ITK tunes IL-4-induced development of innate memory CD8+ T cells in a γδ T and invariant NKT cell-independent manner

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ABSTRACT

True memory CD8+ T cells develop post antigenic exposure and can provide life-long immune protection. More recently, other types of memory CD8+ T cells have been described, such as the memory-like CD8+ T cells (IMP; CD44hiCD122hi) that arise spontaneously in itk−/− mice, which are suggested to develop as a result of IL-4 secreted by NKT-like γδ T cells or PLZF+ iNKT cells found in itk−/− mice. However, we report here that whereas IMP CD8+ T cell development in itk−/− mice is dependent on IL-4/STAT6 signaling, it is not dependent on any γδ T or iNKT cells. Our experiments suggest that the IMP develops as a result of tuning of the CD8+ T cell response to exogenous IL-4 and TCR triggering by ITK and challenge the current model of IMP CD8+ T cell development as a result of NKT-like γδ T or iNKT cells. These findings suggest that some naive CD8+ T cells may be preprogrammed by weak homeostatic TCR signals in the presence of IL-4 to become memory phenotype cells with the ability to elaborate effector function rapidly. The role of ITK in this process suggests a mechanism by which IMP CD8+ T cells can be generated rapidly in response to infection. J. Leukoc. Biol. 96: 55–63; 2014.

Introduction

The development of different types of memory CD8+ T cells has been studied intensely. True memory CD8+ T cells develop in response to antigenic exposure and can provide life-long protection against reinfection [1, 2]. More recently, other types of memory CD8+ T cells have been described, including those generated during homeostatic proliferation in lymphopenic environments, virtual memory cells, or those with IMP cells [3–7]. The routes by which these cells develop seem to vary, and with the exception of true memory cells, their provenance, to date, is unclear. IMP CD8+ T cells (characterized by the phenotype CD44hiCD122hi) express the transcription factor Eomes, carry preformed mRNA for and rapidly produce IFN-γ upon stimulation [3, 5, 8], and can be selected through the hematopoietic HCl, independently of the thymus [3].

The Tec family nonreceptor tyrosine kinase ITK plays an important role in T cell activation and differentiation, and its absence leads to defects in positive selection in the thymus, as well as alterations in Tβ2 and Tβ7 cytokine secretion with reduced Th2/Th17 responses to infection or in diseases, such as allergic asthma [9–15]. However, Itk−/− mice paradoxically carry elevated serum IgE, suggestive of an ongoing Th2 response [10, 11, 16–18]. Itk−/− mice also have elevated numbers of NKT-like CD4+ γδ T cells that express the transcription factor PLZF and secrete IL-4 [16–18]. We and others have shown that the elevated serum IgE, observed in Itk−/− mice, is dependent, in part, on IL-4-secreting, NKT-like γδ T cells in these mice [17, 18]. In addition, Itk−/− iNKT cells exhibit defective maturation and a reduced ability to produce effector cytokines, including IL-4 [19–22].

Mice lacking ITK also develop a population of IMP CD8+ T cells, shown to be dependent on IL-4 and PLZF [23, 24], and it has been proposed that this is a result of the PLZF-dependent, IL-4-producing cells, which act on bystander cells in a cell-extrinsic manner [23]. Indeed, IL-4 can act directly on naive CD8+ T cells to induce IFN-γ and Eomes expression [25], providing support for this model. ITK has been suggested to be required for TCR-induced up-regulation of IRF4, which can suppress the expression of Eomes in CD8+ T cells [26]. Furthermore, it has been speculated that the abundant

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Abbreviations: α-GalCer=α-galactosylceramide, BFA=Brefeldin A, DP=double-positive, Eomes=eomesoderm, FoxP3=forkhead box P3, IMP=innate memory phenotype, iNKT cells=invariant NKT cells, IFN-γ=interferon-γ, ITK=IκB kinase, LPS=lipopolysaccharide, OVA=ovalbumin, PLZF=promyelocytic leukemia zinc finger protein, RAG=recombination-activating gene, SP=single-positive, T-bet=T box expressed in T cells, TCR=T-cell receptor.

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NK-T-like γδ T and immature-iNKT cells that express PLZF may serve as the extrinsic sources of IL-4 [16, 27]. However, we have tested this model and found that whereas IMP CD8+ T cell development in Itk−/− mice is dependent on IL-4/-STAT6 signaling, it is surprisingly not dependent on γδ T cells (including NK-T-like γδ T cells) or iNKT cells. Furthermore, results from partial ITK re-expression in an Itk−/− background model suggest a cell-autonomous role for ITK in this process. We also show that in response to IL-4, ITK negatively tunes the differentiation of CD44+CD122+ CD8+ T cells from naive precursors in a TCR-dependent manner. We also show that although PLZF+ CD4+ thymocytes are generally increased in Itk−/− mice, this population is distinct from the spontaneous IL-4 producers that are non-γδ T, non-iNKT but CD4/CD8 DP cells in the thymus.

**MATERIALS AND METHODS**

**Mice**

All mice were on a C57BL/6 background. WT, Tcrd−/−, Thylα, and CD45.1−/− mice were from The Jackson Laboratory (Bar Harbor, ME, USA). Ja18−/− mice were as described previously [28] and a kind gift from Dr. Masaru Taniguchi (RIKEN Research Center for Allergy and Immunology, Japan) through Dr. Ling Qi (Cornell University, Ithaca, NY, USA) and Dr. Bin Gao (U.S. National Institutes of Health, Bethesda, MD, USA). IIf4−/− mice were a kind gift from Dr. Frank Brombacher (University of Cape Town, South Africa), via Dr. Fred Finkelstein (University of Cincinnati, OH, USA) [29]. Stat6−/− (generated by Grusby’s group [30]) and Il13−/− (from Dr. Alfred Bothwell at Yale University, New Haven, CT, USA) mice were kindly provided by Dr. Margaret S. Bynoe (Cornell University). Itkn−/−, Itk−/− [Tg(hcD2-4b)Itk−/−] mice were as described previously [31]. IIf4−/− crosses were generated by intercrossing IIf4−/− mice with CD45.1−/−, IIf4−/−, IIf4−/−, Stat6−/−, Il13−/−, or Ja18−/− mice. OTI Rag1−/− mice were purchased from Taconic (Hudson, NY, USA). IIf4−/− OTI Rag1−/− mice were generated by crossing IIf4−/− and OTI Rag1−/− mice. All experiments were approved by the Office of Research Protection’s Institutional Animal Care and Use Committee at Cornell University.

**Bone marrow chimeras**

Equally mixed donor bone marrow [Thylα+ (WT) and CD45.1+ (WT or IIf4−/−)] were used to generate bone marrow chimeras, as described [31]. Chimeras were analyzed 4–8 weeks after transplant.

**Antibodies and reagents**

All fluorescent antibodies are listed in “fluorochrome-target” format as follows: eFluor 450-CD122, FITC-TCR-γ, allophycocyanin-CD4, Alexa Fluor 647-IF44, PerCP-eFluor 710-Eomes, PE-Cy7-CD3ε, and PE-Cy7-IgG1-FITC (eBioscience, San Diego, CA, USA); V500-CD44, FITC-TCRβVs5, FITC-Thylα, PE-CD44, PE-IL-4, PE-IL-4Rα, PE-TCRαv2, PE-T-bet, Alexa Fluor 467-IL-4, Alexa Fluor 647-T-bet, allophycocyanin-CD45.2, Alexa Fluor 700-CD4, Alexa Fluor 700-Il4ra, PE-Cy7-CD62 ligand, allophycocyanin-Cy7-TCRβ, allophycocyanin-Cy7-TCRαv2, and allophycocyanin-Cy7-Thylα (BD Biosciences, San Jose, CA, USA); PE-Texas Red-Cd8α (Invitrogen, Carlsbad, CA, USA); FITC-TCR-γ, Alexa Fluor 700-CD45.1, and allophycocyanin-NK1.1 (BioLegend, San Diego, CA, USA). Fc-blocking antibody was from eBioscience. Biotin-conjugated anti-Gr-1, IL-5, TER119, CD122, and CD11b were from BD Biosciences, and anti-B220, F4/80, CD25, CD44, and CD8α were from eBioscience. Purified anti-CD3ε and CD28 antibodies were from BD Biosciences. PE and Alexa Fluor 467-PBS57-loaded CD1d tetramer was from the National Institute of Allergy and Infectious Diseases Tetramer Facility (Atlanta, GA, USA). Anti-IL-4 mAb (Clone 11B11) was from American Type Culture Collection (Manassas, VA, USA). Anti-PLZF antibody (Clone D9, mouse IgG2) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 488 anti-mouse IgG1, was from Life Technologies (Grand Island, NY, USA). Murine rIL-4 and rIL-13 were from Peprotech (Rocky Hill, NJ, USA), and IL-5 was a kind gift from Dr. Judith Appleton (Cornell University).

**T cell stimulation for flow cytometry**

Live cells were blocked and stained immediately after collection. For the IMP CD8+ T cell response, thymocytes and splenocytes were stimulated with P/1 (100 ng/ml/0.5 µM) and BFA (10 µg/ml Sigma-Aldrich, St. Louis, MO, USA) for 6 h, followed by fixation/permeabilization and staining with indicating antibodies using a Foxp3 staining buffer kit (eBioscience). For thymic iNKT stimulation, P/1 was added 1 h before Monensin (Enzo, Farmingdale, NY, USA: 5 µM; 3 h); for peripheral iNKT cell activation, 2 µg/mouse α-GalCer (KRN 7000; Cavanam Chemical, Ann Arbor, MI, USA) was injected i.p., 2 h before collection of splenocytes, followed by a 4-h incubation with BFA; and to detect spontaneous IL-4 production, cells were incubated in Monensin for 4 h, followed by staining for surface markers, fixation with 4% parafomaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), and permeabilization and staining with anti-cytokine antibodies in PBS/0.4% saponin (Sigma-Aldrich). Staining for nuclear factors Eomes, T-bet, IF44, Kit67, and PLZF was performed with a Foxp3 staining buffer kit. All flow cytometry data were acquired on a FACSAria (TreeStar, Ashland, OR, USA).

**Cell purification and real-time quantitative RT-PCR**

Naïve CD8 SP thymocytes (CD4−CD8α−TCRβVs5−CD44+) were purified by flow sorting on a cell sorter. Naïve, splenic CD8+ T cells were purified by flow sorting or magnetic negative selection [staining with biotin-conjugated antibodies against Gr-1, DX5, TER119, CD11b, B220, F4/80, CD25, CD122, CD44, and CD4, followed by anti-biotin MicroBeads (Miltenyi Biotec, Cambridge, MA, USA)]. Naïve CD8+ T cells (CD8α−TCRβVs5−CD44+CD122+) from OTI Rag1−/− or IIf4−/− OTI Rag1−/− mice with purity >92% were used for in vitro stimulation or for mRNA extraction, as described previously [21].

**IL-4 stimulation in vitro and in vivo**

Naïve CD8+ T cells were treated in vitro with IL-4 (40 ng/ml) and/or anti-CD3ε/28 antibodies for 5 days. For in vivo stimulation, 0.4 µg IL-4 antibody (11B11) for 5 min to form a stable IL-4/antibody complex and then given to mice via retro-orbital injection every 24 h. Twenty-four hours post the sixth dose, mice were killed for analysis.

**Statistical analysis**

Unpaired, two-tailed Student’s t-test or two-way ANOVA was performed using GraphPad Prism v5.00 (GraphPad, San Diego, CA, USA), with P < 0.05 considered statistically significant.

**RESULTS AND DISCUSSION**

γδ T cells are dispensable for development of IMP CD8+ T cells in the absence of ITK

We explored whether γδ T cells serve as producers of IL-4 in the place of the defective iNKT cells for induction of IMP CD8+ T cell development in the absence of ITK. We generated IIf4−/− mice that lack the ability to generate γδ T cells by removing the Treg gene. We then analyzed the thymi (Fig. 1A) and spleens (Fig. 1B) of WT, Tcrd−/−, IIf4−/−, and IIf4−/− Tcrd−/− mice for CD8+ T cells that carry the markers of IMP T cells, CD44, and CD122, their ability to produce IFN-γ rapidly, and their expression of transcription factors T-bet and Eomes. To our surprise, we found that the absence of γδ T cells on the IIf4−/− background...
does not affect the development of IMP CD8⁺ T cells (Fig. 1A and B, top), their capacity to produce IFN-γ rapidly (Fig. 1A and B, middle), or their expression of Eomes (Fig. 1A and B, bottom). This suggests that although IL-4-producing γδ T cells are increased in Ilk⁻/⁻ mice, they are dispensable for the excessive development of IMP CD8⁺ T cells in these mice. This is different from their requisite role in the hyper-IgE syndrome in Ilk⁻/⁻ mice [17, 18].

**Persistent deficiency in iNKT development and function in Ilk⁻/⁻ mice lacking γδ T cells**

In Ilk⁻/⁻ mice, there is enhanced development of a subpopulation of γδ T cells that have features of NKT cells, albeit with features of incomplete differentiation [16]. It has been suggested that the absence of ITK favors the development of γδ NKT cells over iNKT cells, as a result of potential competition between the two populations, although recent studies have suggested that such competition may be between the most mature subsets of each cell type [16, 20]. However, this evidence is indirect, and as we did not observe any effect of the absence of γδ T cells on the development of IMP CD8⁺ T cells in Ilk⁻/⁻ mice, we wondered whether the blockade of γδ NKT cell development in Ilk⁻/⁻ Tcrd⁻/⁻ mice would result in an enhancement of iNKT cell development and their ability to produce IL-4. This could also have the effect of influencing development of IMP CD8⁺ T cells in these mice. We therefore analyzed development and function of iNKT cells in the Ilk⁻/⁻ Tcrd⁻/⁻ mice. As reported previously, in the absence of ITK, there is reduced maturation and numbers of the CD1d tetramer-positive iNKT cell population, and these cells are defective in secreting IL-4 and IFN-γ upon stimulation with the iNKT cell ligand αGalCer [16, 20, 21]. Similar to what was observed for Ilk⁻/⁻ iNKT cells, there was reduced percentage, number (Fig. 2A and B, upper), and NK1.1 expression by iNKT cells from Ilk⁻/⁻ Tcrd⁻/⁻ mice (Fig. 2A and B, lower).

The previously defined NK1.1⁻ “immature” iNKT cell has been redefined recently as a sublineage of iNKT cells (NKT2), which express high levels of PLZF and are able to produce IL-4 to modulate IMP CD8⁺ T cell abundance in various mouse strains [27]. By contrast, the PLZF⁻NK1.1⁺ iNKT cells were redefined as NKT1 cells, able to produce IFN-γ [27]. We thus also examined PLZF and NK1.1 expression by these iNKT cells. We found that compared with WT, Ilk⁻/⁻ mice had increased NKT2 and decreased NKT1 iNKT cells, independent of the presence of γδ T cells (Fig. 2C). Whereas the thymic iNKT cells from Ilk⁻/⁻ mice exhibited better IL-4 production in response to P/I, they were significantly defective in IFN-γ (Fig. 2D), which confirmed the biased differentiation of NKT2 over NKT1. However, αGalCer-induced IL-4 and IFN-γ production was reduced significantly by the absence of ITK (Fig. 2E). These cytokine production patterns were retained in Ilk⁻/⁻ Tcrd⁻/⁻ mice (Fig. 2D and E). These data suggest that γδ T cells do not affect iNKT cell development and are dispensable for IMP CD8⁺ T cell development in Ilk⁻/⁻ mice.

**iNKT cells are dispensable for IMP CD8⁺ and NKT-like γδ T cell development in the absence of ITK**

In Ilk⁻/⁻ Tcrd⁻/⁻ mice iNKT cells retain the NKT2 phenotype and the defect in TCR-mediated IL-4 production (Fig. 2 and refs. [19–22]). PLZFhigh immature-like NKT2 iNKT cells seem to be the major IL-4 producers in Ilk⁻/⁻ mice in the steady state, as shown by an IL-4 reporter [16–18]. To investigate whether these iNKT cells are sufficient in inducing IMP CD8⁺ T cell development in Ilk⁻/⁻ mice, we used Ja18⁻/⁻ mice that lack iNKT cells [28]. We found no difference in thymic or splenic IMP CD8⁺ T cells in Ilk⁻/⁻ mice lacking iNKT cells (Ilk⁻/⁻ Ja18⁻/⁻; Fig. 3A and B), suggesting that iNKT cells are dispensable in IMP CD8⁺ T cell development in the absence of ITK. Similarly, the majority of γδ T cells in Ilk⁻/⁻ mice remained NKT-like (CD4⁺), even when iNKT cells were
absent (Fig. 3C). These data support the conclusion that although the NKT-like γδ T cells resemble the immature (or NKT2) iNKT cells, they develop independent of each other, even in the absence of ITK. Most importantly, the dispensable roles of γδ and iNKT cells for IMP CD8+ T cell enrichment in Itk−/− mice suggest the possibility that ITK contributes to a T cell-intrinsic regulation of IMP CD8+ T cell development.

**ITK negatively tunes IMP CD8+ T cell development in an IL-4/STAT6-dependent, cell-intrinsic manner**

Confirming a role for IL-4 in this process, removal of the IL-4Rα or STAT6 reversed the enhanced development of IMP CD8+ T cells in the thymus and spleen in the absence of ITK (Fig. 4A and B). Furthermore, although the IL-4-related cytokine IL-13 shares the IL-4Rα to transduce its signals and can also activate STAT6, the loss of IL-13, along with ITK, has a minor effect on the development of IMP CD8+ T cells, indicating that the requirement is largely, if not uniquely, associated with the IL-4/STAT6 signaling axis (Fig. 4A and B). Indeed, naïve CD8+ T cell uniquely respond to IL-4 but not the other two canonical Th2 cytokines—IL-5 and IL-13—with increased proliferation, capacity for IFN-γ expression, and expression of STAT6 (Fig. 4C–F). With the support of a tuning role for ITK in this process, mice expressing ITK on the Itk−/− background (to 35% of WT levels, ITKtg Itk−/− mice) develop intermediate levels of IMP CD8+ T cells, suggesting an inverse correlation between ITK expression and development of IMP CD8+ T cells (Fig. 4G). We have previously identified ITK as a cell-intrinsic suppressor of NKT-like γδ T cells using a mixed bone marrow chimera approach [17]. With the use of these chimeric models, we further found that Itk−/− origin CD8+ T cells exhibit a higher percentage of the IMP compared with those of WT origin in the same environment (Fig. 4H). These data suggest a cell-intrinsic regulatory role of ITK in IMP CD8+ T cell differentiation, dependent on IL-4 signaling.

**ITK negatively tunes IL-4-induced IMP in naive CD8+ precursors**

The IL-4/STAT6 axis is required for development of IMP CD8+ T cells, and mice lacking ITK, along with all T (and B) cells, except for antigen-specific TCR transgenic cells (OTI T cells, OTI/
**Figure 3.** iNKT cells are dispensable for development of IMP CD8⁺ and NKT-like γδ T cells in Itk⁻/⁻ mice. Expression of memory markers CD44 and CD122 (top), P/L-induced IFN-γ (middle), and transcription factors Eomes/T-bet (bottom) in CD8 SP thymocytes (A) and (B) CD8⁺ splenocytes from WT, Ja18⁻/⁻, Itk⁻/⁻, and Itk⁻/⁻ Ja18⁻/⁻ mice. (C) Percentages of γδ T cell proportions and numbers in the thymi of WT, Ja18⁻/⁻, Itk⁻/⁻, and Itk⁻/⁻ Ja18⁻/⁻ mice; n ≥ 5 from three independent experiments. *P < 0.05, Student’s t-test.

Rag1⁻/⁻/Itk⁻/⁻, have CD8⁺ T cells that do not have the IMP phenotype (Fig. 5A). This indicates that the enhanced IMP CD8⁺ T cell development in the absence of ITK in T cells requires the presence of IL-4 produced by other T (or B) cells. Previous work reported that hematopoietic MHC1 is essential for IMP CD8⁺ T cell development in the absence of ITK [32]. It is therefore likely that ITK regulates IL-4 and TCR signals that contribute to IMP CD8⁺ T cell development. Thus, we next investigated the potential role of ITK downstream of TCR and IL-4. We used naive CD8⁺ T cells isolated from WT or Itk⁻/⁻ mice in the OTI Rag⁻/⁻ background (Fig. 5A) to avoid complicating factors of using CD8⁺ T cells from the Itk⁻/⁻ mice. We examined Eomes expression by naive CD8 SP thymocytes and CD8⁺ T cells from the spleens of these transgenic mice and found that whereas thymic CD8 SP cells express less Eomes mRNA and protein (Fig. 5B, left), splenic, naive Itk⁻/⁻ CD8⁺ T cells had higher levels of Eomes mRNA but not protein compared with WT (Fig. 5B, right). This up-regulation of Eomes mRNA in the peripheral naive Itk⁻/⁻ CD8⁺ T cells may be a result of the T cell-intrinsic functions of ITK during CD8⁺ T cell homeostasis in the periphery.

We cultured naive CD8 SP thymocytes from these mice in vitro in the presence of IL-4 for 5 days, with or without increasing TCR signal. WT CD8 SP thymocytes expanded, accompanied by a reduced proportion of CD44⁺CD122⁺ CD8⁺ T cells, in a TCR signal-dependent manner (Fig. 5C, upper). By contrast, Itk⁻/⁻ CD8 SP precursors maintained or slightly decreased in numbers and exhibited little change in percentage of CD44⁺CD122⁺ IMP-like cells, regardless of TCR signal. IL-4-induced proliferation in WT cells (as indicated by Ki67 expression) is progressively suppressed by increasing TCR signals, whereas Itk⁻/⁻ cells appeared insensitive to increasing TCR signals; by contrast, programmed cell death is constitutively higher in the absence of ITK, suggesting independent patterns of signals regulated by ITK for cell death versus expansion/IMP conversion (Fig. 5C). IL-4 induces IL-4R expression in WT and Itk⁻/⁻ naive CD8 SP cells, and TCR activation dramatically reduces this expression in WT cells but less significantly so in Itk⁻/⁻ cells (higher expression compared with WT), suggesting a mechanism for ITK-mediated suppression of IL-4 signals (Fig. 5C, lower left). IL-4-signalcd WT CD8 SP thymocytes respond to increasing TCR signals with a reduction in Eomes-expressing IFN-γ producers and IFN-γ expression (Fig. 5D). By contrast, IL-4-signalcd Itk⁻/⁻ CD8 SP thymocytes gave rise to similar percentages of Eomes⁺ IFN-γ⁺ subset regardless of TCR signal strength, but exhibited higher expression of both Eomes and IFN-γ in response to increased TCR signals (Fig. 5D). These data suggest that in vitro, ITK inversely tunes the response of CD8 SP thymocytes to IL-4 to develop into IMP-like cells, likely through TCR-mediated signaling. We further tested whether this was the case in vivo, administering IL-4 (in the form of the stabilized IL-4α-IL-4 complex) [33], to determine further the function of ITK in regulating naive CD8⁺ T cell responses to IL-4. We found that 6 days post-in
Figure 4. IL-4/STAT6 signaling axis is required, and ITK plays a cell-autonomous role in development of IMP T cells. Percentage, number of CD44+CD122hi IMP CD8 T cells, percentage of Eomes+ cells, and percentage of P/I-induced IFN-γ-producing cells of CD8 SP thymocytes (A) and (B) CD8 T splenocytes from WT, Itk−/−, 1H13−/−, 1H6−/−, Il4ra−/−, 1H4a−/−, and 1H−/−Stat6−/− mice (n = 2–6) were shown, combined with three independent experiments. (C) Purified WT naive CD8 T cells were stimulated with IL-4 (40 ng/ml), IL-5 (50 ng/ml), or IL-13 (100 ng/ml) for 5 days and then analyzed for proliferative marker Ki67 or expression of IFN-γ, following stimulation with P/I. (E) Number of cells recovered in the presence of indicated cytokines for 5 days. (F) Expression of STAT6, following stimulation with the indicated cytokines (n=5 shown; data represent results of more than three independent experiments. (G) Percentage and number of IMP CD8 T cells in WT (100% ITK expression), ITK−/−Itk−/− (35% ITK expression), and Itk−/− (0% ITK expression) thymocytes and splenocytes (n=8) from four independent experiments. *P < 0.05, Student’s t-test. (H) Thy1a WT bone marrow cells were mixed 1:1 with CD45.1+ WT or Itk−/− cells and transplanted into CD45.2−irradiated WT recipients. Representative flow cytometric analysis of CD44 and CD122 expression by CD8SP thymocytes of WT or Itk−/− origin in the indicated mixed bone marrow chimeras. Data represented results from four to 10 mice in three independent experiments.

vivo IL-4/α-IL-4 administration, WT and Itk−/− CD8 SP thymocytes and CD8+ T cells in the spleens exhibited an IMP-like state, with elevated percentage and number of CD44hiCD122hi cells compared with controls. However, the percentage and number of these cells were significantly higher in the absence of ITK (Fig. 5E). Furthermore, confirming the idea that naive Itk−/− CD8 T cells are more sensitive to IL-4, α-IL-4 alone is able to enhance the percent of CD44hiCD122hi cells in the spleen (Fig. 5E), presumably stabilizing endogenous IL-4 in these mice. Although naive Itk−/− CD8 T cells have higher levels of Eomes mRNA (but not in the thymus; Fig. 5B), it is not translated until IL-4 is provided (Fig. 5F), suggesting that tonic signals received by naive Itk−/− CD8 T cells prime these cells for IL-4 signals toward the IMP. Berg and colleagues [26] have shown recently that inhibition of ITK could synergize with IL-4 to promote Eomes expression, attributed to the role of ITK in inducing the expression of IRF4, a suppressor of Eomes expression. However, to our surprise, we found that in our naive CD8+ T cells, IL-4-induced-IRF4 expression in vivo is higher in Itk−/− CD8+ T cells than in WT (Fig. 5F), suggesting more complex regulation of IRF4 and Eomes expression by ITK and/or IL-4 signals.

Taken together, our data suggest that in naive CD8+ T cells, ITK acts as a cell-intrinsic tuner for IL-4 signals. Downstream of the TCR, ITK acts to tune the IL-4 signals negatively, as in its absence, naive CD8+ T cells exhibit better differentiation to the IMP. It is possible that as has been recently reported for IL-7—where TCR signals tune cytokine signals that cells receive by regulating cytokine receptor expression [34]—reduced TCR signals in the absence of ITK allow enhanced responses to IL-4, in part, by supporting higher IL-4Rα expression. Furthermore, this may be combined with the ability of ITK signals to suppress the expression of Eomes, which may be involved in the development of the IMP state. In peripheral naive CD8+ T cells but not thymocytes,
in the absence of ITK, Eomes mRNA is highly up-regulated but not translated and IL-4 is required to drive the enhanced expression of Eomes protein.

Whereas development of IMP CD8\(^+\) T cells in Itk\(^{-/-}\) mice is dependent on the IL-4/STAT6 signaling axis, this work challenges the prevailing notion that development of these cells is primarily a result of IL-4 production by NKT-like γδ T or PLZF\(^{high}\) NK2 cells [8, 16, 27]. Possible explanations for the continued presence of IMP CD8\(^+\) T cells in Itk\(^{-/-}\)/Tcrd\(^{-/-}\) and Itk\(^{-/-}\)/Ja18\(^{-/-}\) mice include alternative source(s) of IL-4, such as IL-4/CD8 DP. By contrast, the PLZF\(^{high}\) cells are predominantly CD4\(^+\) (Fig. 6D, lower left), and the non-γδ T/non-iNKT cells, PLZF\(^{high}\) CD4\(^+\), which accumulate in Itk\(^{-/-}\) mice, are largely CD4 SP (Fig. 6D, lower right), suggesting that these are separate populations. Note that as a result of technical limitations, we have been unable to determine the IL-4 status of the non-iNKT and non-γδ T/non-iNKT subsets (Fig. 6A and B). The non-γδ T/non-iNKT subset remained as the major PLZF\(^{high}\) CD4 SP thymic population in the absence of ITK, independent of γδ T or iNKT cells (Fig. 6B, bottom), and expressed the αβ TCR (Fig. 6C). However, comparison of the spontaneous IL-4 producers (no stimulation, detected ex vivo) revealed no difference between WT and Itk\(^{-/-}\) thymocytes in the steady state (Fig. 6D, upper). Furthermore, these spontaneous IL-4 producers are largely distinct from iNKT (i.e., are tetramer\(^{+}\)) and γδ T cells (retained in their absence) and are CD4/CD8 DP. By contrast, the PLZF\(^{high}\) cells are predominantly CD4\(^+\) (Fig. 6D, lower left), and the non-γδ T/non-iNKT cells, PLZF\(^{high}\) CD4\(^+\), which accumulate in Itk\(^{-/-}\) mice, are largely CD4 SP (Fig. 6D, lower right), suggesting that these are separate populations. Note that as a result of technical limitations, we were unable to determine the IL-4 status of the PLZF\(^{high}\) population. Thus, whereas there is an IL-4-producing CD4/CD8 DP thymic population at steady state, which may serve as a source of exogenous IL-4 that drives the more-sensitive CD8 SP thymocytes in Itk\(^{-/-}\) mice to the IMP phenotype, this population is not elevated compared with its analogs in WT mice.

As a result of technical limitations, we have been unable to determine whether there is actually a higher level of IL-4 in the thymic niche or the circulation in Itk\(^{-/-}\) mice that stimulates IMP T cell development. Weinreich et al. [24] suggested...
previously that an in vivo environment created by the absence of ITK can influence WT CD8\(^+\) T cells to develop an IMP-like state, and indeed, WT CD8\(^+\) T cells can be skewed toward the IMP state in the presence of IL-4. However, we have found that when the ratio of WT:Itk\(^-\) bone marrow is 1:1, the WT cells are not influenced, whereas the Itk\(^-\) cells retain a better ability to develop into IMP cells. In addition, we have found that Itk\(^-\) cells retain a better ability to develop into IMP cells. We therefore suggest that under WT conditions, CD8\(^+\) T cells that have received weak signals (such as those mimicked by the absence of ITK) may be primed to generate memory phenotype cells under inductive conditions, such as the presence of IL-4. These findings, furthermore, suggest that some naive CD8\(^+\) T cells may be preprogrammed by virtue of weak signals that they received during development or during homeostatic expansion, upon leaving the thymus, to become memory phenotype cells with ability to respond rapidly with effector function.

We have reported previously that IMP CD8\(^+\) T cells can rapidly respond to primary antigens by producing IFN-\(\gamma\) and TNF-\(\alpha\) [3], which can be critical in developing a rapid response or vaccination strategies for emerging pathogens. ITK serves as a CD8\(^+\) T cell-autonomous tuner for IMP differentiation, and the targeting of ITK may enhance selection or expansion of IMP CD8\(^+\) T cells. This would be of tremendous benefit in dealing with emerging infectious diseases.

**AUTHORSHIP**

W.H., A.K.K., F.H., and J.H. conducted experiments. W.H. and A.A. designed the research, analyzed data, and wrote the manuscript.

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DISCLOSURES
The authors declare no competing financial interests.

REFERENCES
ITK tunes IL-4-induced development of innate memory CD8+ T cells in a \( \gamma \delta \) T cell-independent manner

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