



HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2016 November 01.

Published in final edited form as:

J Immunol. 2015 November 1; 195(9): 4273–4281. doi:10.4049/jimmunol.1500939.

PLZF controls the development of fetal-derived IL-17⁺V γ 6⁺ γ δ T-cells[‡]

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Abstract

Expression of promyelocytic leukemia zinc finger (PLZF) protein directs the effector differentiation of invariant NKT (iNKT) cells and IL-4⁺ γ δ NKT cells. We show here that PLZF is also required for the development and function of IL-17⁺ γ δ T-cells. We observed that PLZF is expressed in fetal-derived invariant V γ 5⁺ and V γ 6⁺ γ δ T-cells, which secrete IFN- γ and IL-17 respectively. PLZF-deficiency specifically affected the effector differentiation of V γ 6⁺ cells, leading to reduced numbers of mature CD27⁻CD44⁺ phenotype capable of secreting IL-17. Although PLZF was not required for V γ 5⁺ γ δ T-cells to develop, when these cells were re-programmed into IL-17-secreting cells in SkinT-mutant mice, they required PLZF for their effector maturation, similarly to V γ 6⁺ γ δ T-cells. The impaired effector differentiation of PLZF-deficient V γ 6⁺ γ δ T-cells was not due to increased apoptosis and it was related to reduced proliferation of immature CD27⁻CD44⁻ V γ 6⁺ γ δ T-cells, which was required for their differentiation into mature CD27⁻CD44⁺ IL-17-secreting cells. Thus, this work identifies that PLZF function is not restricted to NKT or IL-4⁺ T-cells but it also controls the development of IL-17⁺ γ δ T-cells.

Keywords

gamma delta T-cells; PLZF; IL-17

INTRODUCTION

Conventional T-cells leave the thymus as naïve cells. Only after recognition of their cognate antigen in the periphery 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 non-lymphoid 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, γ δ NKT-cells (V γ 1.1⁺V δ 6.3⁺ γ δ T-cells), and human mucosal associated invariant T (MAIT) cells, is the expression of the promyelocytic leukemia zinc finger (PLZF) protein

[‡]This work was funded by NIH intramural support, NCI investigator-initiated intramural Research Projects (ZIA) # 1ZIABC011429

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(3–7). PLZF is considered a determinant of innate-like differentiation as it is necessary for the acquisition of effector functions in iNKT cells and $\gamma\delta$ NKT cells. PLZF-deficient iNKT 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 iNKT cells (4, 8, 9).

During fetal hematopoiesis $\gamma\delta$ T-cell subsets with innate-like properties are generated, such as invariant $V\gamma 5^+$ and $V\gamma 6^+\gamma\delta$ T-cells. Hematopoietic progenitors that enter the thymus at approximately embryonic day 14 (E14) first rearrange the $V\delta 1$ and $V\gamma 5$ TCR loci. Successful rearrangement allows the development of dendritic epidermal T-cells (DETC), which secrete IFN- γ and migrate to the intraepithelial compartment of the skin (10, 11). In contrast, upon unproductive $V\gamma 5$ rearrangements, cells attempt to rearrange the $V\gamma 6$ loci, leading to the generation of invariant $V\gamma 6V\delta 1$ $\gamma\delta$ T-cells, which secrete IL-17 and colonize the epithelium of the uterus, tongue, lungs and peritoneum. IL-17 $^+\gamma\delta$ T-cells are broadly identified as CD27-negative cells in peripheral tissues (12, 13) and preferentially proliferate in response to IL-7 signals, allowing their self-renewal in peripheral tissues (14). IL-17 $^+\gamma\delta$ 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 $\gamma\delta$ T-cells in response to IL-1 and IL-23 was shown to mediate autoimmune inflammation in an experimental autoimmune encephalomyelitis (EAE) model (17).

In this study, we have uncovered a novel function of PLZF in the development of innate-like IL-17 $^+V\gamma 6^+$ $\gamma\delta$ T-cells. Although PLZF is expressed in both $V\gamma 5^+$ and $V\gamma 6^+$ fetal thymic precursors, it is specifically required for the development of $V\gamma 6^+$ $\gamma\delta$ T-cells, allowing their normal maturation, expansion, and acquisition of IL-17 secretion after selection. Interestingly, despite PLZF expression in $V\gamma 5^+$ $\gamma\delta$ T-cells, PLZF-deficiency didn't affect their development or their ability to colonize the skin. The inability of PLZF-deficient $V\gamma 6^+\gamma\delta$ T-cells to expand intrathymically was not due to increased apoptosis, but to an impairment of $V\gamma 6^+\gamma\delta$ T-cells to proliferate and acquire a mature CD27 $^-$ CD44 $^+$ phenotype and function.

MATERIAL AND METHODS

Materials and Mice

C57BL/6 mice were purchased from Jackson laboratories. PLZF-deficient (PLZF KO) mice have been described (3). Lck-Bcl2 TG mice were previously described (18). Rag2/ γ c-deficient mice (Rag2-gc KO) were purchased from Taconic. FVB/N mice from Taconic farms, which present the Skin1 mutation, were crossed with PLZF $^{-/-}$ mice to generate compound PLZF $^{-/-}$ Skint1-mutant mice. A similar cross was performed between FVB/N mice from Jackson labs, which does not contain the Skint1 mutation, with PLZF $^{-/-}$ mice to obtain PLZF $^{-/-}$ Skint1-wild type mice. All animals were bred in-house and surgeries performed under approved animal protocols by the NCI animal care and use committee.

Tissue dissections, antibodies and flow cytometry

Pregnant day 16 mothers were euthanized by bottled CO₂, fetus were isolated and placed on ice for 30–60 minutes. Neonatal day1 pups were similarly sacrificed by hypothermia. Fetus and neonates were washed in PBS and tail samples digested for genotyping. Their thymuses extracted under a magnifier and dispersed under a 70 µm cell strainer (Falcon) to obtain single cell suspensions. $\gamma\delta$ T-cells from uterus and skin were isolated by Liberase (Roche) and DNaseI (Sigma) digestion of the tissues, wash 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 antibody. Antibody staining was performed for 30 min on ice and FACs analysis was done using BD LSRFortessa cell analyzer (BD Biosciences). The following antibody clones were used: Lin-biotin panel (TER-119, RB6-8C5, RA3-6B2, M1/70, 145-2C11), c-kit (2B8), CD44(IM7), CD25(PC61), TCR $\gamma\delta$ (GL3), CD3 ϵ (145-2C11), CD27(16.7F9/LG.7F9), V γ 5(536), CCR6(140706), CD127(A7R34), CD62L(MEL-14). Alpha-galcer-(aGC)-CD1d tetramers were obtained from the NIH tetramer core facility. Identification of V γ 6⁺ $\gamma\delta$ T-cells was performed by prestaining with GL3 antibody, followed by staining with the unconjugated rabbit 17D1 IgM antibody and a labeled secondary anti-IgM antibody (Jackson ImmunoResearch). When identifying V γ 6⁺ $\gamma\delta$ T-cells, a simultaneous staining with V γ 5-specific antibody was performed to exclude V γ 5⁺ cells from the analysis.

Intranuclear PLZF staining was performed after surface staining using the Foxp3 transcription factor staining buffer set (eBioscience) followed by staining with the anti-PLZF-PE antibody (Mags.21F7) or isotype control (eBiosciences). Analysis of IL-17⁺ $\gamma\delta$ T-cells was performed by sorting $\gamma\delta$ T-cells according to surface markers using the FACsaria cell sorter and stimulation for 4 hours with PMA plus ionomycin and Brefeldin-A, followed by surface staining, fixation and permeabilization of cells using the intracellular staining kit (BD Biosciences) and staining with the anti-IL-17a antibody (TC11-18H10.1, BioLegend). FACS was performed using FITC, PE, PERCP5.5, PEcy7, APC and Pacific Blue dyes. Dead cells were excluded by PI staining or LIVE/DEAD Fixable Dead Cell Stain Kits (Life technologies) and doublets were excluded based on FCS-W and size distribution.

BrdU labeling

Analysis of *in vivo* proliferation was performed by a single intraperitoneal injection of BrdU (10mg/ml) in PBS to the mother. 24hs later, mothers were sacrificed and fetuses dissected to analyze BrdU incorporation in thymic $\gamma\delta$ T-cells by FACs analysis

RT-PCR

qPCR was analyzed using SYBR green in 7900KT Fast-Real-time PCR system. The following primers were used: *Zbtb16* forward, TCTGACAAAGATGGGGATGATCC, reverse, CAGTATTCCTGCAGATGGTACAC; *Bgl2* forward, GGTGGGGTCATGTGTGTGG, reverse, CGGTTCAAGTACTCAGTCATCC; V γ 6 forward, GAAGCCCGATGCATACATAC, and reverse, GGGAAATGTCTGCATCAAGC. V γ 5 forward, GAGGATCCCGCTTGAAATGGATGAGA, reverse, CTTATGGAGATTTGTTTCAGC; *Sox13* forward, TACTCACAGACTGATCCCCAG, reverse, AAGTGCAGTTTCACCCATCAC; *Runx1* forward,

TGAACCACTCCACTGCCTTTAA, reverse, AATGGGTGTCGCTGGGTG; *Rorc* forward, TGCAAGACTCATCGACAAGGC, reverse, AGCTTTTCCACATGTTGGCTG; *Hprt* forward, TCAGTCAACGGGGGACATAAA, reverse, GGGGCTGTACTGCTTAACCAG.

Statistics

Plotting of the data and statistic analysis was performed using the Graphpad Prism software using unpaired standard T-test to compare two populations. The significance obtained is represented in the graphs.

RESULTS

PLZF is expressed in fetal V γ 5⁺ and V γ 6⁺ γ δ T-cells

PLZF is required for the innate-like differentiation of iNKT and V γ 1.1V δ 6.3 γ δ T-cells. As fetal hematopoiesis preferentially gives rise to innate-like subsets, we assessed whether PLZF expression is also shared with other innate-like T-cells of fetal origin. To this end, we analyzed intracellular PLZF levels in different thymic subsets in embryonic day 16 (E16) and day one (d1) neonatal thymi. PLZF was present in early thymic progenitors (ETP) of fetal origin, increasing at the DN2a stage and then gradually decreasing as cells progressed towards the DN3 and DN4 stages. Analysis of PLZF mean fluorescence intensity (MFI) levels in fetal and neonatal γ δ T-cells identified approximate 50% lower levels in d1 neonatal cells as compared to fetal γ δ T-cells (Figure 1a). We next analyzed whether the presence of PLZF protein correlated with increased expression by measuring *Zbtb16* (PLZF) mRNA transcripts in fetal-derived γ δ T-cells. We included CD4 single positive (CD4SP) thymocytes, iNKT and γ δ T-cells from adult thymus as reference controls for the analysis. *Zbtb16* mRNA levels were high in fetal (E16) γ δ T-cells and were reduced eighty-fold in neonatal (d1) γ δ T-cells (Figure 1b), indicating that high PLZF expression is restricted to γ δ T-cell subsets that develop at the earliest fetal stages. The low *Zbtb16* mRNA levels that we detected in adult γ δ T-cells may correspond to the PLZF⁺V γ 1.1V δ 6.3 γ δ T-cell subset that develops after birth (6). Interestingly, *Zbtb16* mRNA levels in fetal γ δ T-cells were approximately four-fold higher than those of adult iNKT cells. Furthermore, the high levels of PLZF in DN2 thymocytes in the fetal thymus, which are the precursors of all T-cells and have not been committed to the γ δ T-cell lineage, indicated that PLZF expression occurs before γ δ T-cell selection.

To investigate the γ δ T-cell subsets that express PLZF in the fetal (E16) thymus, we set up to identify V γ 5⁺ and V γ 6⁺ γ δ T-cell subsets by FACs analysis. We used the 17D1 antibody that recognizes the V γ 5V δ 1 γ δ TCR but can also recognize the V γ 6V δ 1 γ δ TCR if cells are pre-stained with the anti- γ δ TCR GL3 antibody clone (19). In order to verify the specificity of the 17D1 antibody for V γ 6⁺ γ δ T-cells, we first performed a double staining with an anti-V γ 5 antibody and 17D1. We observed that 17D1⁺ cells were V γ 5⁺ as well as V γ 5⁻ cells; that some V γ 5⁺ cells didn't present 17D1 staining; and that many γ δ T-cells didn't present V γ 5 or 17D1 staining (Figure 1c). V γ 5⁻17D1⁻ γ δ T-cells corresponded mostly to the V γ 4 γ δ T-cell subset (Supplementary Figure 1). In order to clearly identify which of these γ δ T-cell subpopulations corresponded to the V γ 6 and V γ 5 subsets, we sorted the different γ δ T-cell subpopulation and analyzed V γ 5 and V γ 6 transcripts by RT-PCR. As expected, V γ 5

transcripts were present only in $V\gamma 5^+$ $\gamma\delta$ T-cells irrespective of staining with the 17D1 antibody while expression of the $V\gamma 6$ TCR was restricted to $V\gamma 5^-17D1^+$ $\gamma\delta$ T-cells (Figure 1d), indicating that the combination of 17D1 and $V\gamma 5$ staining could be used to specifically identify $V\gamma 6^+$ $\gamma\delta$ T-cells. Sequence analysis of the purified $V\gamma 6$ RT-PCR product further confirmed that it corresponded to the canonical invariant $V\gamma 6^+$ $\gamma\delta$ TCR (**data not shown**). High *Zbtb16* transcript levels were found in both $V\gamma 5^+$ and $V\gamma 6^+$ but not in $V\gamma 5^-V\gamma 6^-$ $\gamma\delta$ T-cell subsets in the E16 thymus (Figure 1d), suggesting that PLZF expression may play a role in the development of these lineages.

In order to evaluate whether $V\gamma 5^+$ and $V\gamma 6^+$ $\gamma\delta$ T-cells found in adult mice were exclusively derived from PLZF-expressing cells, we performed fate mapping using compound PLZF-CRE transgenic x 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 $V\gamma 5^+$ $\gamma\delta$ T-cell in the adult skin and 100% of $V\gamma 6^+$ $\gamma\delta$ T-cells in the adult uterus were derived from PLZF-expressing cells, while approximately 30% of $\gamma\delta$ T-cells found in the spleen were derived from PLZF-expressing cells (Figure 1e). This 30% Tdtomato label in adult $\gamma\delta$ T-cells is consequence of PLZF expression in hematopoietic stem cells, which also leads to a 30% Tdtomato label in $CD4^-CD8^-$ double negative (DN) and $CD4^+CD8^+$ double positive (DP) thymocytes as well as in $CD4$ and $CD8$ T-cells in spleen (20) (Supplementary Figure 2).

Thus, PLZF is expressed in $V\gamma 5^+$ and $V\gamma 6^+$ $\gamma\delta$ T-cells in the fetal thymus.

PLZF is required for normal development of $V\gamma 6^+$ but not of $V\gamma 5^+$ $\gamma\delta$ T-cells

Based on the data presented above, we were interested in determining whether PLZF plays similar or different roles in the development of $V\gamma 6^+$ and $V\gamma 5^+$ $\gamma\delta$ T-cells. PLZF-deficiency didn't affect the number of $V\gamma 5^+$ or $V\gamma 6^+$ $\gamma\delta$ 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 $V\gamma 6^+$ $\gamma\delta$ T-thymocytes was reduced by approximately four-fold (Figure 2a and 2b and 2c; $p < 0.0001$), suggesting that the amplification of $V\gamma 6^+$ $\gamma\delta$ T-cells between the fetal to the neonatal stage required PLZF. Analysis of $V\gamma 6^+$ $\gamma\delta$ T-cell numbers in lungs and utero of adult mice didn't identify a significant reduction in PLZF-deficient mice (Figure 2d), suggesting that self renewal in peripheral tissues may compensate their impaired development. Interestingly, $V\gamma 5^+$ $\gamma\delta$ T-cell numbers in the fetal thymus and adult skin were not affected by PLZF deficiency (Figure 2e), strongly suggesting that PLZF specifically contributes to the development of $V\gamma 6^+$ but not $V\gamma 5^+$ $\gamma\delta$ T cells.

To determine whether PLZF deficiency affects the phenotype as well as the expansion of $V\gamma 6^+$ $\gamma\delta$ T cells, we assessed their phenotype. $\gamma\delta$ T-cells that secrete IL-17 are identified as $CD27^-CD44^+$ in the periphery, but within the fetal thymus, this subset represented less than 10% of $V\gamma 6^+$ $\gamma\delta$ cells and was not affected by PLZF-deficiency (Figure 2f). Notably though, by day 1 of life, the vast majority of $V\gamma 6^+$ $\gamma\delta$ T-cells in wild type mice were $CD27^-CD44^+$ (77%, range 74-81%), indicating that $V\gamma 6^+$ $\gamma\delta$ T-cells acquire this phenotype during the late fetal stages. PLZF-deficiency led to a decrease in both the absolute numbers of $V\gamma 6^+$ $\gamma\delta$ T-

cells as well as in the percentage of $V\gamma 6^+$ cells with a $CD27^-CD44^+$ phenotype (50%, range 34-64%)(Figure 2f). This change in phenotype resulted in reduced numbers of $CD27^-CD44^+ V\gamma 6^+ \gamma\delta T$ -cells in PLZF-deficient mice and similar numbers of $CD27^+CD44^- V\gamma 6^+ \gamma\delta T$ -cells in wild type and PLZF-deficient mice (Figure 2g). These results suggest that PLZF specifically regulates the generation of the $CD27^-CD44^+$ subset from immature precursors.

PLZF is required for the IL-17 effector differentiation of $V\gamma 6^+ \gamma\delta T$ -cells

Our results indicated that intrathymic $V\gamma 6^+ \gamma\delta T$ -cells can be subdivided into different subpopulations based on the expression of CD27 and CD44. They also showed that the predominant $V\gamma 6^+ \gamma\delta T$ -cell subset in the fetal E16 thymus was $CD27^+CD44^-$ while a $CD27^-CD44^+$ 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 $V\gamma 6^+ \gamma\delta T$ -cells from the fetal thymus had the capacity to secrete IL-17 and this proportion increased to approximately 80% in the neonatal thymus. Interestingly, PLZF-deficiency led to a reduced proportion and numbers of $IL-17^+ V\gamma 6^+ \gamma\delta T$ -cells in both the fetal and neonatal thymus (Figure 2h and 2i), indicating that in the absence of PLZF, the effector maturation of $V\gamma 6^+ \gamma\delta T$ -cells was affected. The impaired acquisition of IL-17 secretion in PLZF-deficient $V\gamma 6^+ \gamma\delta T$ -cells did not correlate with increased IFN- γ secretion as shown by intracellular staining (Supplementary Figure 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 $V\gamma 6^+ \gamma\delta T$ -cells during the fetal period. Impaired effector-maturation of PLZF-deficient $V\gamma 6^+ \gamma\delta T$ -cells was also evident as cells failed to downregulate CD62L levels, a common characteristic of maturation in innate-like T-cells (Figure 2j).

In Sum, these data indicate that PLZF expression is required for the acquisition of IL-17-secretion in $V\gamma 6^+ \gamma\delta T$ -cells, as well as their maturation into a $CD62L^-CD27^-CD44^+$ phenotype.

PLZF is required for immature $CD27^+CD44^- V\gamma 6^+ \gamma\delta T$ -cells to develop into mature $CD27^-CD44^+$ IL-17 secreting cells

To specifically determine whether $CD27^+CD44^- V\gamma 6^+ \gamma\delta T$ -cells found in the E16 fetal thymus were the precursors of $CD27^-CD44^+$ cells, we sorted $CD27^+CD44^- V\gamma 6^+ \gamma\delta T$ -cell subsets from wild type and PLZF-deficient E16 thymus and assessed their differentiation potential *in vitro* in co-cultures with the stroma cell line OP9-DL1 for 3 days. Wild type $CD27^+CD44^- V\gamma 6^+ \gamma\delta T$ -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, $CD27^+CD44^- V\gamma 6^+ \gamma\delta T$ -cells were unable to generate the $CD27^-CD44^+$ subset in the absence of PLZF, revealing a requirement for PLZF at this maturation step (Figure 3a). Thus, PLZF is required for the maturation of $CD27^+CD44^- V\gamma 6^+ \gamma\delta T$ -cells to a $CD27^-CD44^+$ phenotype.

We next assessed whether PLZF-deficient immature CD27⁺CD44⁻ V γ 6⁺ γ δ T-cells from fetal E16 thymus could acquire IL-17 secretion after *in vitro* culture. Approximately 20% of sorted immature CD27⁺CD44⁻ E16 V γ 6⁺ wild type γ δ T-cells were capable of secreting IL-17 and this proportion increased to 50% after a 3-day *in vitro* co-culture 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⁻ V γ 6⁺ γ δ 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 (Figure 3b). During these *in vitro* differentiation co-cultures we did not detect a significant increase in apoptosis in either wild type or PLZF-deficient V γ 6⁺ γ δ T-cells (Figure 3c), suggesting that PLZF is not providing a pro-survival role.

Interestingly, analysis of proliferation by dilution of cell trace label identified that PLZF-deficient CD27⁺CD44⁻ V γ 6⁺ γ δ T-cells proliferated at a lower rate than wild type cells during the OP9-DL1 co-cultures (Figure 3d). We also observed that PLZF-deficient E16 V γ 6⁺ γ δ T-cells incorporated lower levels of BrdU twenty four hours after BrdU injection into the mother (Figure 3e). These results indicate that PLZF is required for the proliferation of immature V γ 6⁺ γ δ T-cells, opening the possibility that lack of proliferation may be a causal mechanism impairing the maturation of PLZF-deficient cells

To test if impaired proliferation was sufficient to prevent the maturation of V γ 6⁺ γ δ T-cells, we sorted wild type CD27⁺CD44⁻ V γ 6⁺ γ δ T-cells and co-cultured 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 100nM treatment led to stronger inhibition of proliferation than 50nM treatment, as observed by dilution of cell trace violet (Figure 3f). Interestingly, 50nM and 100nM PD0332991 treatment led to approximately 20% and 65% inhibition in the generation of mature CD27⁻CD44⁺ V γ 6⁺ γ δ T-cells from immature CD27⁺CD44⁻ progenitors respectively (Figure 3g), strongly suggesting that the inability of PLZF-deficient CD27⁺CD44⁻ V γ 6⁺ γ δ 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 V γ 6⁺ γ δ T-cells (Figure 3h). Impaired IL-17 effector maturation of PLZF-deficient V γ 6⁺ γ δ T-cells correlated with impaired expression of *Sox13*, *Rorc* and *Runx1*, which control the IL-17 program in γ δ T-cells and Th17 cells (Figure 3i).

Altogether, these results show that PLZF-deficient CD27⁺CD44⁻ V γ 6⁺ γ δ T-cells fail to proliferate and acquire a mature CD27⁻CD44⁺ phenotype with increased IL-17 secretion.

Transgenic Bcl2 expression does not rescue V γ 6⁺ γ δ T-cell development in PLZF-deficient mice

Although we didn't observe increased apoptosis of PLZF-deficient V γ 6⁺ γ δ T-cells *in vitro*, reduced survival of PLZF-deficient V γ 6⁺ cells *in vivo* could still contribute to their impaired development. To evaluate this hypothesis, we generated compound PLZF-deficient x lck-Bcl-2 transgenic mice because transgenic expression of Bcl2 has been shown to prevent the apoptosis of lymphocytes to different stimuli (21). While Bcl2 expression in sorted V γ 6⁺

$\gamma\delta$ T-cells from transgenic neonatal thymus was increased by twenty-fold (Figure 4a), there was no rescue of $V\gamma 6^+$ $\gamma\delta$ T-cells in the Bcl2-transgenic PLZF-deficient neonatal thymus (Figure 4b and 4c). The reduced proportion of IL-17⁺ $V\gamma 6^+$ $\gamma\delta$ T-cell in PLZF-deficient neonates was also not rescued by transgenic Bcl2 expression (Figure 4d and 4e). These results suggest that increased apoptosis is not the underlying mechanism responsible for the developmental impairment of PLZF-deficient $V\gamma 6^+$ $\gamma\delta$ T-cells.

PLZF-deficiency impairs the IL-17 effector maturation of Skint1-mutant $V\gamma 5^+$ $\gamma\delta$ T-cells

Skint-1 is an immunoglobulin family gene expressed in Thymic epithelial cells (TECs) and keratinocytes. Interestingly, in Skint-1 mutant mice, $V\gamma 5^+$ $\gamma\delta$ 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 $V\gamma 6^+$ $\gamma\delta$ T-cells (22). Based on this, it was postulated that the IL-17 program in $\gamma\delta$ T-cells is a default pathway that occurs under weak or absent TCR signals. In order to interrogate if 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 Skint1-deficient mice. To evaluate this, we generated compound Skint1-mutant x PLZF-deficient mice and analyzed the phenotype of $V\gamma 5^+$ $\gamma\delta$ T-cells in the fetal E16 and neonatal d1 thymus. Fetal E16 wild type and PLZF-deficient Skint1-mutant $V\gamma 5^+$ $\gamma\delta$ T-cells remained CD27⁺CD44⁻, phenotypically resembling $V\gamma 6^+$ $\gamma\delta$ T-cells. Interestingly, while some $V\gamma 5^+$ $\gamma\delta$ T-cells acquired a CD27⁻CD44⁺ phenotype in Skint1-mutant neonates, this differentiation was completely abrogated in compound Skint1-mutant x PLZF-deficient neonates (Figure 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 Skint1-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. Skint1-mutant x PLZF-deficient $V\gamma 5^+$ $\gamma\delta$ T-cells had a significantly reduced proportion of IL-17⁺ cells as compared to Skint1-mutant x PLZF-wild type $V\gamma 5^+$ $\gamma\delta$ T-cells (35 \pm 0.5 % vs 15 \pm 2.2 %; $p < 0.0001$) (Figure 5b and 5c).

Altogether, these results indicate that the requirement of PLZF expression for the development and effector maturation of $V\gamma 6^+$ $\gamma\delta$ T-cells is associated with the IL-17 program and it 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 Skint1-mutant mice.

DISCUSSION

The mechanisms controlling the intrathymic effector differentiation of innate-like IL-17⁺ $\gamma\delta$ T-cells are different to 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 it has been proposed for $\gamma\delta$ NKT cells (5).

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 HSCs 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⁺ $\gamma\delta$ T-cells, it is possible that PLZF is positively regulated or maintained by IL-7 signals. Although, PLZF expression already occurs before $\gamma\delta$ T-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 V γ 6⁺ $\gamma\delta$ T-cells occurs in the absence of TCR engagement (22), however, later work identified that V γ 6⁺ $\gamma\delta$ T-cells have received TCR signals during development (31), therefore, it is possible that TCR signals maintain PLZF expression in V γ 6⁺ $\gamma\delta$ T-cells.

We also show that PLZF specifically allows the differentiation of fetal-derived IL-17⁺ $\gamma\delta$ T-cells, but not IFN- γ ⁺ $\gamma\delta$ 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⁺ $\gamma\delta$ T-cells.

We have observed that PLZF is required for both the development and acquisition of IL-17-secretion in V γ 6⁺ $\gamma\delta$ T-cells as well as in V γ 5⁺ $\gamma\delta$ 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 V γ 5⁺ $\gamma\delta$ 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 naïve 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⁺ $\gamma\delta$ T-cells in peripheral tissues (12, 13). However, in the fetal thymus, some V γ 6⁺ $\gamma\delta$ 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 V γ 6⁺ $\gamma\delta$ T-cells and have shown that CD27⁺CD44⁻ V γ 6⁺ $\gamma\delta$ T-cells are the precursors of CD27⁻CD44⁺ V γ 6⁺ $\gamma\delta$ 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⁺ V γ 6⁺ $\gamma\delta$ T-cells was highly reduced in the neonatal thymus and PLZF-deficient immature CD27⁺CD44⁻ V γ 6⁺ $\gamma\delta$ T-cells did not proliferate and generate mature CD27⁻CD44⁺ V γ 6⁺ $\gamma\delta$ T-cells *in vitro*. PLZF-mediated proliferation of immature CD27⁺CD44⁻ V γ 6⁺ $\gamma\delta$ T-cells is required for the

effector maturation into a CD27⁻CD44⁺IL-17⁺ phenotype as block of proliferation in wild type immature CD27⁺CD44⁻ V γ 6⁺ γ δ T-cells also prevented their maturation. Impaired maturation of PLZF-deficient V γ 6⁺ γ δ T-cells was also evident as inefficient CD62L downregulation, a phenotype associated with the maturation of “innate-like” T-cells that is disrupted in PLZF-deficient iNKT and γ δ NKT cells (4, 6). It is not clear how a defect in proliferation leads to a maturation block in V γ 6⁺ γ δ T-cells. However, previous reports identified a requirement of proliferation for developmental progression in T-cells. The effector maturation of helper T-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 required for the generation of DP thymocytes (36). Presumably, epigenetic changes during cell cycle may be required for developmental progression (37).

It is also not clear how PLZF controls the proliferation of immature V γ 6⁺ γ δ 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 V γ 6⁺ γ δ T-cells (**data not shown**), indicating that PLZF is required for other mechanisms of cell cycle control in V γ 6⁺ γ δ T-cells.

Despite the deficient development of V γ 6⁺ γ δ T-cells in the absence of PLZF, V γ 6⁺ γ δ T-cells were present in the lungs and uterus of adult mice, indicating that PLZF-deficient V γ 6⁺ γ δ T-cells are able to self-renew in the periphery.

Although transgenic PLZF expression protects from super-antigen mediated negative selection in conventional T-cells (33), a pro-survival function does not seem to be the main role of PLZF in controlling the effector differentiation of V γ 6⁺ γ δ T-cells. We did not detect increased apoptosis of PLZF-deficient V γ 6⁺ γ δ T-cells, also transgenic Bcl2 expression, which protects against several apoptotic stimuli in thymocytes, didn't rescue the development of PLZF-deficient V γ 6⁺ γ δ 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.

In Sum, we have identified that PLZF not only controls the effector maturation of NKT and IL-4⁺ γ δ T-cells but also that of “innate-like” IL-17⁺ γ δ T-cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Dr. Naomi Taylor and Dr. Avinash Bhandoola for critically reviewing the manuscript. Dr. Derek Sant'Angelo for providing the PLZF-CRE x Rosa-Tdtomato strain. Dr. Robert Tigelaar for the 17D1 antibody and Dr. Zuniga-Pflucker for the OP9-DL1 cell line.

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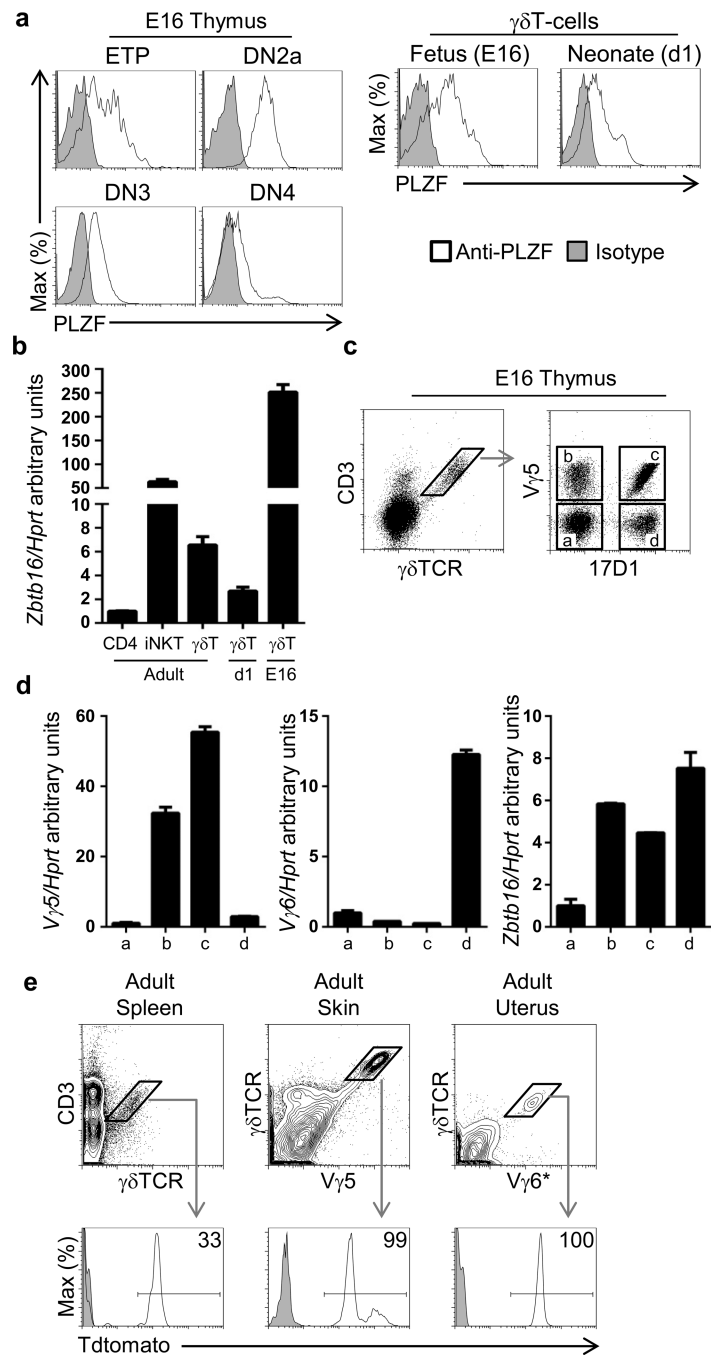


Figure 1. PLZF is expressed in fetal-derived V γ 5⁺ and V γ 6⁺ $\gamma\delta$ T-cells

a). PLZF intracellular staining of C57BL/6 fetal embryo day 16 (E16) and neonatal d1 thymus. Double negative (DN) thymocytes: ETP (CD4⁻CD8⁻Lin⁻CD44⁺ckit^{hi}CD25⁻); DN2a (CD4⁻CD8⁻Lin⁻CD44⁺ckit^{hi}CD25⁺); DN3 (CD4⁻CD8⁻Lin⁻CD44⁻CD25⁺); DN4 (CD4⁻CD8⁻Lin⁻CD44⁻CD25⁻) and $\gamma\delta$ T-cells (GL3⁺, 2C11⁺). **b)** RT-PCR of sorted T-cell subsets from adult, neonatal day 1 (d1) and fetal day 16 (E16) thymus. **c)** Gating strategy used to sort subpopulations of $\gamma\delta$ T-cells based on V γ 5 and 17D1 staining. **d)** RT-PCR from sorted $\gamma\delta$ 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 x Rosa-Tdtomato mice. (a and e) are representative of three independent experiments, (b, c, and d) are representative of two independent experiments. * $V\gamma 6^+$ cells are identified as $CD45.2^+ GL3^+ 2C11^+ V\gamma 5^- 17D1^+$ cells.

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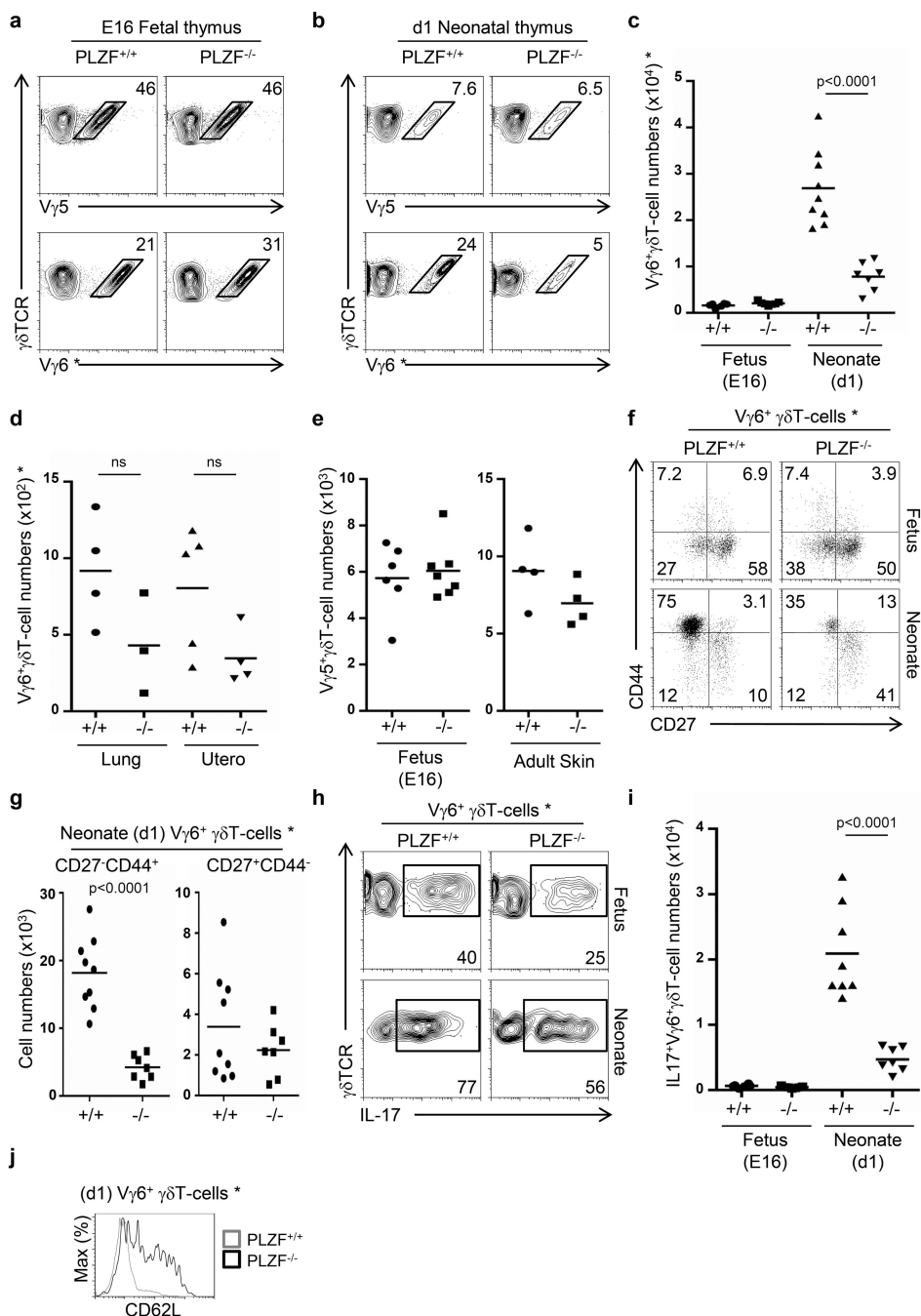


Figure 2. PLZF is required for the generation of V γ 6⁺ γ δ T-cells with a mature CD27⁻CD44⁺ phenotype and it does not affect the development of V γ 5⁺ γ δ T-cells
 FACs analysis showing the proportion of V γ 5⁺ and V γ 6⁺ γ δ T-cells in fetal day 16 (E16) (a) and neonatal day 1 thymus (b). Gram plots showing thymic V γ 6⁺ γ δ T-cell numbers (c), and V γ 6⁺ γ δ T-cell numbers in Lung and uterus (d) comparing wild type (+/+) and PLZF-deficient (-/-) cells. e) Number of V γ 5⁺ γ δ T-cells in the Fetal (E16) thymus and in adult skin. f) FACs profile of V γ 6⁺ γ δ T-cells in the fetal (E16) and neonatal (d1) thymus. g) Gram plot indicating the cell numbers of subsets of V γ 6⁺ γ δ T-cells identified in panel (f). h) FACs

analysis for intracellular IL-17 staining in $V\gamma 6^+\gamma\delta T$ -cells. i) Gram plot indicating the number of IL-17 $^+$ $V\gamma 6^+\gamma\delta T$ -cells. j) Histogram analysis of $V\gamma 6^+\gamma\delta T$ -cells in the neonatal thymus. The numbers within the dot-plots represent the proportion of events (a, b, f, h). Data correspond to two and three independent experiments. Each dot in the gram plots represents data from a fetus or neonate and horizontal lines represent the mean value. p values corresponding to the significance using T-test are shown. * $V\gamma 6^+$ cells are identified as $GL3^+2C11^+V\gamma 5^-17D1^+$ cells.

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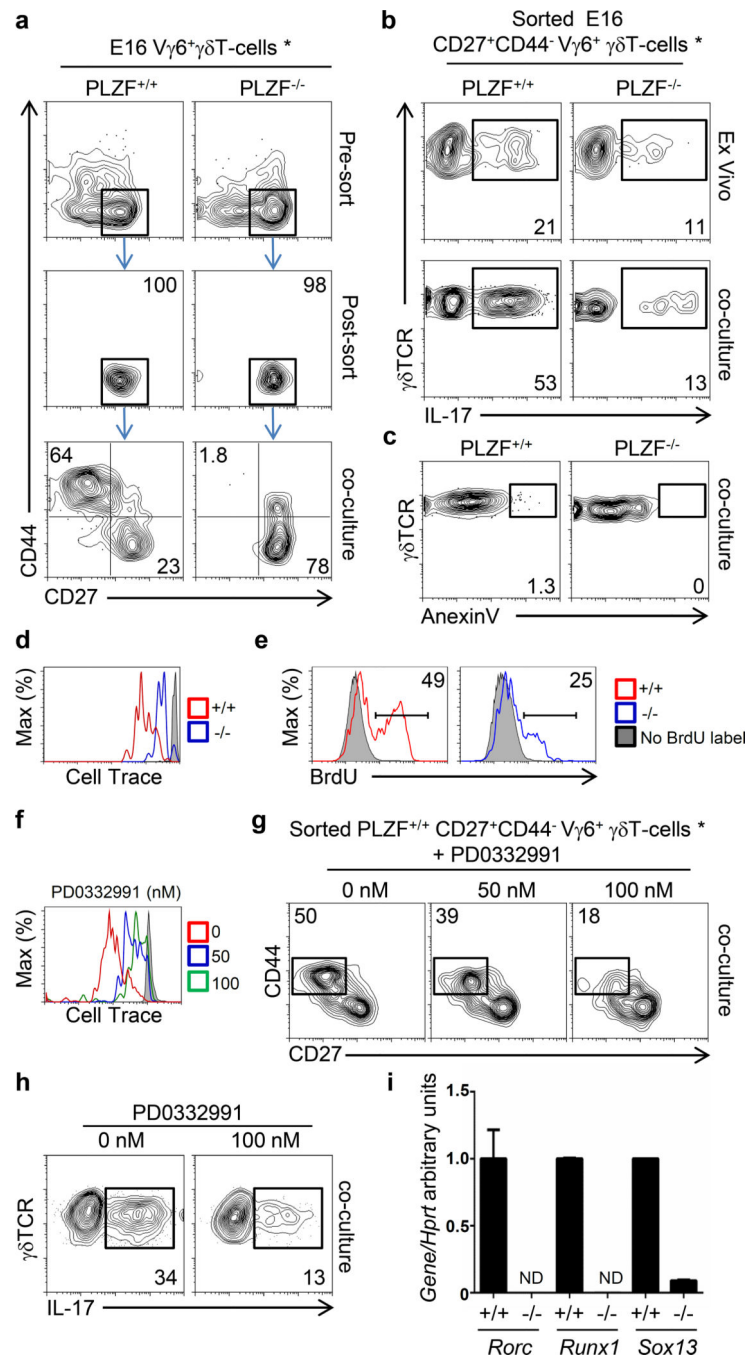


Figure 3. PLZF is required for the effector maturation of $V\gamma 6^+$ $\gamma\delta$ T-cells *in vitro*
a) Gating strategy indicating the subpopulations of $V\gamma 6^+$ $\gamma\delta$ T-cells that were sorted and placed for 3 days in OP9-DL1 co-cultures in the presence of IL-7 to evaluate their ability to generate different subpopulations. FACS analysis showing the phenotypic profile of sorted cells post-sort and after co-culture. 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⁻ $V\gamma 6^+$ $\gamma\delta$ T-cells from fetal (E16) thymus after sort (Ex vivo) or after a 3 day differentiation in OP9-DL1 co-cultures as in panel (a). The percentage

of IL-17⁺ cells is indicated. **e)** FACs analysis of Annexin V positive cells after 3 day OP9-DL1 co-culture. The percentage of annexinV positive cells is indicated. **d)** Histogram showing the dilution of cell trace violet on sorted wild type (+/+) and PLZF-deficient (-/-) CD27⁺CD44⁻ V γ 6⁺ γ δ T-cells after 3 day co-culture as in (a). The gray histogram represent non-proliferating T-cells. **e)** histograms showing the incorporation of BrdU in fetal E16 V γ 6⁺ γ δ T-cells 24 hours after a single BrdU injection to the mother. **f)** Histogram showing the dilution of cell trace violet on sorted wild type CD27⁺CD44⁻ V γ 6⁺ γ δ T-cells after co-culture in the presence of the CDK4/6 inhibitor (PD0332991). **g)** FACs analysis showing the phenotypic profile of sorted cells after co-culture. **h)** FACs analysis showing intracellular IL-17 staining in sorted cells after co-culture. The numbers indicate the proportion of cells within the quadrants. **i)** RT-PCR analysis of sorted immature E16 V γ 6⁺CD27⁺CD44⁻ γ δ T-cells for the indicated genes. Data correspond to two independent experiments. * V γ 6⁺ cells are identified as GL3⁺2C11⁺V γ 5⁻17D1⁺ cells.

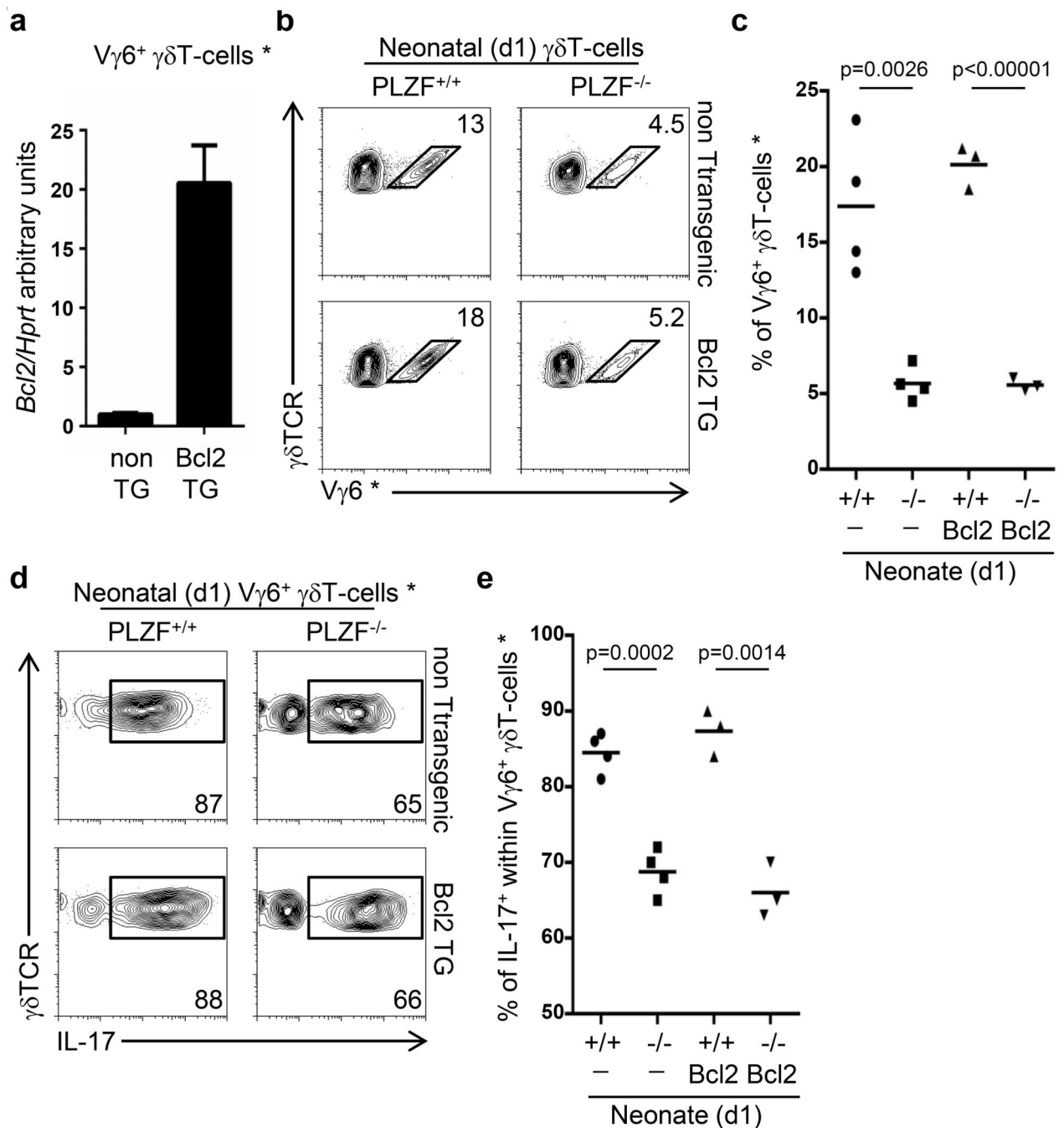


Figure 4. Transgenic Bcl2 expression fails to rescue the effector maturation of PLZF-deficient $V\gamma 6^+ \gamma\delta T\text{-cells}$

a RT-PCR from wild type and Bcl2-TG neonatal $V\gamma 6^+ \gamma\delta T\text{-cells}$. **b** FACS analysis on $\gamma\delta T\text{-cells}$ from PLZF-wild type or PLZF-deficient neonatal thymus that are also transgenic for Bcl2 (Bcl2 TG). The numbers indicate the percentage of cells within the gate. **c** Gram plot indicating the percentage of $V\gamma 6^+ \gamma\delta T\text{-cells}$ represented in panel (b). **d** FACS analysis for intracellular IL-17 staining after PMA plus Ionomycin stimulation of $V\gamma 6^+ \gamma\delta T\text{-cells}$ indicated in panel (b). Numbers represent the percentage of events within the gate. **e** Gram

plot of the percentage of IL-17⁺ V γ 6⁺ γ δ T-cells as represented in panel (d). Data is representative of two independent experiments. Each dot in the gram plots represents data from a neonate and horizontal lines represent the mean value. p values corresponding to the significance using T-test are shown. * V γ 6⁺ cells are identified as GL3⁺2C11⁺V γ 5⁻17D1⁺ cells.

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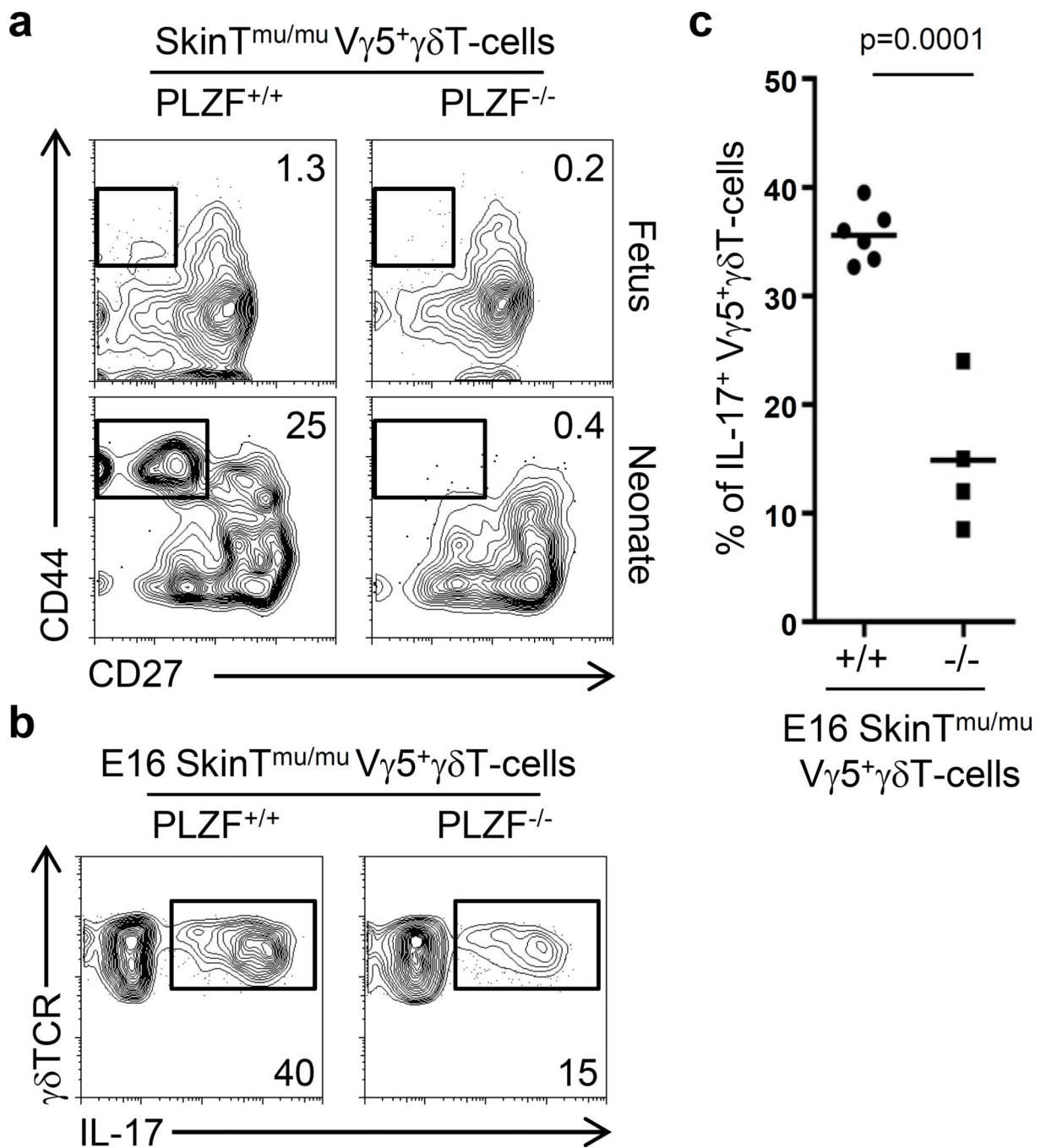


Figure 5. Impaired IL-17 effector maturation of PLZF-deficient SkinT-mutant V γ 5⁺ γ δ T-cells
a) FACS analysis of gated V γ 5⁺ γ δ T-cells from fetal (E16) and neonatal (d1) thymus in the indicated strain. The numbers indicate the percentage of cells within the gate. **b**) FACS analysis for intracellular IL-17 staining after PMA plus Ionomycin stimulation of V γ 5⁺ γ δ T-cells in Skint1-mutant (E16) thymus. **c**) Gram plot of the percentage of IL-17⁺ V γ 5⁺ γ δ T-cells as represented in panel (b). Data is representative of two independent experiments.

Each dot in the gram plots represents data from a fetus and horizontal lines represent the mean value. p values corresponding to the significance using T-test are shown.

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