3A). Furthermore, the frequency and number of Eomes$^+$ CD8$^+$ T cells were significantly decreased (Fig. 3B), and lack of MHC class II expression led to a near disappearance of $itk^{-/-}$ Eomes$^+$ CD4$^+$ SP thymocytes (Fig. 3C). These latter data support the conclusion that $itk^{-/-}$ Eomes$^+$ CD4$^+$ SP thymocytes are conventional CD4$^+$ T cells that are converted to an innate-like phenotype. The reason for the reduced numbers of $itk^{-/-}$

**FIGURE 1.** Mature Eomes$^+$ CD8 and CD4 T cells develop in the absence of Itk. Thymocytes from WT and $itk^{-/-}$ mice were isolated and stained with CD1d-tetramer and Abs to CD4, CD8, TCR$\beta$, TCR$\delta$, HSA (CD24), CD44, CD62L, CD122 (IL-2R$\beta$), CXCR3, CD124 (IL-4R$\alpha$), and Eomes. **(A)** CD4/CD8 ratio of total thymocytes in WT versus $itk^{-/-}$ mice. **(B and C)** Eomes versus CD44 staining of CD8$^+$ SP (B) and CD4$^+$ SP (C) thymocytes, gated as indicated. The numbers indicate the percentages of CD44$^{hi}$Eomes$^+$ cells in each subset. The graphs below show compilations of all data indicating percentages and absolute numbers of Eomes$^+$ cells in each subset. Results are representative of more than five independent experiments ($n$ = 20–22 mice/group). Statistical analysis was done using a Mann–Whitney $U$ test. **(D and E)** Histograms show staining of CD62L, CD122, CD124, and CXCR3 on mature CD8$^+$ SP (D) or mature CD4$^+$ SP (E) thymocytes. WT thymocytes are shown in gray-filled histograms; $itk^{-/-}$ thymocytes are shown in black. (D) WT and $itk^{-/-}$ thymocytes are gated on TCR$\beta^{hi}$CD24lo CD8$^+$ SP cells. (E) WT thymocytes gated on CD4$^+$ SP TCR$\beta^{hi}$CD1d-tetramer$^{lo}$ HSA$^{lo}$ thymocytes, $itk^{-/-}$ thymocytes gated on CD4$^+$ SP TCR$\delta^{hi}$CD1d-tetramer$^{hi}$ HSA$^{lo}$Eomes$^+$ thymocytes. Results are representative of at least three independent experiments.
inmate-like CD8SP in mice lacking conventional αβ CD4+ T cells is unclear, but it may reflect an unforeseen contribution of IL-4 from this conventional T cell subset. Alternatively, itk−/− CD4SP thymocytes may promote the expansion of thymocytes during the double-negative to double-positive transition because we saw a significant decrease in thymic cellularity.

**Eomesαβ T cells develop independently of MAIT cells**

The data shown above indicate that both conventional CD8αβ and CD4αβ T cells aberrantly upregulate Eomes expression in itk−/− mice. For the CD8SP thymocytes, this altered development is dependent on excess IL-4 in the environment. One potential source of IL-4 is a subset of innate T, that is, MAIT cells. MAIT cells express an invariant TCR (mouse Vα19-Jα33, human Vα7.2-Jα33) that recognizes the nonclassical MHC class Ib molecule MR1; furthermore, these cells preferentially home to the mesenteric lymph node or gut lamina propria (23). Vα19 transgenic cells can produce copious amounts of IL-4, consistent with the finding that human Vα7.2αβ cells express the transcription factor PLZF, a key regulator of cytokine production by iNKT cells (24, 25). As shown in Supplemental Fig. 2, a deficiency in MR1, which has been shown to block the development of MAIT cells (23), had no effect on the numbers of itk−/− total thymocytes or on the numbers of CD8SP or CD4SP thymocytes expressing high levels of Eomes.

**iNKT cells promote the development of Eomesαβ T cells**

A second potential source of excess IL-4 in itk−/− mice is iNKT cells. Although mature iNKT cells predominantly produce IFN-γ, with little IL-4 production, immature iNKT cells produce high levels of IL-4 (26). Additionally, previous studies showed that iNKT cell maturation in the absence of Itk is impaired, leading to increased proportions of the immature IL-4-producing subset in these mice (27, 28). To test for a role of iNKT cells, we crossed itk−/− mice to CD1d-deficient mice and examined the thymocytes in these mice. In the absence of iNKT cells, although we observed no overall decrease in thymic cellularity between itk−/− and itk−/−cd1d−/− mice (Fig. 4A), we did observe a slight decrease in the frequency and number of CD8SP Eomesαβ cells (Fig. 4B). These data indicate that whereas iNKT cells do not appear to contribute to the development of Eomes+CD8αβ thymocytes, iNKT cells may promote the expansion of itk−/−Eomes+ CD8αβ thymocytes. In contrast, CD4SP Eomesαβ cells were substantially reduced in Itk/CD1d double-deficient mice when compared with Itk-deficient mice (Fig. 4C). Furthermore, Itk/CD1d double-deficient CD4SP thymocytes resembled WT and CD1d-deficient CD4SP thymocytes, with no statistical significance found between these three groups (Fig. 4C). Detailed analysis revealed that the percentages and absolute numbers of total CD4SP thymocytes were identical between itk−/− and itk−/−cd1d−/− mice (Supplemental Fig. 3A, 3B), indicating that iNKT cells contributed to the conversion of conventional CD4SP to Eomes+CD8αβ, and not to their development per se. These data show that iNKT cells are significantly promoting Eomes upregulation in conventional CD4+ T cells, but iNKT cells are not contributing significantly to the development of itk−/−EomesαβCD8αβ thymocytes.

If iNKT cells are contributing significant amounts of IL-4 to the thymic environment of itk−/− mice, then IL-4Rα (CD124) expression should also be decreased on itk−/− CD8SP and CD4SP thymocytes in the absence of iNKT cells, as IL-4 signaling is known to induce a positive feedback loop leading to upregulation of the IL-4R (29). Although there was a slight decrease in the geometric mean fluorescence intensity of IL-4Rα on mature Itk/CD1d double-deficient CD8SP and CD4SP cells compared with those in itk−/− mice, IL-4Rα was still highly expressed on itk−/−mature SP thymocytes in the absence of iNKT cells (Supplemental Fig. 3C, 3D). One possible explanation for these data is that the loss of IL-4 normally contributed by iNKT cells in itk−/− mice is not sufficient to impact CD124 expression, but it is enough to prevent Eomes upregulation. Alternatively, the absence of αβ iNKT cells may enhance the development of γδ NKT cells, which has been demonstrated in WT mice (30). Indeed, we saw that PLZFγδ T cells were increased in both percentage and number.
in the absence of αβ iNKT cells (Supplemental Fig. 3E, 3F), which may account for the maintained high expression of IL-4Rα. Therefore, iNKT cells may regulate innate T cell development in itk<sup>−/−</sup> mice by a mechanism independent of IL-4 production.
Eomes+ T cells develop independently of T \( \gamma \delta \) cells

The data shown above indicate that iNKT cells are essential for the development of innate Eomes+CD4+ T cells in \( \text{itk}^{-/-} \) mice. Nonetheless, the \( \text{Itk/CD1d} \) double-deficient mice still had a substantial population of Eomes+CD8+ T cells. In addition to \( \alpha \beta \) iNKT cells, a closely related subset of NKT cells has been described that expresses an invariant \( \gamma \delta \) TCR, utilizing V\( \gamma 1 \)/V\( \delta 6.3 \) (30, 31). Recent studies have reported a dramatic expansion in this population in \( \text{itk}^{-/-} \) mice, as well as in other lines of mice that develop Eomes+ innate CD8+ T cells, including SLP-76(Y145F) mice (6, 7, 11, 12). \( \gamma \delta \) NKT cells also produce copious amounts of IL-4 in response to stimulation (11, 12) and have been

**FIGURE 4.** \( \alpha \beta \) NKT cells promote the development of Eomes+ T cells. Thymocytes from WT, \( \text{itk}^{-/-} \), \( \text{cd1d}^{-/-} \), and \( \text{itk/CD1d} \) double-deficient mice were stained with CD1d-tetramer and Abs against CD4, CD8, TCR\( \beta \), HSA, CD44, and Eomes. Dot plots show Eomes versus CD44 staining, and graphs show compilations of the percentages and absolute numbers of Eomes+ cells. (A) Total thymic cellularity. Significant differences were seen between WT and CD1d KO mice \((p < 0.05)\). (B) Gated on CD8SP TCR\( \beta^{hi} \) HSA\( ^{lo} \) thymocytes. (C) Gated on CD4SP TCR\( \beta^{lo} \) TCR\( \beta^{hi} \) HSA\( ^{lo} \)CD1d-tetramer\( ^{lo} \) thymocytes. Results are from four independent experiments \((n = 7-9 \text{ mice/group})\). Statistical analysis performed using a one-way ANOVA. \(*p < 0.05, **p < 0.0005, ***p < 0.0001.\)
proposed as a possible cellular source of the excess IL-4 driving innate T cell development in both *itk*−/− and SLP-76 (Y145F) mice (13).

To address the role of γδ T cells, we crossed *itk*−/− mice to TCRδ-deficient (tcrd−/−) mice. To our surprise, thymocytes in the *itk*−/− mice lacking γδ T cells were indistinguishable...
from those in single itk−/− mice. Overall thymic cellularity and both the CD8SP and CD4SP subsets had comparable proportions and numbers of Eomes+ cells when itk−/− thymocytes were compared with itk−/− tcrd−/− thymocytes (Fig. 5). Although we did see significant increases in the frequency and cell number of αβ iNKT cells in tcrd−/− mice, there were no differences in αβ iNKT cells between itk−/− and itk−/− tcrd−/− mice (Supplemental Fig. 4A, 4B). Thus, in contrast to recent studies in WT mice (30), it seems unlikely that itk−/− αβ iNKT cells are overcompensating in the absence of itk−/− γδ NKT cells. Similar findings were observed in SLP-76(Y145F) mice that lacked γδ T cells, such that the frequencies and numbers of Eomes+ CD8SP and CD4SP

\[\text{Eomes}^+\] T cells are dependent on IL-4. Thymocytes from WT, itk−/−, il4ra−/−, and itk−/− il4ra−/− mice were stained with Abs to CD4, CD8, TCRβ, HSA, CD44, and Eomes. Dot plots show Eomes versus CD44 staining, and graphs show compilations of the percentages and absolute numbers of Eomes+ cells. (A) Total thymic cellularity. Significant differences were detected between WT and itk−/− (p < 0.0001), WT and itk−/− il4ra−/− (p < 0.0005), itk−/− and il4ra−/− (p < 0.0005), and il4ra−/− and itk−/− il4ra−/− (p < 0.005). (B) Gated on CD8SP TCRβ highCD24 (HSA) low thymocytes. (C) Gated on CD4SP TCRβ highCD24 (HSA) low thymocytes. Results are representative of three independent experiments (n = 3–6 mice/group). Statistical analysis was performed using one-way ANOVA. ***p < 0.0001.

\[\text{CD4}^+\] and CD8^+ Eomes+ T cells are dependent on IL-4. Thymocytes from WT, itk−/−, il4ra−/−, and itk−/− il4ra−/− mice were stained with Abs to CD4, CD8, TCRβ, HSA, CD44, and Eomes. Dot plots show Eomes versus CD44 staining, and graphs show compilations of the percentages and absolute numbers of Eomes+ cells. (A) Total thymic cellularity. Significant differences were detected between WT and itk−/− (p < 0.0001), WT and itk−/− il4ra−/− (p < 0.0005), itk−/− and il4ra−/− (p < 0.0005), and il4ra−/− and itk−/− il4ra−/− (p < 0.005). (B) Gated on CD8SP TCRβ highCD24 (HSA) low thymocytes. (C) Gated on CD4SP TCRβ highCD24 (HSA) low thymocytes. Results are representative of three independent experiments (n = 3–6 mice/group). Statistical analysis was performed using one-way ANOVA. ***p < 0.0001.
thymocytes were similar in SLP-76(Y145F) mice and 

\textbf{SLAM family receptor adapter protein–dependent PLZF$^+$ CD1d-tetramer$^-$ αβ T cells develop in the absence of Itk.} 

Previous studies have shown a dependence of \( \text{itk}^{-/-} \) Eomes$^+$ innate CD8SP thymocytes on IL-4 for their development (8). However, we were unsure whether the same was true for \( \text{itk}^{-/-} \) Eomes$^+$ innate CD4SP thymocytes, because we observed differential requirements for IL-15 signaling (Supplemental Fig. 1) and the presence of iNKT cells (Fig. 4) between these two subsets. To further examine the dependence on IL-4 for the development of \( \text{itk}^{-/-} \) Eomes$^+$ innate CD4SP thymocytes, Itk-deficient mice were crossed to mice deficient in IL-4R$^+$ (\( \chi \text{D}124 \)). Although we saw reduced thymic cellularity in \( \text{itk}^{-/-} \) and \( \text{itk}^{-/-} \) il4ra$^{-/-}$ mice, these populations did not differ significantly from each other (Fig. 6A). As previously seen for innate CD8SP thymocytes, the Eomes-expressing population was eliminated in the absence of IL-4 signaling (Fig. 6B). Eomes$^+$ innate CD4SP thymocytes also failed to develop in the absence of IL-4 signaling (Fig. 6C). Thus, although Eomes$^+$ innate CD8SP and CD4SP thymocytes do have some differential requirements for their development, both populations require IL-4.

The data presented above ruled out a role for well-defined IL-4–producing CD4$^+$ T cells populations, such as γδ NKT cells and MR1-dependent MAIT cells, in the development of Eomes$^+$ αβ

\*Graphs (right) show frequencies and absolute numbers of CD4SP TCR$^+$ HSA$^+$ PLZF$^+$ thymocytes. Results are from independent experiments (\( n = 20-23 \) mice/group). Statistical analysis was performed using a Mann–Whitney Student \( t \) test. **** \( p < 0.0001 \).

\*Flow cytometry plots are gated on CD4 SP TCR$^+$ HSA$^+$ CD1d-tetramer$^+$ thymocytes. Results are representative of four independent experiments involving two to three mice per group per experiment. (C and D) Thymocytes from WT or Itk KO mice were harvested, processed, and stimulated with PMA and ionomycin (right) or remained unstimulated (left) in the presence of brefeldin A and monensin for 5–6 h at 37°C before staining with CD1d tetramer and with Abs against CD4, CD8, Eomes, PLZF, IFN-γ, and IL-4. (C) Gated on CD4SP CD1d-tetramer$^+$ Eomes$^+$ thymocytes. (D) Gated on CD4SP CD1d-tetramer$^+$ PLZF$^+$ thymocytes. Results are representative of two independent experiments involving five to seven mice per group.
T cells in *itk*−/− mice. Additionally, the data indicated that CD1d-dependent αβ iNKT cells were not essential. This led us to consider whether *itk*−/− mice might have an additional population of PLZF+ cells capable of producing IL-4. Therefore, we examined mature CD4SP αβ thymocytes (TCRδ−, TCRβhi, HSAlo, CD1d-tetramer−) and found an increased population of PLZF+ cells compared with WT mature CD4SP thymocytes (Fig. 7A). Furthermore, when Itk knockout mice were crossed to IL-4 reporter mice that have a bicistronic promoter adding an IRES-GFP component to the untranslated region of the IL-4 locus (32), we found an increased population of mature CD4SP PLZF+CD1d-tetramer− cells expressing PLZF (left) or Eomes (right). Results are representative of four independent experiments (n = 10–12 mice/group). Statistical analysis was performed using a one-way ANOVA. ****p < 0.0001.

T cells in *itk*−/− mice. Additionally, the data indicated that CD1d-dependent αβ iNKT cells were not essential. This led us to consider whether *itk*−/− mice might have an additional population of PLZF+ cells capable of producing IL-4. Therefore, we examined mature CD4SP αβ thymocytes (TCRδ−, TCRβhi, HSAlo, CD1d-tetramer−) and found an increased population of PLZF+ cells compared with WT mature CD4SP thymocytes (Fig. 7A). Furthermore, when Itk knockout mice were crossed to IL-4 reporter mice that have a bicistronic promoter adding an IRES-GFP component to the untranslated region of the IL-4 locus (32), we found an increased population of mature CD4SP thymocytes expressing IL-4 mRNA (Fig. 7B). IL-4 mRNA appears to only be present in cells expressing PLZF because CD4SP CD1d-tetramer− thymocytes expressing PLZF produce IL-4 and IFN-γ in response to stimulation whereas CD4SP CD1d-tetramer− thymocytes expressing Eomes only produce IFN-γ (Fig. 7C, 7D). Thus, in the absence of Itk, a population of mature CD4SP PLZF+ αβ T cells with capabilities of producing IL-4 develops in the thymus.

Eomes+ αβ T cells in *itk*−/− mice are dependent on SLAM family receptor adapter protein (SAP) for their development (6, 33), an observation that has been linked to the requirement for SAP in the development of PLZF+ cells in other mouse models (6). To determine whether *itk*−/− CD4SP PLZF+ CD1d-tetramer− αβ thymocytes were also dependent on SAP, we examined Itk/SAP double-deficient mice. As shown in Fig. 8, SAP was essential for the development of PLZF+ CD4SP CD1d-tetramer− αβ thymocytes in *itk*−/− mice. Furthermore, all *itk*−/− Eomes+ CD4SP thymocytes reverted to a conventional CD4SP phenotype in the absence of SAP. These data identify a novel population of SAP-dependent PLZF+ CD4+ T cells that is essential for the development of innate-like Eomes+ αβ T cells in *itk*−/− mice.

**FIGURE 8.** Development of all innate T cells in *itk*−/− is dependent on SAP. Thymocytes from WT, *itk*−/−, *sh2d1a*−/−, and *itk*−/− *sh2d1a*−/− mice were stained with CD1d-tetramer and Abs against CD4, CD8, TCRβ, HSA, Eomes, and PLZF. (A) Total thymic cellularity. Significant differences were seen between WT and *itk*−/− mice (p < 0.005), WT and *sh2d1a*−/− mice (p < 0.05), and WT and *itk*−/− *sh2d1a*−/− mice (p < 0.0005). (B) Dot plots show PLZF versus Eomes staining on CD4SP TCRβhiHSAloCD1d-tetramer− thymocytes. (C and D) Graphs show frequencies (C) and absolute numbers (D) of CD4SP TCRβhiHSAloCD1d-tetramer− cells expressing PLZF (left) or Eomes (right). Results are representative of four independent experiments (n = 10–12 mice/group). Statistical analysis was performed using a one-way ANOVA. ****p < 0.0001.
Discussion

The data presented in this study show that innate T cell development is not restricted to the CD8⁺ T cell subset in itk⁻/⁻ mice. We demonstrate that a sizeable proportion of itk⁻/⁻ CD4SP thymocytes develop as innate-like cells expressing high levels of Eomes. Although there is a sizeable population of Eomes⁺ innate itk⁻/⁻ CD4SP thymocytes, we found that only a small proportion (5.5–11.4%) of these cells produce IFN-γ upon stimulation. However, this finding is similar to results obtained with mature innate itk⁻/⁻ CD8SP thymocytes where only a portion (~13%) produce IFN-γ upon stimulation, despite the fact that the vast majority express Eomes (8, 15). We also saw a sizeable proportion of Eomes⁺ CD4SP thymocytes that produced IFN-γ (34). This is most likely due to an increase in PLZF⁺ CD4SP thymocytes, because PLZF allows β T NK cells to produce both IL-4 and IFN-γ (25). Collectively, our data support the conclusion that the Eomes⁺ CD4SP cells in itk⁻/⁻ mice are conventional MHC class II–dependent cells that have been induced to upregulate Eomes by the cell-extrinsic factor IL-4. When considering both the CD4⁺ and the CD8⁺ subsets of Eomes-expressing innate T cells in itk⁻/⁻ mice, we have identified two independent cell populations that are critical for the dramatic conversion of conventional T cells into innate T cells in these mice.

First, we discovered that IL-4 signaling is crucial for the development of Eomes⁺ innate CD4SP thymocytes, similarly to what has been previously demonstrated for itk⁻/⁻ CD8SP thymocytes (8). Furthermore, we found that a SAP-dependent population of PLZF⁺ T cells is essential for the development of Eomes⁺ CD8⁺ T cells in itk⁻/⁻ mice, data that are consistent with several previous studies showing that both SAP and PLZF are necessary for this process (6, 8). Second, our data reveal a surprising role for iNKT cells in the development of itk⁻/⁻ Eomes⁺ CD4⁺ T cells but not CD8⁺ T cells. This latter finding indicates that the two subsets of Eomes⁺ innate T cells in itk⁻/⁻ mice have distinct requirements and/or signaling pathways that regulate their development. One possibility is that CD4SP thymocytes need a higher concentration of IL-4 to induce Eomes upregulation relative to CD8SP thymocytes. If true, this might necessitate the combined cytokine production of several PLZF⁺ populations to achieve this threshold. Although possible, this scenario seems unlikely, as we observed no effect of eliminating γδ T cells on the Eomes⁺ CD4SP population, despite the fact that the itk⁻/⁻ γδ iNKT cells produce enough IL-4 to induce a hyper-IgE phenotype in these mice (11, 12). An alternative possibility is that different cell populations reside in distinct compartments in the thymus, thus limiting access of cytokines to cells in the immediate environment. Thus, Eomes⁺ CD4SP thymocytes in itk⁻/⁻ mice might colocalize with iNKT cells to a greater extent than CD8SP thymocytes. However, although we find that iNKT cells do not appear to regulate the development of itk⁻/⁻ Eomes⁺ CD8SP thymocytes, iNKT cells may contribute to the expansion of this innate T cell population. Furthermore, it appears that conventional CD4SP thymocytes may also have a role in the expansion of itk⁻/⁻ Eomes⁺ CD8SP thymocytes. Our laboratory has previously described that Itk-deficient mice have a slight proliferative defect at the double-negative to double-positive transitions (35). Thus, SP thymocytes may aid in the proliferation of developing thymocytes via cytokine secretion.

One unexpected finding from our studies was the lack of importance of γδ NKT cells in the development of innate Eomes⁺ CD4⁺ and CD8⁺ T cells in itk⁻/⁻ mice. Because this cell population is greatly expanded in numbers in itk⁻/⁻ mice and is a significant producer of IL-4, it was a reasonable hypothesis that these cells would contribute to, or possibly be completely responsible for, the IL-4 production that generates Eomes⁺ innate T cells. However, we observed no detectable change in either CD4SP or CD8SP thymocytes in itk⁻/⁻ mice lacking γδ T cells, a result that was confirmed in SLP-76(Y145F) mice. Instead, our data suggest that the PLZF⁺ T cells responsible for converting itk⁻/⁻ CD4⁺ and CD8⁺ thymocytes into innate Eomes-expressing cells are primarily β TCR⁺ cells. Furthermore, for the development of itk⁻/⁻ CD8⁺ innate T cells, our data rule out a role for the other known subsets of PLZF⁺ T cells, such as iNKT cells and MAIT cells. Although it is possible that these subsets could be sufficient to induce the development of memory/innate-like thymocytes in some contexts, our findings indicate that these subsets are not required. Instead, our data argue for a novel PLZF⁺CD4⁺ αβ T cell subset capable of producing IL-4 that is expanded in itk⁻/⁻ mice, similar to the situation for γδ NKT cells.

One interesting possibility is that the population of itk⁻/⁻ PLZF⁺CD4⁺ αβ T cells responsible for inducing innate T cell development is related to the “T-CD4” cells found in the thymus of mice engineered to express MHC class II proteins on their thymocytes (36, 37). These “T-CD4” cells develop as a result of SAP-dependent thymocyte–thymocyte interactions that lead to upregulation of PLZF and to an innate capacity to secrete IL-4 (38–40). Additionally, the CD8SP thymocytes in mice with T-CD4 develop as innate cells expressing high levels of Eomes (40). Furthermore, these mice also have a population of CD4SP thymocytes expressing Eomes (40). Although there does appear to be few mature CD4SP thymocytes expressing Eomes in WT mice, we did find significant differences in a population of mature PLZF⁺CD4⁺ αβ T cells between WT and SAP-deficient mice (p < 0.0002 using a Mann–Whitney test). Therefore, development of PLZF⁺ thymocytes may be tightly regulated in WT mice to control the development of innate-like lymphocytes expressing Eomes. Furthermore, unlike mouse thymocytes, human thymocytes express MHC class II molecules, correlating with a substantial number of Eomes⁺ CD8⁺ T cells in human fetal thymus and spleen. Based on these findings, Min et al. (40) have suggested that these innate T cells may function in protective immunity during the perinatal period. Thus, it is possible that a normal developmental pathway for human T cells has been revealed by the genetic ablation of key T cell signaling proteins and transcription factors in mice.

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Disclosures

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References
