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Requirements for Eomesodermin and Promyelocytic Leukemia Zinc Finger in the Development of Innate-Like CD8⁺ T Cells

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Conventional and nonconventional T cell development occur in the thymus. Nonconventional thymocytes that bear characteristics typically associated with innate immune cells are termed innate-like lymphocytes (ILLs). Mice harboring a tyrosine to phenylalanine mutation in the adaptor protein Src homology 2 domain-containing leucokocyte protein of 76 kDa at residue 145 (Y145F mice) develop an expanded population of CD8⁺CD122⁺CD44⁺ ILLs, typified by expression of the T-box transcription factor eomesodermin. Y145F mice also have an expanded population of γδ T cells that produce copious amounts of IL-4 via a mechanism that is dependent on the BTB-ZF transcription factor promyelocytic leukemia zinc finger. Using mice with T cell-specific deletion of Eomes, we demonstrate that this transcription factor is required for CD8⁺ IIL development in Y145F as well as wild-type mice. Moreover, we show that promyelocytic leukemia zinc finger and IL-4 are also required for the generation of this ILL population. Taken together, these data shed light on the cell-intrinsic and cell-extrinsic factors that drive CD8⁺ ILL differentiation. The Journal of Immunology, 2011, 186: 4573–4578.

The T cell compartment largely consists of conventional CD4⁺ and CD8⁺ T cells. These cells develop in the thymus and exit to peripheral lymphoid organs as naïve T cells. Additional populations of thymically derived T cells have also been identified, including those with regulatory or innate-like functions. T cells constituting this latter subset are termed innate-like lymphocytes (ILLs) because they acquire effector function during development, prior to peripheral activation. Other characteristics of ILLs include specificity for nonclassical MHC molecules, selection on hematopoietic cells, and expression of molecules typically associated with activated or memory T cells, such as CD44, CD122 (the β-chain of the IL-15 and IL-2 receptors), and NK1.1 (reviewed in Ref. 1). NKT cells and a variety of CD8⁺ populations, including H2-M3–specific T cells, CD8αα⁺ T cells, and mucosal-invariant T cells, have been classified as ILLs (1). ILLs have been found to participate in host defense against bacteria and viruses, and additional studies have suggested that NKT cells can aid in antitumor responses (2–5). In some contexts, however, ILL participation during an immune response may be disadvantageous. Several reports implicate these cells in asthma and autoimmunity, whereas others have linked them to negative outcomes following infection with particular organisms (5, 6).

Several transcription factors regulate thymocyte commitment to distinct T cell lineages. GATA3 and Th-POK regulate CD4⁺ T cell differentiation, Runt domain transcription factors (Runx) are critical for CD8⁺ T lineage commitment (7, 8), and Foxp3 is required for the development of CD4⁺ T regulatory cells (9, 10). Transcriptional control of nonconventional T cell development has also been studied, but our knowledge is limited largely to the NKT lineage. NKT cells are dependent on several transcription factors including, but not limited to, promyelocytic leukemia zinc finger (PLZF), Runx1, ETS1, ELF4, c-myc, RelA, and T-bet (reviewed in Ref. 11). Transcription factors that regulate CD8⁺ ILL development are less well defined.

Src homology 2 domain-containing leucokocyte protein of 76 kDa (SLP-76) is an adaptor protein critical for thymocyte development due to its role in TCR signal transduction (12). Through its protein–protein interaction domains, SLP-76 nucleates a signaling complex containing linker of activation of T cells, growth factor receptor-bound protein 2-related adaptor downstream of SHc, phospholipase C-γ1, and the Tec family tyrosine kinase II-2-inducible T cell kinase (Itk) (13). We recently provided evidence that mutation of tyrosine 145 of SLP-76 (Y145F) results in altered thymocyte selection and in development of a population of nonconventional CD8 single-positive (SP) thymocytes (14). In addition to exhibiting such ILL hallmarks as enhanced expression of CD44 and CD122, Y145F CD8⁺ ILLs express elevated mRNA levels of eomesodermin (Eomes) (14), a T-box transcription factor...
that regulates differentiation during embryogenesis and directs fate and function of effector and memory T cells (14–17). Y145F mice also have an expanded population of γδ T cells that express high levels of PLZF, a member of BTB-ZF family of transcription factors (18). These PLZFγδ T cells produce copious amounts of IL-4 (18), which has been implicated in driving Eomes expression in CD8SP thymocytes (19).

In this study, we find that Eomes is essential for CD8+ ILL development in Y145F as well as wild-type (WT) mice. We also observe that, in the presence of Y145F-derived bone marrow (BM), WT thymocytes differentiate into Eomes+CD8+ ILLs, indicating that non–cell-autonomous factors direct CD8+ ILL development. Development of this population is also dependent on PLZF and IL-4, as Y145F CD8+ ILLs do not develop in the absence of PLZF and are significantly diminished upon administration of a blocking Ab to IL-4. Taken together, our findings highlight key cell-intrinsic and cell-extrinsic factors that control the development of CD8+ ILLs.

Materials and Methods

Mice

Y145F, PLZF−/−, and Cd4-cre Eomes+/− mice have been described (13, 14, 20, 21). Animals were housed at the University of Pennsylvania or Sloan-Kettering and experiments were performed in accordance with protocols approved by their Institutional Animal Care and Use Committees.

Flow cytometry and real-time PCR

Cells were stained and analyzed as described (13, 18). Abs to the following proteins were from BD Pharmingen unless otherwise noted: CD3 Alexa Fluor 700, CD4 PerCP-Cy5.5, PE-Cy7 (BioLegend), or allophycocyanin-PE Fluor 780 (eBioscience), CD8a FITC, allophycocyanin, Alexa Fluor 700 (eBioscience), Pacific Blue (BioLegend), or PE-Cy7 (eBioscience), CD44 FITC, CD45.1 PE-Cy7 (eBioscience), CD45.2 FITC (eBioscience), CD122 PE or biotin, streptavidin PE-Texas Red, CD124 PE, TCRγδ PE or allophycocyanin (eBioscience), CXCRC3 PE, Eomes AF647 (eBioscience), mouse anti-mouse PLZF (Santa Cruz Biotechnology) followed by anti-mouse IgG1 FITC, IFN-γ Pacific Blue (eBioscience), IL-4 PE-Cy7 (eBioscience). Aqua Live/Dead (Invitrogen) was used to gate out dead cells. Real-time PCR was performed as described (14).

Ex vivo stimulation of thymocytes

Thymocytes were isolated and stimulated directly ex vivo at 37°C for 5 h with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 1 μg/ml brefeldin A. Cells were then assayed for cytokine production by intracellular staining and flow cytometric analysis.

BM chimeras

C57BL/6-SJL (CD45.1) mice were irradiated with 950 rad and injected i.v. with a 1:1 mixture of T cell-depleted (magnetic bead depletion; Qiagen) BM from C57BL/6-SJL (CD45.1) mice and WT C57BL/6 (CD45.2) or Y145F (CD45.2) mice. Recipients were reconstituted with 4 × 10^6 cells. Recipients were analyzed 9–10 wk after transplantation.

In vivo IL-4 blockade

Y145F mice (2–3 wk age) were injected i.p. with either PBS or 1 mg/mouse neutralizing anti–IL-4 Ab (11B11) a total of three times over the course of 2 wk.

Statistical analysis

Prism (GraphPad Software) was used for statistical analysis. A Student t test was used to assess statistical significance. For linear regression analysis, p values indicate the significance of a slope being non-0, and r^2 represents the goodness of fit.

Results

SLP-76 Y145F mice generate CD8+ ILLs

Mice bearing a tyrosine to phenylalanine mutation at residue 145 of SLP-76 (Y145F) have defective positive and negative selection of conventional T cells (14). Additionally, these mice have a diminished CD4/CD8 ratio, owing to a >2-fold increase in the absolute number of CD8SP thymocytes, accompanied by a more modest decrease in CD4SP T cell numbers (Fig. 1A). Further analysis of the CD8SP population revealed that this compartment is largely composed of CD122+CD44+ cells (Ref. 14 and Fig. 1B). Compared to WT CD8SP thymocytes, Y145F CD8SP cells express elevated levels of Eomes protein and perforin mRNA, a direct target of Eomes (16). Moreover, Y145F CD8SP thymocytes produce IFN-γ following direct ex vivo stimulation, another hallmark of Eomes’ lymphocytes (Fig. 1C). Taken together, these data indicate that most CD8SP thymocytes from Y145F mice are ILLs.

CD8+ ILL fate in Y145F mice is conferred by cell-extrinsic factors

Development of conventional CD8+ thymocytes depends on both cell-intrinsic transcriptional programs and cell-extrinsic factors, such as cytokines (22). To determine whether CD8+ ILL development in Y145F mice is driven by cell-intrinsic or cell-extrinsic factors, mixed BM chimeras were generated in which WT (CD45.1) and Y145F (CD45.2) BM were mixed and transplanted into lethally irradiated WT (CD45.1) hosts. WT (CD45.1): WT (CD45.2) mixed BM chimeras served as controls. Percentage chimerism was defined as the percentage CD45.2+CD4−CD8− thymocytes of total double-negative thymocytes. When mixed with Y145F donor BM, the percentage of Eomes+ CD8SP thymocytes derived from WT BM was equivalent to the percentage derived from Y145F donor BM (Fig. 2A, 2B). Thus, Y145F-derived cells are capable of directing WT thymocytes to adopt an ILL phenotype. The extent of CD8+ ILL development in the mixed BM chimeras positively correlated with the relative con-
Y145F (CD45.2+) CD8SP thymocytes (panels). Analysis of mixed BM chimeras (n = 7). A, Eomes expression in WT (CD45.1+) or WT (CD45.2+) CD8SP thymocytes (left panels) and in WT (CD45.1+) or Y145F (CD45.2+) CD8SP thymocytes from all chimeras. B, Percentage Eomes+ of WT (CD45.1+) or Y145F (CD45.2+) CD8SP thymocytes from all chimeras. C, Linear regression analysis of the percentage Eomes+ of CD8SP thymocytes in WT- or Y145F-derived populations as a function of the percentage Y145F chimerism, defined as percentage of CD45.2+CD4+CD8− thymocytes. The $r^2$ values describe the linear relationship (goodness of fit) between the percentage Y145F chimerism and CD8+ ILL development. The $p$ values indicate whether the slope of the regression line is significantly different from 0. D, PLZF and γδ TCR expression in WT (CD45.1+) or WT (CD45.2+) thymocytes (left panels) and in WT (CD45.1+) or Y145F (CD45.2+) thymocytes (right panels). E, Percentage PLZF+ γδ+ of total WT (CD45.1+) or Y145F (CD45.2+) thymocytes from all chimeras. F, Linear regression analysis of the percentage PLZF+ γδ+ thymocytes in WT- or Y145F-derived populations as a function of the percentage Y145F chimerism, as defined in C.

FIGURE 2. Cell-extrinsic factors govern CD8+ ILL development, but cell-intrinsic factors control enhanced PLZF+ γδ T cell generation in Y145F mice. Analysis of mixed BM chimeras (n = 7). A, Eomes expression in WT (CD45.1+) or WT (CD45.2+) CD8SP thymocytes (left panels) and in WT (CD45.1+) or Y145F (CD45.2+) CD8SP thymocytes from all chimeras. B, Percentage Eomes+ of WT (CD45.1+) or Y145F (CD45.2+) CD8SP thymocytes from all chimeras. C, Linear regression analysis of the percentage Eomes+ of CD8SP thymocytes in WT- or Y145F-derived populations as a function of the percentage Y145F chimerism, defined as percentage of CD45.2+CD4+CD8− thymocytes. The $r^2$ values describe the linear relationship (goodness of fit) between the percentage Y145F chimerism and CD8+ ILL development. The $p$ values indicate whether the slope of the regression line is significantly different from 0. D, PLZF and γδ TCR expression in WT (CD45.1+) or WT (CD45.2+) thymocytes (left panels) and in WT (CD45.1+) or Y145F (CD45.2+) thymocytes (right panels). E, Percentage PLZF+ γδ+ of total WT (CD45.1+) or Y145F (CD45.2+) thymocytes from all chimeras. F, Linear regression analysis of the percentage PLZF+ γδ+ thymocytes in WT- or Y145F-derived populations as a function of the percentage Y145F chimerism, as defined in C.

One explanation for our observation that Y145F thymocytes induce the ILL fate in WT CD8+ thymocytes in a dose-dependent fashion could be the presence of a cytokine-producing "inducer" cell that is more abundant among Y145F than WT thymic cells. IL-4 has been previously found to positively regulate Eomes expression (23). Eomes+ CD8SP cells from Y145F mice express high levels of CXCR3 and IL-4 receptor (Supplemental Fig. 1), consistent with data implicating IL-4 signaling in upregulation of these receptors (19, 24). Additionally, Y145F mice contain an expanded population of γδ T cells that produce IL-4 in a PLZF-dependent manner (18, 25). These data demonstrate that PLZF is essential for the development of CD8+ ILLs in Y145F mice.

Next, we directly addressed whether IL-4 regulates CD8+ ILL development in Y145F mice. To this end we dampened IL-4 signaling in vivo by treating mice with neutralizing Abs against IL-4. Compared to Y145F mice injected with PBS, Y145F mice receiving anti–IL-4 Ab treatment exhibited an increase in the thymic CD4/CD8 ratio (Fig. 4A). IL-4 blockade also resulted in a decreased percentage of CD8SP thymocytes expressing classic markers of ILLs, including CD44, CD122, and Eomes (Fig. 4B, 4C). Taken together, these data are consistent with a model in which IL-4, likely derived from a PLZF+ T cell population, in-
Eomes expression on CD8SP thymocytes from indicated mice. CD8SP thymocytes from indicated mice.

It was previously demonstrated that mutation of this study that IL-4 is required for Eomes upregulation and development of the CD8+ ILL phenotype. These results are consistent with the extrinsic nature of CD8+ ILL generation (Fig. 2) and the reported role of IL-4 in Eomes regulation (23). Our findings are also in agreement with recent reports suggesting that IL-4 production by PLZF+ nonconventional T cells results in elevated

due to an Eomes-independent effect of the Y145F mutation on the selection of conventional CD4SP thymocytes.

Within the CD8SP compartment, it was the selective loss of CD8+CD122+CD44+ cells that account for the decrease in the total number of CD8SP cells in Y145F EomesF/FCd4-Cre mice (Fig. 5B, Supplemental Fig. 2C). Additionally, ILL generation was sensitive to Eomes hemizygosity, as Y145F YomesF/FCd4-Cre mice have an intermediate phenotype with respect to CD8+ ILL numbers (Supplemental Fig. 2A–C). The loss of CD8+ ILLs correlated with a loss of IFN-γ-producing CD8SP thymocytes in the absence of Eomes (Fig. 5C). These data support a requirement for Eomes in CD8+ ILL development in Y145F mice.

Although CD8+ ILLs are clearly apparent and dependent on Eomes in Y145F mice, it remained to be seen whether this population represented an Eomes-dependent population found normally in the thymus of WT mice. To address this issue, we examined thymi of WT and EomesF/FCd4-Cre mice for the presence of CD8+ ILLs. We found a significant decrease in the percentage of CD8+CD122+CD44+ ILLs in EomesF/FCd4-Cre thymi, relative to WT thymi (Fig. 6A, 6B). The percentage of IFN-γ-producing CD8+ cells was also significantly decreased in Eomes-deficient thymocytes, compared with their WT counterparts (Fig. 6C). Taken together, these data are consistent with an essential role for Eomes in the development of CD8SP cells with phenotypic and functional properties of ILLs in WT mice.

Discussion
In this study, we have used a model of altered T cell signaling to highlight a population of CD8+ ILLs found normally in WT mice. We show that development of the CD8+ ILLs present in Y145F mice require Eomes and PLZF and propose that IL-4 may serve as the CD8+ ILL-extrinsic factor linking the function of these two transcription factors. We previously demonstrated that mutation of Y145 of SLP-76 drives the enhanced development of a population of γδ T cells that express high levels of PLZF, and that PLZF is responsible for IL-4 production by these cells (18). We show in this study that IL-4 is required for Eomes upregulation and development of the CD8+ ILL phenotype. These results are consistent with the extrinsic nature of CD8+ ILL generation (Fig. 2) and the reported role of IL-4 in Eomes regulation (23). Our findings are also in agreement with recent reports suggesting that IL-4 production by PLZF+ nonconventional T cells results in elevated

FIGURE 4. IL-4 blockade diminishes the CD8+ ILL compartment in Y145F mice. A, Flow cytometric analysis of thymi from Y145F animals treated with PBS or with blocking anti–IL-4 Ab (n = 4 mice/group for A–C). B, Flow cytometric analysis for CD44 and CD122 expression on CD8SP thymocytes from indicated mice. C, Flow cytometric analysis for Eomes expression on CD8SP thymocytes from indicated mice.

Eomes is required for CD8+ ILL development in Y145F and WT mice
Given the role of Eomes in directing cell fates (16, 17), EomesF/F Cd4-Cre+ mice were bred to Y145F mice to test whether ILL development in Y145F mice is dependent on Eomes. Deletion of Eomes in the T cell compartment of Y145F mice restored the percentage and number of Y145F CD8SP thymocytes to levels seen in WT mice (Fig. 5A, Supplemental Fig. 2A). The CD4/CD8 ratio in Y145F EomesF/F Cd4-Cre+ mice was increased compared with Y145F mice, although it was not restored to WT levels (Supplemental Fig. 2B). Failure of Y145F EomesF/F Cd4-Cre+ mice to achieve a completely normalized CD4/CD8 ratio is likely


FIGURE 6. Eomes regulates CD8+ ILL development in WT mice. A, Flow cytometric analysis of thymi from WT and EomesF/F Cd4-Cre+ mice (n = 6 mice/group for A–C). B, Percentage CD122+CD44+ cells of total CD8SP thymocytes from indicated genotypes. C, Percentage IFN-γ-producing cells of total CD8SP thymocytes from indicated genotypes after stimulation with PMA/ionomycin. Expression of IFN-γ was assessed by flow cytometric analysis.
numbers of CD8+ ILLs in the setting of Id3 or KLF2 deletion (26, 27). Additionally, in an independent study, Eomes was shown to be required for IL-4–mediated upregulation of CXCR3 in by-stander CD8SP thymocytes developing in the context of Klf2 deficiency, suggesting a cell-intrinsic requirement for Eomes during CD8+ ILL development (19). We find that Eomes and PLZF expression are mutually exclusive in WT and Y145F thymocytes and that PLZF expression is maintained in Eomes<sup>−/−</sup> Cd4-Cre<sup>−</sup> mice (Supplemental Fig. 3). Preliminary data also indicate that PLZF expression is not altered in Y145F Eomes<sup>−/−</sup> Cre<sup>−</sup> mice, consistent with a CD8+ ILL intrinsic role for Eomes (S. M. Gordon, unpublished observations). However, it remains to be formally tested whether Eomes is required for the development or survival of an IL-4–producing “inducer cell.” Similarly, although we have not observed expression of PLZF in CD8+ ILLs in Y145F mice (data not shown), we have not formally ruled out the possibility that a precursor of the CD8+ ILL expresses and is dependent on PLZF.

Our proposed mechanism of ILL development in Y145F mice—a pathway intrinsically dependent on Eomes and extrinsically driven by a PLZF-dependent IL-4–producing population—may be more universal, as similar findings have been observed in Itk<sup>−/−</sup> and conditionally Chp<sup>−/−</sup> mice, which also develop a preponderance of CD8+ ILLs (26, 28–30). The link between the SLIP-76 mutant and Itk-deficient phenotypes may rest in the functional relationship between these two molecules, as SLIP-76 Y145 is a binding site for Itk, and this tyrosine is critical for inducible Itk activation following TCR ligation (31). In addition to Y145F and Itk<sup>−/−</sup> mice having increased numbers of CD8+ ILLs, the frequency of PLZF<sup>−/−</sup> γδ thymocytes capable of producing IL-4 is increased in both mouse strains (14, 18, 32, 33). Our studies have demonstrated that the loss of PLZF or blockade of IL-4 signaling in Y145F mice impairs CD8+ ILL development. Taken together, these data are consistent with the possibility that PLZF<sup>−/−</sup> γδ cells serve as the source of IL-4 that drives the ILL phenotype, although formal testing of the hypothesis is required. Moreover, how mutation of the SLP-76/Itk signaling axis influences PLZF<sup>−/−</sup> γδ T cell development remains to be elucidated.

Eomes regulates several of the cell surface phenotypes and functions that define CD8+ ILLs, such as CD122 and perforin expression, as well as IFN-γ production (16, 34). One prediction for these studies was that deletion of Eomes in Y145F mice would not alter the number of CD8SP thymocytes, only their phenotype. However, we found that Eomes deficiency resulted in not only the loss of CD8SP thymocytes with an innate-like phenotype, but also a decrease in the absolute number of CD8SP cells. This result is similar to the impact IL-15 deficiency has on CD8+ ILL numbers in Itk<sup>−/−</sup> mice (28). IL-15 signaling is dependent on CD122, and thus it is possible that Eomes regulates the survival of CD8+ ILLs through its regulation of CD122 expression. Alternatively, other Eomes-regulated genes may control the differentiation and/or survival of this innate-like population.

ILLs contribute both positively and negatively to the outcome of immune responses. CD8+ ILLs generated in Itk<sup>−/−</sup> mice have been shown to contribute to the low bacterial burden observed in these mice following Listeria infection (35). How the CD8+ ILL population from Y145F mice and other gene deletion models influences additional immune responses is unknown. Studies focused on this question and how Eomes contributes to these outcomes may provide insight into how the immune response can be manipulated to enhance its effectiveness. Additionally, understanding Eomes and PLZF regulation during thymocyte development will aid in our understanding of conventional and nonconventional T cell development.

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Disclosures
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