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

J Immunol 2014; 193:688-699; Prepublished online 18 June 2014;
doi: 10.4049/jimmunol.1302059
http://www.jimmunol.org/content/193/2/688

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/06/18/jimmunol.1302059.DCSupplemental

References
This article cites 40 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/193/2/688.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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 thymocytes, in several strains of mice harboring mutations in T cell signaling proteins or transcriptional regulators, conventional CD8+ T cells develop as innate cells with characteristics of memory T cells. Thus, in Itk-deficient mice, mature CD4+CD8+ (CD8 single-positive [SP]) thymocytes express high levels of the transcription factor eomesodermin (Eomes) and are dependent on IL-4 being produced in the thymic environment by a poorly characterized subset of CD4+ thymocytes expressing the transcriptional regulator promyelocytic leukemia zinc finger. In this study, we show that a sizeable proportion of mature CD4+CD8+ (CD4SP) thymocytes in *itk*−/− mice also develop as innate Eomes-expressing T cells. These cells are dependent on MHC class II and IL-4 signaling for their development, indicating that they are conventional CD4+ T cells that have been converted to an innate phenotype. Surprisingly, neither CDMP nor CD8SP innate Eomes+ thymocytes in *itk*−/− or SLP-76(Y145F) mice are dependent on γδ T cells for their development. Instead, we find that the predominant population of Eomes+ innate *itk*−/− CD4SP thymocytes is largely absent in mice lacking CD1d-specific invariant NKT cells, with no effect on innate *itk*−/− CD8SP thymocytes. In contrast, both subsets of innate Eomes+ T cells require the presence of a novel promyelocytic leukemia zinc finger–expressing, SLAM family receptor adapter protein–dependent thymocyte population that is essential for the conversion of conventional CD4+ and CD8+ T cells into innate T cells with a memory phenotype.
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 mice (4get) mice were a gift from Markus Mohrs (Trudeau Institute, Saranac Lake, NY) and were crossed to itk−/− mice at the University of Massachusetts Medical School. The cd1d−/− mice were a gift from the laboratory of Raymond Welsh and were crossed to itk−/− mice at the University of Massachusetts Medical School. The mrl−/− and il15−/− mice were a gift from Joonsoo Kang and were also crossed to itk−/− mice at the University of Massachusetts Medical School. The sh2d1a−/− mice were previously described (18) and were crossed to itk−/− mice at the University of Massachusetts Medical School. H2-K1a/Ki mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were crossed to itk−/− mice at the University of Massachusetts Medical School. The tcrd−/− mice were a gift from the laboratory of Raymond Welsh and were crossed to itk−/− mice at the University of Massachusetts Medical School as previously described (11). SLP-76(Y146F) mice were previously described (19) and housed at the University of Pennsylvania in accordance with their Institutional Animal Care and Use Committee. The tcrd−/− mice were purchased from The Jackson Laboratory and crossed with SLP-76(Y146F) mice to generate Y146F/tcrd−/− mice. The Itk−/− mice were a gift from Kristin Hoggquist (University of Minnesota, Minneapolis, MN) and were bred to itk−/− mice at the National Institutes of Health in accordance with their Institutional Animal Care and Use Committee to generate itk−/−Itk−/− mice.

Cell preparation

Thymi were harvested from mice and stored in RPMI 1640 (Life Technologies by Invitrogen, Grand Island, NY) supplemented with FBS, 1-glutamine, penicillin, streptomycin, 2-ME, and HEPEs (RPMI 10). Thymi were processed using forceps and frosted microscope slides. Thymocytes were lysed with RBC lysis buffer prior to single-cell suspension with RPMI 10.

Cell stimulations

Cells were plated at 105–106 cells/well prior to stimulating with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 6 h at 37°C in the presence of brefeldin A and monensin.

Extracellular/intracellular staining

Cells were plated at 105–106 cells per well prior to washing with FACS buffer (1% PBS supplemented with 2% FBS). Fc receptors were blocked using supernatant from 2.4G2 hybridoma grown in the laboratory prior to staining with the CD1d tetramer loaded with PBSt7 (a gift from the National Institutes of Health). Cells were stained with various combinations of the cell surface Abs against CD4 (RM-4.5), CD8 (53-67.6 or 5H10), TCRβ (H57-597), TCRα (GL3), heat-stable Ag (HSA, CD24) (30-F1 or M169), CD44 (IM7), CD62L (ME-14), CD124 (IL-4Rα) (mIL-4R-M1), CD122 (IL-2Rβ) (SH4 or TM-B1), and CCR3 (CXC3R1-173). Cells were then permeabilized using the E Bioscience (San Diego, CA) Foxp3/transcription factor staining kit according to the manufacturer’s protocol prior to staining with Abs against the transcription factors Eomes (Dan11mag) and PLZF (D-9, IgG1; A85-1), as well as the cytokines IL-4 (BVD6-24G2) and IFN-γ (XMG1.2). Abs were purchased from BD Biosciences (San Jose, CA), eBioscience, or Santa Cruz Biotechnology (Dallas, TX).

Statistical analysis

Statistics were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA) using a Student t test or one-way ANOVA as applicable.

Results

CD4+Eomes+ αβ T cells develop in the absence of Itk

As previously described (15, 20), thymocytes from itk−/− mice have an increased frequency of CD4+ CD8+ (CD8 single-positive [SP]) cells compared with WT thymocytes, leading to a decreased ratio of CD4/CD8 SP thymocytes (Fig. 1A). Additionally, the predominant populations of CD8SP thymocytes in itk−/− mice express high levels of Eomes owing to increased IL-4 signaling (8, 15) (Fig. 1B). itk−/− CD8SP thymocytes also express increased levels of the IL-4Rα (CD124) and have a memory-like phenotype characterized by high expression of CD44, CD62L, IL-2Rβ (CD122), and CCR3 (Fig. 1B, 1D).

To determine whether itk−/− CD4+ αβ T cells also underwent altered development, we characterized CD4+CD8− (CD4SP) thymocytes from itk−/− mice. We found that the distribution of conventional, mature (TCRβ−/−/−/−) and nonconventional, immature (TCRβ+−/−/−) CD4+CD8− thymocytes were similar in WT and itk−/− mice (Fig. 1B). Moreover, we found that the frequency and number of Eomes+CD4+CD8− thymocytes were also similar in WT and itk−/− mice (Fig. 1C).

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−/− 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 ($H2^{dAb1-Ea}$). 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 $itk^{-/-}$
produce copious amounts of IL-4, consistent with the finding that lymph node or gut lamina propria (23). Furthermore, these cells preferentially home to the mesenteric that recognizes the nonclassical MHC class Ib molecule MR1; itk subset in these mice (27, 28). To test for a role of iNKT cells, we leading to increased proportions of the immature IL-4–producing that iNKT cell maturation in the absence of Itk is impaired, high levels of IL-4 (26). Additionally, previous studies showed IFN- innate-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.

Eomes+ αβ T cells develop independently of MAIT cells

The data shown above indicate that both conventional CD8+ and CD4+ αβ 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 Vα7.2+ 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 Eomes+ αβ 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 thymiccellularity between itk−/− and itk−/−cd1d−/− mice (Fig. 4A), we did observe a slight decrease in the frequency and number of CD8SP Eomes+ cells (Fig. 4B). These data indicate that whereas iNKT cells do not appear to contribute to the development of Eomes+CD8+ thymocytes, iNKT cells may promote the expansion of itk−/−Eomes+CD8+ thymocytes. In contrast, CD4SP Eomes+ 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+CD4SP, 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−/−Eomes+CD8+ 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 PLZF+ γδ 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 \( \text{itk}^{-/-} \) mice by a mechanism independent of IL-4 production.
Eomes+ T cells develop independently of T γδ cells

The data shown above indicate that iNKT cells are essential for the development of innate Eomes+CD4+ T cells in Itk2/2 mice. Nonetheless, the Itk/CD1d double-deficient mice still had a substantial population of Eomes+ innate CD8+ T cells. In addition to αβ iNKT cells, a closely related subset of NKT cells has been described that expresses an invariant γδ TCR, utilizing Vγ1.1/Vδ6.3 (30, 31). Recent studies have reported a dramatic expansion in this population in Itk2/2 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). γδ NKT cells also produce copious amounts of IL-4 in response to stimulation (11, 12) and have been
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

---

**FIGURE 5.** Eomes+ T cells develop independently of γδ T cells. Thymocytes from WT, *itk*−/−, *tcrd*−/−, and *itk*−/−*tcrd*−/− mice were stained with CD1d-tetramer and 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. No significant differences were detected. (B) Gated on CD8SP TCRβhi HSAlo thymocytes. (C) Gated on CD4SP TCRβhi HSAlo CD1d-tetramerhigh thymocytes. Results are representative of two independent experiments (*n* = 4–7 mice/group). Statistical analysis was performed using a one-way ANOVA. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.
from those in single \( \textit{itk}^{-/-} \) mice. Overall thymic cellularity and both the CD8SP and CD4SP subsets had comparable proportions and numbers of Eomes cells when \( \textit{itk}^{-/-} \) thymocytes were compared with \( \textit{itk}^{-/-} \textit{il4ra}^{-/-} \) thymocytes (Fig. 5). Although we did see significant increases in the frequency and cell number of \( \alpha \beta \iNKT \) cells in \( \textit{tdr}^{-/-} \) mice, there were no differences in \( \alpha \beta \iNKT \) cells between \( \textit{itk}^{-/-} \) and \( \textit{itk}^{-/-} \textit{tdr}^{-/-} \) mice (Supplemental Fig. 4A, 4B). Thus, in contrast to recent studies in WT mice (30), it seems unlikely that \( \textit{itk}^{-/-} \alpha \beta \iNKT \) cells are overcompensating in the absence of \( \textit{itk}^{-/-} \gamma \delta \) NKT cells. Similar findings were observed in SLP-76(Y145F) mice that lacked \( \gamma \delta \) T cells, such that the frequencies and numbers of Eomes' CD8SP and CD4SP
thymocytes were similar in SLP-76(Y145F) mice and tcrd−/− mice (Supplemental Fig. 4C, 4D). Additionally, the absence of gd NKT cells did not influence the frequency or number of ab NKT cells in SLP-76(Y145F) mice (Supplemental Fig. 4E). Interestingly, although gd T cells constitute half of the PLZF+ thymocytes in SLP-76(Y145F) mice, the total number of PLZF+ thymocytes was not reduced in tcrd−/− SLP-76(Y145F) mice (Supplemental Fig. 4F), leaving open the possibility that the contribution of gd T cells to the development of innate-like thymocytes might be masked by a compensatory increase in other PLZF+ populations. Thus, these data demonstrate that although gd NKT cells contribute significantly to the hyper-IgE syndrome seen in Itk-deficient mice (11, 12), these cells are not required for the development of Eomes+ ab T cells.

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 itk−/− Eomes+ innate CD4SP thymocytes on IL-4 for their development (8). However, we were unsure whether the same was true for 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 itk−/− Eomes+ innate CD4SP thymocytes, Itk-deficient mice were crossed to mice deficient in IL-4Rα (XαD124). Although we saw reduced thymic cellularity in itk−/− and 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 gd NKT cells and MR1-dependent MAIT cells, in the development of Eomes+ αβ
In itk<sup>−−</sup> mice, T cells in CD4SP TCR<sup>b</sup><sup>hi</sup> HSA<sup>lo</sup> CD1d-tetramer<sup>−−</sup> thymocytes were found to be PLZF+ and capable of producing IL-4. This led to the consideration that these cells might be essential for the development of innate-like Eomes<sup>+</sup> T cells. To further investigate this, 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. The resulting mice showed an increased population of mature CD4SP PLZF+ cells compared with WT mice (Fig. 7B). Additionally, all itk<sup>−−</sup> Eomes<sup>+</sup> CD4SP thymocytes reverted to a conventional CD4SP phenotype in the absence of SAP. These data identify a novel population of SAP-dependent PLZF<sup>+</sup>CD4<sup>+</sup> T cells that is essential for the development of innate-like Eomes<sup>+</sup> T cells in itk<sup>−−</sup> mice.
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 ββ NK T 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+ CD4SP 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 NK T cells on the Eomes+ CD4SP population, despite the fact that the itk/-/ γδ NK T 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 IκB-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 NK T 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 ββ 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+ ββ T cell subset capable of producing IL-4 that is expanded in itk/-/ mice, similar to the situation for γδ iNKT cells.

One interesting possibility is that the population of itk/-/ PLZF+CD4+ ββ 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 cells 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+ ββ 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.

Acknowledgments

We thank Regina Whitehead and Sharlene Hubbard for technical assistance and the National Institutes of Health Tetramer Core Facility for use of the mCD1d tetramer.

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Data

A) Thymic Cellularity
- WT: $1.58 \times 10^6$
- Itk KO: $1.03 \times 10^6$
- IL-15 KO: $1.39 \times 10^6$
- Itk/IL-15 DKO: $7.49 \times 10^7$
- Itk KO: $\leq 6.12 \times 10^7$
- IL-15 KO: $\leq 3.14 \times 10^7$
- Itk/IL-15 DKO: $\leq 2.07 \times 10^7$

B) CD8 SP TCRβ<sup>hi</sup> HSA<sup>lo</sup>

- WT
- Itk KO
- IL-15 KO
- Itk/IL-15 DKO

C) CD4 SP TCRβ<sup>neg</sup> TCRβ<sup>hi</sup> HSA<sup>lo</sup> CD1d tetramer<sup>neg</sup>

- WT
- Itk KO
- IL-15 KO
- Itk/IL-15 DKO
Supplemental Figure 1: IL-15 regulates the expansion of Eomesodermin$^+$ αβ T cells.

Thymocytes from WT, $itk^{-/-}$, $il15^{-/-}$, and $itk^{-/-}il15^{-/-}$ mice were stained with CD1d-tetramer and antibodies to CD4, CD8, TCRδ, TCRβ, HSA (CD24), CD44, and Eomesodermin. 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 $itk^{-/-}$ mice (p < 0.005) and between $il15^{-/-}$ and $itk^{-/-}il15^{-/-}$ mice (p < 0.05).

(B) Gated on CD8SP TCR$^+$ high HSA$^+$ low thymocytes.

(C) Gated on CD4SP TCR$^+$ neg TCRβ$^+$ high HSA$^+$ low CD1d-tetramer$^+$ neg thymocytes.

$n = 6-7$ mice per group. Results are from three independent experiments. Statistical analysis performed using a one-way ANOVA. *p < 0.05 **p < 0.05 ***p < 0.0005 ****p < 0.0001
A) Thymic Cellularity

<table>
<thead>
<tr>
<th>Condition</th>
<th>WT</th>
<th>Itk KO</th>
<th>MR1 KO</th>
<th>Itk/MR1 DKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>$1.27 \times 10^8$</td>
<td>$7.42 \times 10^7$</td>
<td>$1.58 \times 10^8$</td>
<td>$7.81 \times 10^7$</td>
</tr>
<tr>
<td>Variance</td>
<td>$\pm 5.23 \times 10^7$</td>
<td>$\pm 2.14 \times 10^7$</td>
<td>$\pm 7.62 \times 10^7$</td>
<td>$\pm 3.70 \times 10^7$</td>
</tr>
</tbody>
</table>

B) CD8 SP TCR$^{\beta}$high HSA$^{low}$

C) CD4 SP TCR$^{\delta}$neg TCR$^{\beta}$high HSA$^{low}$ CD1d Tetramer$^{neg}$
Supplemental Figure 2: *itk*⁻/⁻ Eomesodermin⁺ αβ T cells develop independently of MAIT cells.

Thymocytes from WT, *itk*⁻/⁻, *MR1*⁻/⁻, and *itk*⁻/⁻ *MR1*⁻/⁻ mice were stained with CD1d-tetramer and antibodies to CD4, CD8, TCRδ, TCRβ, HSA, CD44, and Eomesodermin. 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 *itk*⁻/⁻ and *MR1*⁻/⁻ mice (p < 0.05) and between *MR1*⁻/⁻ and *itk*⁻/⁻ *MR1*⁻/⁻ mice (p < 0.05).

(B) Gated on CD8SP TCRβʰigh HSAʰlow thymocytes.

(C) Gated on CD4SP TCRδⁿeg TCRβʰigh HSAʰlow CD1d-tetramerⁿeg thymocytes.

n = 5-8 mice per group. Results are representative of three independent experiments. Statistical analysis performed using a one-way ANOVA. **p < 0.005 ****p < 0.0001
Supplemental Figure 3: $\alpha\beta$ NKT cells promote the development of Eomesodermin$^+$ T cells.

Thymocytes from WT, itk$^{-/-}$, cd1d$^{-/-}$, and itk$^{-/-}$cd1d$^{-/-}$ mice were stained with CD1d-tetramer and antibodies against CD4, CD8, TCR$\delta$, TCR$\beta$, HSA, IL-4R$\alpha$ (CD124), and Eomesodermin.

(A-B) Graphs show a compilation of four independent experiments indicating the percentage (A) and the number (B) of CD4 SP thymocytes. $n = 7$-9 mice per group.

(C) Histograms of IL-4R$\alpha$ (CD124) expression on mature CD8 SP thymocytes (CD8 SP TCR$\beta^{\text{high}}$ HSA$^{\text{low}}$). Graphs represent one experiment with 2-3 mice per group.

(D) Histograms of IL-4R$\alpha$ (CD124) expression on mature CD4 SP thymocytes (CD4 SP TCR$\delta^{\text{neg}}$ TCR$\beta^{\text{high}}$ HSA$^{\text{low}}$ CD1d-tetramer$^{\text{neg}}$). Graphs represent one experiment with 2-3 mice per group.

(E-F) Graphs show a compilation of four independent experiments indicating the percentage (E) and the number (F) of CD4SP TCR$\delta^{\text{pos}}$ TCR$\beta^{\text{neg}}$ PLZF$^+$ ($\gamma\delta$ NKT) thymocytes. $n = 7$-9 mice per group.

Statistical analysis performed using a one-way ANOVA. *$p < 0.05$ **$p < 0.005$ ***$p < 0.0005$ ****$p < 0.0001$
Supplemental Figure 4: Eomesodermin+ T cells develop independently of γδ T cells.

(A-B) Thymocytes from WT, itk−/−, tcrd−/−, and itk−/−tcrd−/− mice were stained with CD1d-tetramer and antibodies to CD4 and CD8. Graphs show compilations of the percentages (A) and absolute numbers (B) of αβ iNKT cells that are gated on CD4 SP CD1d-tetramer+ thymocytes. n = 4-7 mice per group. Results are representative of two independent experiments. Statistical analysis was performed using a one-way ANOVA. *p < 0.05, ****p < 0.0001

(C-F) Thymocytes from SLP-76(Y145F) and SLP-76(Y145F)tcrd−/− mice were stained with a cell viability dye, CD1d-tetramer, and antibodies to CD4, CD8, TCRβ, and Eomesodermin. n = 5 mice per group. Results are representative of two independent experiments. Statistical significance was analyzed in GraphPad Prism using a student’s t test.

Dot-plots show (C) Eomes versus CD8 staining or (D) Eomes versus CD4, and graphs show compilations of the percentages and absolute numbers of Eomes+ cells.

(C) Gated on CD8SP TCRβhigh thymocytes.

(D) Gated on CD4SP TCRβhigh CD1d-tetramerneg thymocytes.

(E) Graphs show the frequency (right) and number (left) of αβ iNKT cells (Gated on live thymocytes that are TCRβhigh and CD1d-tetramerpos).

(F) Graphs show the frequency (right) and number (left) of total CD4+ PLZF+ thymocytes (Gated on live PLZF+ thymocytes).