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PLZF Controls the Development of Fetal-Derived IL-17⁺Vγ6⁺ γδ T Cells

Ying Lu,¹ Xin Cao,¹ Xianyu Zhang, and Damian Kovalovsky

Expression of promyelocytic leukemia zinc finger (PLZF) protein directs the effector differentiation of invariant NKT (iNKT) cells and IL-4⁺ γδ NKT cells. In this study, we show that PLZF is also required for the development and function of IL-17⁺ γδ T cells. We observed that PLZF is expressed in fetal-derived invariant Vγ5⁺ and Vγ6⁺ γδ T cells, which secrete IFN-γ and IL-17, respectively. PLZF deficiency specifically affected the effector differentiation of Vγ6⁺ T cells, leading to reduced numbers of mature CD27⁺CD44⁺ phenotype capable of secreting IL-17. Although PLZF was not required for Vγ5⁺ γδ T cells to develop, when these cells were reprogrammed into IL-17-secreting cells in Skint-1 mutant mice, they required PLZF for their effector maturation, similarly to Vγ6⁺ γδ T cells. The impaired effector differentiation of PLZF-deficient Vγ6⁺ γδ T cells was not due to increased apoptosis and it was related to reduced proliferation of immature CD27⁺CD44⁻ Vγ6⁺ γδ T cells, which was required for their differentiation into mature CD27⁺CD44⁺ IL-17-secreting cells. Thus, the present study identifies that PLZF function is not restricted to NKT or IL-4⁺ T cells, but it also controls the development of IL-17⁺ γδ T cells. The Journal of Immunology, 2015, 195: 4273–4281.

Conventional T cells leave the thymus as naïve cells. Only after recognition of their cognate Ag in the periphery do they differentiate into cytokine-secreting cells belonging mainly to the Th1, Th2, and Th17 T cell subsets. “Innate-like” T cells acquire cytokine-secreting functions within the thymus and preferentially migrate to nonlymphoid tissues, providing a first line of defense against invading pathogens (1, 2).

A common feature of different subsets of innate-like T cells within the adult T cell compartment, including invariant NKT (iNKT) cells, γδ NKT cells (Vγ1.1⁺Vδ6.3⁺ γδ T cells), and human mucosal-associated invariant T cells, is the expression of the promyelocytic leukemia zinc finger (PLZF) protein (3–7). PLZF is considered a determinant of innate-like differentiation, as it is necessary for the acquisition of effector functions in iNK cells and γδ NKT cells. PLZF-deficient iNK cells have a naïve phenotype, preferentially secrete IL-2, and migrate to lymph nodes (3, 4, 6). Conversely, transgenic PLZF expression during T cell development is sufficient to provide some “innate” effector functions to conventional T cells. These are the acquisition of a memory/activated phenotype, the ability to co-secrete IL-4 and IFN-γ, and preferential migration to the liver microvasculature, unique characteristics of iNK cells (4, 8, 9).

During fetal hematopoiesis γδ T cell subsets with innate-like properties are generated, such as invariant Vγ5⁺ and Vγ6⁺ γδ T cells. Hematopoietic progenitors that enter the thymus at approximately embryonic day 14 (E14) first rearrange the Vγ6 TCR loci. Successful rearrangement allows the development of dendritic epidermal T cells, which secrete IFN-γ and migrate to the intraepithelial compartment of the skin (10, 11). In contrast, upon unproductive Vγ5 rearrangements, cells attempt to rearrange the Vγ6 loci, leading to the generation of invariant Vγ6Vδ1 γδ T cells, which secrete IL-17 and colonize the epithelium of the uterus, tongue, lungs, and peritoneum. IL-17⁺ γδ T cells are broadly identified as CD27⁺ cells in peripheral tissues (12, 13) and preferentially proliferate in response to IL-17 signals, allowing their self-renewal in peripheral tissues (14). IL-17⁺ γδ T cells are a major source of innate IL-17 in several mucosal infection models such as Mycobacterium tuberculosis, Escherichia coli, and Listeria monocytogenes infections (15, 16). Innate IL-17 and IL-21 secretion by γδ T cells in response to IL-1 and IL-23 was shown to mediate autoimmune inflammation in an experimental autoimmune encephalomyelitis model (17).

In this study, we have uncovered a novel function of PLZF in the development of innate-like IL-17⁺Vγ6⁺ γδ T cells. Although PLZF is expressed in both Vγ5⁺ and Vγ6⁺ fetal thymic precursors, it is specifically required for the development of Vγ6⁺ γδ T cells, allowing their normal maturation, expansion, and acquisition of IL-17 secretion after selection. Interestingly, despite PLZF expression in Vγ5⁺ γδ T cells, PLZF deficiency did not affect their development or their ability to colonize the skin. The inability of PLZF-deficient Vγ6⁺ γδ T cells to expand intrathymically was not due to increased apoptosis, but to an impairment of Vγ6⁺ γδ T cells to proliferate and acquire a mature CD27⁺CD44⁺ phenotype and function.

Materials and Methods

Materials and mice
C57BL/6 mice were purchased from The Jackson Laboratory. PLZF-deficient (PLZF knockout [KO]) mice have been described (3). Lck-Bcl2 transgenic mice were previously described (18). Rag2γc-deficient mice were purchased from Taconic. FVB/N mice from Taconic, which present the Skin-1 mutation, were crossed with PLZF−/+ mice to generate compound PLZF−/−/Skin-1 mutant mice. A similar cross was performed between FVB/N mice from The Jackson Laboratory, which does not contain the Skin-1 mutation, with PLZF−/− mice to obtain PLZF−/−/Skin-1 wild-type mice. All animals were bred in-house and surgeries were performed for all experiments described in this manuscript.

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Abbreviations used in this article: DN, double-negative; E, embryonic day; ETP, early thymic progenitor; iNKT, invariant NKT; KO, knockout; PLZF, promyelocytic leukemia zinc finger.

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performed under approved animal protocols by the National Cancer Institute Animal Care and Use Committee.

**Tissue dissections, Abs, and flow cytometry**

Pregnant day 16 mothers were euthanized by bottled CO2, and fetuses were isolated and placed on ice for 30–60 min. Neonatal day 1 pups were similarly sacrificed by hypothermia. Fetuses and neonates were washed in PBS and tail samples were digested for genotyping. Thymuses were extracted under a magnifier and dispersed under a 70-µm cell strainer (Falcon) to obtain single-cell suspensions, γδ T cells from uterus and skin were isolated by Liberase (Roche) and DNase (Sigma-Aldrich) digestion of the tissues, washing, and filtration through a 70-µm cell strainer (Falcon). Immune cells in these cell suspensions were identified by staining with an anti CD45.2 (104)-specific Ab. Ab staining was performed for 30 min on ice and FACS analysis was done using a BD LSRRosetta cell analyzer (BD Biosciences). The following Ab clones were used: lineage biotin with an anti-CD45.2 (104)-specific Ab. Ab staining was performed for 30 min. Immune cells in these cell suspensions were identified by staining with the anti–PLZF-PE Ab (Mabs.21F7) or isotype control (TC11-18H10.1, BioLegend). FACS was performed using FITC, PE, PerCP5.5, PE-Cy7, allophycocyanin, and Pacific Blue dyes. Dead cells were excluded by propidium iodide staining or Live/Dead fixable dead cell staining kit (BD Biosciences) and staining with the anti–IL-17a Ab (BioLegend). Analysis of in vivo proliferation was performed by a single i.p. injection of BrdU (10 mg/ml) in PBS to the mother. Twenty-four hours later, mothers were sacrificed and fetuses dissected to analyze BrdU incorporation in γδ T cells (Fig. 1). To verify the specificity of the 17D1 Ab for γδ TCR we used the 17D1 Ab that recognizes the Vγ5Vβ6 γδ TCR but can also recognize the Vγ6Vβ1 γδ TCR when cells are prestained with the anti–γδ TCR GL3 Ab clone (19). To verify the specificity of the 17D1 Ab for Vγ6γδ T cells, we first performed a double staining with an anti–Vγ5 Ab and 17D1. We observed that 17D1+ cells were Vγ5+ as well as Vγ5- cells, that some Vγ5+ cells did not present 17D1 staining, and that many γδ T cells did not present Vγ5 or 17D1 staining (Fig. 1C). Vγ5-17D1+ γδ T cells corresponded mostly to the Vγ4 γδ T cell subset (Supplemental Fig. 1). To clearly identify which of these γδ T cell subpopulations corresponded to the Vγ6 and Vγ5 subsets, we sorted the different γδ T cell subpopulation and analyzed Vγ5 and Vγ6 transcripts by RT-PCR. As expected, Vγ5 transcripts were present only in Vγ5+ γδ T cells irrespective of staining with the 17D1 Ab whereas expression of the Vγ5Vβ6 γδ TCR was restricted to Vγ5-17D1+ γδ T cells (Fig. 1D), indicating that the combination of 17D1 and Vγ5 staining could be used to specifically identify Vγ6+ γδ T cells. Sequence analysis of the purified Vγ6 RT-PCR product further confirmed that it corresponded to the canonical invariant Vγ6+ γδ TCR (data not shown). High Zbtb16 transcript levels were found in both Vγ5+ and Vγ6+ but not in Vγ5- Vγ6+ γδ T cell subsets in the E16 thymus (Fig. 1D), suggesting that PLZF expression may play a role in the development of these lineages.

To evaluate whether Vγ5+ and Vγ6+ γδ T cells found in adult mice were exclusively derived from PLZF-expressing cells, we performed fate mapping using compound PLZF-CRE transgenic × Rosa-Tdtomato mice. In this strain, expression of CRE is controlled by the PLZF promoter, leading to the excision of a stop cassette in the Rosa promoter and to the constitutive expression of Tdtomato. Therefore, even the transient expression of PLZF leads to the permanent label of cells and their progeny. Using this fate-mapping model we observed that 100% of Vγ5+ γδ T cells in the adult skin and 100% of Vγ6+ γδ T cells in the adult uterus were derived from PLZF-expressing cells, whereas ~30% of γδ T cells found in the spleen were derived from PLZF-expressing cells (Fig. 1E). This 30% Tdtomato label in adult γδ T cells is a consequence of PLZF expression in hematopoietic stem cells, which also leads to a 30% Tdtomato label in Cd4+ CD8- DN and Cd4+...
FIGURE 1. PLZF is expressed in fetal-derived Vγ5+ and Vγ6+ γδ T cells. (A) PLZF intracellular staining of C57BL/6 fetal E16 and neonatal day 1 thymus. DN thymocytes include ETP (CD4−CD8−Lin−CD44+c-Kit−CD25−), DN2a (CD4−CD8−Lin−CD44+c-Kit+CD25+), DN3 (CD4−CD8−Lin−CD44−CD25+), DN4 (CD4−CD8−Lin−CD44−CD25−), and γδ T cells (GL3+, 2C11+). (B) RT-PCR of sorted T cell subsets from adult, neonatal day 1, and E16 thymus. (C) Gating strategy used to sort subpopulations of γδ T cells based on Vγ5 and 17D1 staining. (D) RT-PCR from sorted γδ T cell subsets indicated in (C). (E) FACS analysis of Tdtomato label in the subpopulations indicated from different tissues. The numbers indicate the percentage of events within the gate from compound PLZF-CRE × Rosa-Tdtomato mice. (A) and (E) are representative of three independent experiments; (B)–(D) are representative of two independent experiments. *Vγ6+ cells are identified as CD45.2+GL3+2C11+Vγ5+17D1+ cells. d1, day 1.
CD8+ double-positive thymocytes as well as in CD4 and CD8 T cells in spleen (20) (Supplemental Fig. 2).

Thus, PLZF is expressed in Vγ5+ and Vγ6+ γδ T cells in the fetal thymus.

PLZF is required for normal development of Vγ6+ but not of Vγ5+ γδ T cells

Based on the data presented above, we were interested in determining whether PLZF plays similar or different roles in the development of Vγ6+ and Vγ5+ γδ T cells. PLZF deficiency did not affect the number of Vγ5+ or Vγ6+ γδ T cells in the fetal thymus at the time point at which they are first selected (E16). However, by day 1 of life, the number of PLZF-deficient Vγ6+ γδ T thymocytes was reduced by ~4-fold (Fig. 2A–C; \( p < 0.0001 \)), suggesting that the amplification of Vγ6+ γδ T cells between the fetal and neonatal stages required PLZF. Analysis of Vγ6+ γδ T cell numbers in lungs and in utero of adult mice did not identify a significant reduction in PLZF-deficient mice (Fig. 2D), suggesting that self renewal in peripheral tissues may compensate their impaired development. Interestingly, Vγ5+ γδ T cell numbers in the fetal thymus and adult skin were not affected by PLZF deficiency (Fig. 2E), strongly suggesting that PLZF specifically contributes to the development of Vγ6+ but not Vγ5+ γδ T cells.

To determine whether PLZF deficiency affects the phenotype as well as the expansion of Vγ6+ γδ T cells, we assessed their phenotype. γδ T cells that secrete IL-17 are identified as CD272 CD44+ in the periphery, but within the fetal thymus, this subset represented \( \sim 10\% \) of Vγ6+ γδ T cells and was not affected by PLZF deficiency (Fig. 2F). Notably, however, by day 1 of life, the vast majority of Vγ6+ γδ T cells in wild-type mice were CD272 CD44+ (77%, range 74–81%), indicating that Vγ6+ γδ T cells acquire this phenotype during the late fetal stages. PLZF deficiency led to a decrease in both the absolute numbers of Vγ6+ γδ T cells as well as in the percentage of Vγ6+ cells with a CD272 CD44+ phenotype.
PLZF is required for the IL-17 effector differentiation of Vγ6+γδ T cells

Our results indicated that intrathymic Vγ6+γδ T cells can be subdivided into different subpopulations based on the expression of CD27 and CD44. They also showed that the predominant Vγ6+γδ T cell subset in the fetal E16 thymus was CD27CD44+ whereas a CD27CD44− phenotype was predominant in the neonatal thymus. We therefore assessed whether these phenotypes corresponded to differences in the ability to secrete IL-17 and, furthermore, whether PLZF was required for the acquisition of this effector function. Approximately 40% of wild-type Vγ6+γδ T cells from the fetal thymus had the capacity to secrete IL-17 and this proportion increased to ~80% in the neonatal thymus. Interestingly, PLZF deficiency led to a reduced proportion and numbers of IL-17+Vγ6+γδ T cells in both the fetal and neonatal thymus (Fig. 2H, 2I), indicating that in the absence of PLZF, the effector maturation of Vγ6+γδ T cells was affected. The impaired acquisition of IL-17 secretion in PLZF-deficient Vγ6+γδ T cells did not correlate with increased IFN-γ secretion as shown by intracellular staining (Supplemental Fig. 3), indicating that PLZF does not prevent an alternative differentiation fate into IFN-γ-secreting cells. These results show that PLZF is required for the acquisition of IL-17 effector functions in Vγ6+γδ T cells during the fetal period. Impaired effector maturation of PLZF-deficient Vγ6+γδ T cells was also evident, as cells failed to downregulate CD62L levels, a common characteristic of maturation in innate-like T cells (Fig. 2J).

Collectively, these data indicate that PLZF expression is required for the acquisition of IL-17 secretion in Vγ6+γδ T cells, as well as their maturation into a CD62L−CD27+CD44+ phenotype.

PLZF is required for immature CD27+CD44−Vγ6+γδ T cells to develop into mature CD27+CD44+ IL-17–secreting cells

To specifically determine whether CD27+CD44−Vγ6+γδ T cells found in the E16 fetal thymus were the precursors of CD27+CD44+ cells, we sorted CD27+CD44−Vγ6+γδ T cell subsets from wild-type and PLZF-deficient E16 thymuses and assessed their differentiation potential in vitro in cocultures with the stroma cell line OP9-DL1 for 3 d. Wild-type CD27+CD44−Vγ6+γδ T cells gave rise to a majority of CD27+CD44+ cells after culture, indicating that CD27+CD44− cells are the precursors of CD27+CD44+ cells. Importantly, CD27+CD44− Vγ6+γδ T cells were unable to generate the CD27+CD44+ subset in the absence of PLZF, revealing a requirement for PLZF at this maturation step (Fig. 3A). Thus, PLZF is required for the maturation of CD27+CD44−Vγ6+γδ T cells to a CD27+CD44+ phenotype.

We next assessed whether PLZF-deficient immature CD27−CD44− Vγ6+γδ T cells from fetal E16 thymus could acquire IL-17 secretion after in vitro culture. Approximately 20% of sorted immature CD27−CD44− E16 Vγ6+γδ wild-type γδ T cells were capable of secreting IL-17, and this proportion increased to 50% after a 3-d in vitro coculture on OP9-DL1 stroma, indicating that maturation into a CD27+CD44+ phenotype correlates with the acquisition of IL-17 secretion. Immature PLZF-deficient CD27−CD44− Vγ6+γδ T cells already presented a reduced IL-17 secretion profile and, consistent with their lack of maturation in the OP9-DL1 system, the proportion of IL-17+ cells did not increase (Fig. 3B). During these in vitro differentiation cocultures we did not detect a significant increase in apoptosis in either wild-type or PLZF-deficient Vγ6+γδ T cells (Fig. 3C), suggesting that PLZF is not providing a prosurvival role.

Interestingly, analysis of proliferation by dilution of cell trace label identified that PLZF-deficient CD27+CD44+ Vγ6+γδ T cells proliferated at a lower rate than did wild-type cells during the OP9-DL1 cocultures (Fig. 3D). We also observed that PLZF-deficient E16 Vγ6+γδ T cells incorporated lower levels of BrdU 24 h after BrdU injection into the mother (Fig. 3E). These results indicate that PLZF is required for the proliferation of immature Vγ6+γδ T cells, opening the possibility that lack of proliferation may be a causal mechanism impairing the maturation of PLZF-deficient cells.

To test whether impaired proliferation was sufficient to prevent the maturation of Vγ6+γδ T cells, we sorted wild-type CD27+CD44− Vγ6+γδ T cells and cocultured them with OP9-DL1 stroma in the presence of different concentrations of a cyclin kinase 4/6 inhibitor (PD0332991) to inhibit their proliferation. We observed that 100 nM treatment led to stronger inhibition of proliferation than did 50 nM treatment, as observed by dilution of CellTrace Violet (Fig. 3F). Interestingly, 50 and 100 nM PD0332991 treatment led to ~20 and 65% inhibition in the generation of mature CD27+CD44+ Vγ6+γδ T cells from immature CD27−CD44− progenitors, respectively (Fig. 3G), strongly suggesting that the inability of PLZF-deficient CD27+CD44+ Vγ6+γδ T cells to proliferate is causative of their maturation defect. In correlation with the requirement of proliferation for cells to acquire a mature CD27+CD44+ phenotype, inhibition of proliferation during the in vitro cultures also led to a reduction of IL-17 secretion in wild-type Vγ6+γδ T cells (Fig. 3H). Impaired IL-17 effector maturation of PLZF-deficient Vγ6+γδ T cells correlated with impaired expression of Sox13, Rorc, and Runx1, which control the IL-17 program in γδ T cells and Th17 cells (Fig. 3I).

 Altogether, these results show that PLZF-deficient CD27+CD44+ Vγ6+γδ T cells fail to proliferate and acquire a mature CD27+CD44+ phenotype with increased IL-17 secretion.

Transgenic Bcl2 expression does not rescue Vγ6+γδ T cell development in PLZF-deficient mice

Although we did not observe increased apoptosis of PLZF-deficient Vγ6+γδ T cells in vitro, reduced survival of PLZF-deficient Vγ6+γδ cells in vivo could still contribute to their impaired development. To evaluate this hypothesis, we generated compound PLZF-deficient × lck−Bcl-2 transgenic mice because transgenic expression of Bcl2 has been shown to prevent the apoptosis of lymphocytes to different stimuli (21). Whereas Bcl2 expression in sorted Vγ6+γδ T cells from transgenic neonatal thymus was increased by 20-fold (Fig. 4A), there was no rescue of Vγ6+γδ T cells in the Bcl2-transgenic PLZF-deficient neonatal thymus (Fig. 4B, 4C). The reduced proportion of IL-17+Vγ6+γδ T cells in PLZF-deficient neonates was also not rescued by transgenic Bcl2 expression (Fig. 4D, 4E). These results suggest that increased apoptosis is not the underlying mechanism responsible for the developmental impairment of PLZF-deficient Vγ6+γδ T cells.

PLZF deficiency impairs the IL-17 effector maturation of Skint-1 mutant Vγ5+γδ T cells

Skint-1 is an Ig family gene expressed in thymic epithelial cells and keratinocytes. Interestingly, in Skint-1 mutant mice, Vγ5+γδ T cells do not acquire the ability to secrete IFN-γ and instead acquire a IL-17–secreting fate and migrate to the epithelia of the uterus instead of the skin, similar to Vγ6+γδ T cells (22). Based on this, it was postulated that the IL-17 program in γδ T cells is...
FIGURE 3. PLZF is required for the effector maturation of Vγ6+γδ T cells in vitro. (A) Gating strategy indicating the subpopulations of Vγ6+γδ T cells that were sorted and placed for 3 d in OP9-DL1 cocultures in the presence of IL-7 to evaluate their ability to generate different subpopulations. FACS analysis shows the phenotypic profile of sorted cells after sort and after coculture. The numbers indicate the proportion of cells within the quadrants. (B) FACS analysis for intracellular IL-17 staining after PMA plus ionomycin stimulation of sorted CD27+CD44+Vγ6+γδ T cells from fetal (E16) thymus after sort (ex vivo) or after a 3 d differentiation in OP9-DL1 cocultures as in (A). The percentage of IL-17+ cells is indicated. (C) FACS analysis of annexin V+ cells after 3 d OP9-DL1 coculture. The percentage of annexin V+ cells is indicated. (D) Histogram showing the dilution of CellTrace Violet on sorted wild-type (+/+ ) and PLZF-deficient (−/−) CD27+CD44+Vγ6+γδ T cells after 3 d coculture as in (A). The gray histogram represents nonproliferating T cells. (E) Histograms showing the incorporation of BrdU in fetal E16 Vγ6+γδ T cells 24 h after a single BrdU injection to the mother. (F) Histogram showing the dilution of CellTrace Violet on sorted wild-type CD27+CD44+Vγ6+γδ T cells after coculture in the presence of the CDK4/6 inhibitor (PD0332991). (G) FACS analysis showing the phenotypic profile of sorted cells after coculture. (H) FACS analysis showing intracellular IL-17 staining in sorted cells after coculture. The numbers indicate the proportion of cells within the quadrants. (I) RT-PCR analysis of sorted immature E16 Vγ6+CD27+CD44+γδ T cells for the indicated genes. Data correspond to two independent experiments. *Vγ6+ cells are identified as GL3+2C11+Vγ517D1+ cells.
a default pathway that occurs under weak or absent TCR signals. To interrogate whether the requirement of PLZF expression for the effector maturation of $V_{\gamma}6^+\gamma\delta T$ cells was related to the specificity of the TCR or to the IL-17 program, we assessed whether PLZF may also contribute to the IL-17 effector maturation of $V_{\gamma}5^+\gamma\delta T$ cells in Skint-1–deficient mice. To evaluate this, we generated compound Skint-1 mutant $3^{rd}$ PLZF-deficient mice and analyzed the phenotype of $V_{\gamma}5^+\gamma\delta T$ cells in the fetal E16 and neonatal day 1 thymus. Fetal E16 wild-type and PLZF-deficient Skint-1 mutant $V_{\gamma}5^+\gamma\delta T$ cells remained CD27$^+$CD44$^-$, phenotypically resembling $V_{\gamma}6^+\gamma\delta T$ cells. Interestingly, whereas some $V_{\gamma}5^+\gamma\delta T$ cells acquired a CD27$^+$CD44$^+$ phenotype in Skint-1 mutant neonates, this differentiation was completely abrogated in compound Skint-1 mutant $3^{rd}$ PLZF-deficient neonates (Fig. 5A). Thus, under conditions where $V_{\gamma}5^+\gamma\delta T$ cells are programmed to differentiate into IL-17–secreting cells, PLZF is required for the acquisition of a CD27$^+$CD44$^+$ phenotype. To directly assess the role of PLZF in the IL-17 effector differentiation of Skint-1 mutant $V_{\gamma}5^+\gamma\delta T$ cells, we analyzed the proportion of IL-17$^+\gamma\delta T$ cells within the $V_{\gamma}5^+\gamma\delta T$ cells by intracellular staining. Skint-1 mutant $\times$ PLZF-deficient $V_{\gamma}5^+\gamma\delta T$ cells had a significantly reduced proportion of IL-17$^+ T$ cells as compared with Skint-1 mutant $\times$ PLZF-wild type $V_{\gamma}5^+\gamma\delta T$ cells (35 ± 0.5 versus 15 ± 2.2%; $p < 0.0001$) (Fig. 5B, 5C).

Altogether, these results indicate that the requirement of PLZF expression for the development and effector maturation of $V_{\gamma}5^+\gamma\delta T$ cells is associated with the IL-17 program and is independent of the $V_{\gamma}6$ TCR, as a similar phenotype is observed in $V_{\gamma}5^+\gamma\delta T$ cells that are forced to acquire an IL-17 phenotype in Skint-1 mutant mice.

**Discussion**

The mechanisms controlling the intrathymic effector differentiation of innate-like IL-17$^+\gamma\delta T$ cells are different from those required for the differentiation of Th17 cells in the periphery (23–27). We have identified a novel requirement of PLZF for the development of IL-17$^+V_{\gamma}6^+\gamma\delta T$ cells. We show that PLZF expression precedes the selection of innate-like IL-17$^+\gamma\delta T$ cells, suggesting that PLZF expression is not induced downstream of TCR signals in these cells as has been proposed for $\gamma\delta$ NKT cells (5).
FIGURE 5. Impaired IL-17 effector maturation of PLZF-deficient Skint-1 mutant Vγ6+ γδ T cells. (A) FACS analysis of gated Vγ5+ γδ T cells from fetal (E16) and neonatal (day 1) thymus in the indicated strain. The numbers indicate the percentage of cells within the gate. (B) FACS analysis for intracellular IL-17 staining after PMA plus ionomycin stimulation of Vγ5+ γδ T cells in Skint-1 mutant (E16) thymus. (C) Scatter plot of the percentage of IL-17+Vγ5+ γδ T cells as represented in (B). Data are representative of two independent experiments. Each dot in the scatter plots represents data from a fetus and horizontal lines represent the mean value. The p values corresponding to significance using a t-test are shown.

Although PLZF is not expressed at the earliest stages of T cell development in the adult thymus, we have found that ETPs in the fetal thymus are PLZF-expressing cells. A unique characteristic of fetal hematopoietic stem cells is the expression of Lin28b, which blocks the production of mature Let-7 microRNAs (28). As PLZF was recently shown to be regulated by Let-7 microRNAs (29), it is reasonable to speculate that the increase of PLZF levels during fetal hematopoiesis might be due to increased Lin28b and reduced Let-7 levels in fetal progenitors. We have also observed that PLZF levels are further increased in DN2 thymocytes in the fetal thymus. Interestingly, Zbtb16 (PLZF) expression has been previously identified in DN2 thymocytes of adult mice (30), indicating that PLZF may be induced as cells develop into DN2a thymocytes. As IL-7 plays a critical role for the generation of DN2a thymocytes as well as in the maintenance of IL-17+ γδ T cells, it is possible that PLZF is positively regulated or maintained by IL-7 signals. Although, PLZF expression already occurs before γδT cell selection in the fetal thymus, a role of TCR signals in regulating PLZF expression cannot be ruled out. It was originally postulated that the IL-17 program in fetal Vγ6+ γδ T cells occurs in the absence of TCR engagement (22); however, later work identified that Vγ6+ γδ T cells have received TCR signals during development (31), and therefore it is possible that TCR signals maintain PLZF expression in Vγ6+ γδ T cells.

We also show that PLZF specifically allows the differentiation of fetal-derived IL-17+ γδ T cells, but not IFN-γ+ γδ T cells, despite sharing a common PLZF+ precursor. Therefore, PLZF-driven innate-like differentiation is not exclusive to NKT cells or IL-4+ T cell subsets (5, 32), but it also controls the differentiation of innate-like IL-17+ γδ T cells.

We have observed that PLZF is required for both the development and acquisition of IL-17 secretion in Vγ6+ γδ T cells as well as in Vγ5+ γδ T cells when redirected into a IL-17-secreting phenotype in Skint-1 mutant mice, indicating that the requirement of PLZF expression for the development of IL-17-secreting cells is independent of the specificity of the TCR. Interestingly, development of Vγ5+ γδ T cells was not affected by the absence of PLZF in Skint-1 wild-type mice when acquiring their IFN-γ program, suggesting that TCR signals during differentiation may compensate the requirement of PLZF expression. In fact, the ability of transgenic PLZF expression to confer effector functions to the conventional naive CD4 T cell population in the absence of TCR stimulation suggests that PLZF and TCR signals may play redundant roles in providing the effector maturation to T cells (8, 33).

Lack of surface CD27 identifies IL-17+ γδ T cells in peripheral tissues (12, 13). However, in the fetal thymus, some Vγ6+ γδ T cells acquired IL-17 secretion but have not yet downregulated CD27 levels, indicating that downregulation of CD27 is not required for their effector maturation.

We have characterized the different developmental stages of Vγ6+ γδ T cells and have shown that CD27+CD44+ Vγ6+ γδ T cells are the precursors of CD27−CD44+ Vγ6+ γδ T cells, which are the major subset present in the neonatal day 1 thymus and secrete IL-17. PLZF was required for this developmental step, as the number of mature CD27−CD44+ Vγ6+ γδ T cells was highly reduced in the neonatal thymus, and PLZF-deficient immature CD27+CD44− Vγ6+ γδ T cells did not proliferate and generate mature CD27+CD44+ Vγ6+ γδ T cells in vitro. PLZF-mediated proliferation of immature CD27+CD44− Vγ6+ γδ T cells is required for the effector maturation into a CD27−CD44+IL-17+ phenotype, as blocking proliferation in wild-type immature CD27+CD44− Vγ6+ γδ T cells also prevented their maturation. Impaired maturation of PLZF-deficient Vγ6+ γδ T cells was also evident as insufficient CD62L downregulation, a phenotype associated with the maturation of innate-like T cells that is disrupted in PLZF-deficient iNKT and γδ NKT cells (4, 6). It is not clear how a defect in proliferation leads to a maturation block in Vγ6+ γδ T cells. However, previous reports identified a requirement of proliferation for developmental progression in T cells. The effector maturation of Th cells into IFN-γ−, IL-4−, and IL-10−secreting cells was shown to be dependent on proliferation (34, 35), and, more recently, β-selection-induced proliferation was shown to be required for the generation of double-positive thymocytes (36). Presumably, epigenetic changes during the cell cycle may be required for developmental progression (37).

It is also not clear how PLZF controls the proliferation of immature Vγ6+ γδ T cells. Although overexpression of PLZF leads to a block of cell cycle progression in a myelomonocytic cell line (U937T) (38), it was recently described that PLZF expression may stimulate proliferation in NIH3T3 cells by repressing expression of Cdkn1a (p21), a negative regulator of cell cycle progression (39); however, we did not find increased Cdkn1a levels in PLZF-deficient Vγ6+ γδ T cells (data not shown), indicating that PLZF is required for other mechanisms of cell cycle control in Vγ6+ γδ T cells.

Despite the deficient development of Vγ6+ γδ T cells in the absence of PLZF, Vγ6+ γδ T cells were present in the lungs and uterus of adult mice, indicating that PLZF-deficient Vγ6+ γδ T cells are able to self-renew in the periphery.

Although transgenic PLZF expression protects from superantigen-mediated negative selection in conventional T cells (33), a prosurvival
function does not seem to be the main role of PLZF in controlling the effector differentiation of Vγ6γδ T cells. We did not detect increased apoptosis of PLZF-deficient Vγ6γδ T cells; also, transgenic Bcl2 expression, which protects against several apoptotic stimuli in thymocytes, did not rescue the development of PLZF-deficient Vγ6γδ T cells.

Interestingly, a recent report identified that PLZF is expressed in human Th17 cells, binds to the CCR6 promoter, and regulates the expression of IL-17-associated genes (40), suggesting that PLZF may control IL-17 effector maturation in other cell types. Collectively, we have identified that PLZF not only controls the effector maturation of NKT and IL-4γδ T cells but also that of innate-like IL-17γδ T cells.

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Disclosures

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References


