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PLZF Induces the Spontaneous Acquisition of Memory/Effector Functions in T Cells Independently of NKT Cell-Related Signals

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The broad complex, tramtrack, bric-a-brac–zinc finger (BTB-ZF) transcription factor promyelocytic leukemia zinc finger (PLZF) is required for development of the characteristic innate/effector functions of NKT cells. In this study, we report the characterization and functional analysis of transgenic mouse T cells with forced expression of PLZF. PLZF expression was sufficient to provide some memory/effector functions to T cells without the need for Ag stimulation or proliferation. The acquisition of this phenotype did not require the proliferation typically associated with T cell activation. Furthermore, PLZF transgenic cells maintained a diverse TCR repertoire, indicating that there was no preferential expansion of specific clones. Functionally, PLZF transgenic CD4 and CD8 lymphocytes were similar to wild type memory cells, in that they had similar requirements for costimulation and exhibited a similar pattern of cytokine secretion, with the notable exception that transgenic T cells produced significantly increased levels of IL-17. Whereas transgene-mediated PLZF expression was not sufficient to rescue NKT cell development in Fyn- or signaling lymphocytic activation-associated protein (SAP)-deficient mice, the acquisition of memory/effector functions induced by PLZF in conventional T cells was independent of Fyn and SAP. These data show that PLZF is sufficient to promote T cell effector functions and that PLZF acts independently of SAP- and Fyn-mediated signaling pathways. The Journal of Immunology, 2010, 184: 000–000.

Invariant NKT lymphocytes have unique characteristics that place them at the interface of the innate and adaptive immune systems. One defining characteristic is the use of a highly restricted TCR that is composed of an invariant Vα14-Jα18 TCRα-chain that associates with Vβ8, Vβ7, and Vβ2 chains in mice. This TCR recognizes glycolipids presented by the nonclassical MHC-I molecule CD1d on cortical CD4+CD8+ double positive (DP) thymocytes, triggering the NKT cell differentiation pathway (1, 2). Homotypic interactions of Slamf1 and Slamf6 receptors (3) between cortical DP thymocytes and NKT cell precursors trigger signals through the adaptor signaling lymphocytic activation (Slam)-associated protein (SAP) and the Src kinase Fyn, which contribute to the positive selection of NKT cells (4–8). Downstream of these signaling pathways, activation of PKCζ, Bcl-10 and NF-kBp50 have been implicated in the differentiation and survival of NKT cells (9–11). After positive selection, NKT cell precursors undergo a proliferative burst (12) in the thymus that depends on the activity of c-myc (13, 14), followed by the acquisition of effector/memory functions. In contrast to conventional T cells, NKT cells that egress from the thymus can rapidly respond to stimulation without the need for Ag education in the periphery. Their invariant TCRs subunit, memory phenotype, presence of NK markers, ability to cosecrete IFN-γ and IL-4 among other cytokines, and preformed cytotoxic granzyme B+ granules are unique characteristics that distinguish NKT cells from conventional T cells (15).

We and others recently identified that promyelocytic leukemia zinc finger (PLZF), a member of the bric-a-brac, tramtrack, broad complex-poxvirus zinc finger (BTB-ZF) family of transcription factors, is highly expressed during the differentiation of NKT cells and is essential for the acquisition of effector/memory innate-like functions (16, 17). Lack of PLZF expression severely affected NKT cell development and function. PLZF-deficient NKT cells lose their characteristic memory phenotype, accumulated in lymph nodes instead of the liver, and their function was greatly impaired. In particular, PLZF-deficient NKT lymphocytes lose their unique ability to cosecrete IL-4 and IFN-γ upon ex vivo stimulation and did not express NK cell-specific markers (16, 17). The failure to produce cytokines was not a general defect in the cells; however, upon secondary activation, PLZF-deficient NKT cells produced cytokines comparable to wild type (WT) cells (16). Therefore, in the absence of PLZF, NKT cells do not acquire innate effector functions, but instead exhibit behaviors similar to naive conventional T cells. The loss of PLZF did not in any measurable way affect the function of conventional T cells (16).
High PLZF expression was also recently shown in a subset of γδ T cells (18–20). These γδ1.1V66.3 T cells exhibit innate T cell-like features similar to NKT cells (21) and these innate T cell characteristics are dependent on PLZF expression (18, 20). Interestingly, the frequency of these innate γδ T cells was dramatically increased in IL-2-inducible T cell kinase (ITK)-deficient mice (19). The increased frequency of the γδ1.1V66.3 T cells was subsequently shown to be a direct consequence of reduced TCR signaling that was controlled by inhibitor of DNA binding 3 (Id3) (20). Interestingly, these γδ T cells were also shown to require the CD4 T cell determinant, ThPOK, for normal development (20), suggesting functional interplay between these two B2TZ-FPs.

Ectopic expression of PLZF confers an effector/memory phenotype in CD4 T cells, associated with the acquisition of double IFN-γ/IL-4 secretion (17). Similarly, transgenic PLZF expression in CD8 lymphocytes leads to increased IFN-γ secretion (22). Although these studies establish that PLZF expression is sufficient to confer an NKT cell-like memory phenotype to T cells, it is not clear whether this transformation corresponds to a lineage change or to a lower threshold for peripheral activation and acquisition of memory as it occurs in WT lymphocytes. Therefore, PLZF transgenic T cells might be functionally equivalent to WT memory cells with the amplification of specific T cell clones. PLZF transgenic T cells might be functionally equivalent to WT memory T cells. Moreover, it is also not clear whether PLZF functions depend on the Slam/Fyn/Sap signals that are essential for the development of NKT cells and other innate T lymphocytes, but not conventional T cell lineages (3, 5).

In this work, we characterized a transgenic mouse model in which PLZF is expressed in all T cells. We observed that ectopic expression of PLZF in T cells led to the acquisition of a memory-like phenotype. These memory-like T cells accumulated in the liver and acquired high CXCR3 expression. Notably, PLZF-expressing T cells did not induce the upregulation of NK markers, and only a small proportion of CD4 lymphocytes became double IFN-γ/IL-4 secreting, indicating that PLZF is not sufficient to transform all T cells into NKT cell or innate-like T cell functional equivalents. Acquisition of the memory effector phenotype was spontaneous and not correlated with increased T cell proliferation in vivo or the amplification of specific T cell clones. PLZF transgenic T cells had a cytokine secretion profile similar to WT memory cells with the exception of a bias toward increased IL-17 secretion. PLZF transgenic T cells were also more sensitive to TCR stimulation than were WT T cells. Finally, transgenic PLZF expression induced a memory-like (innate-like) phenotype in conventional T cells in the absence Fyn or Sap expression. Therefore, PLZF functions independently of NKT cell-related signaling pathways.

Materials and Methods

Mice

The LCK-PLZF transgene was constructed by inserting the ~2kb gene into the LCK vector as described (23). Transgene DNA was injected into fertilized C57BL/6 eggs by the Memorial Sloan-Kettering Cancer Center (MSKCC) Mouse Genetics Core Facility. All animal work was done in compliance with the MSKCC Institutional Animal Care and Use Committee guidelines. PLZF-deficient, Fyn-deficient, and Sap-deficient mice have been previously described (5, 7, 24).

Flow cytometry

Surface staining was performed in FACS buffer (PBS with 2% heat inactivated FBS) for 20 min on ice using the indicated surface Abs. Data acquisition was performed on an LSRII cytometer (BD Biosciences, San Jose, CA), and exclusion of dead cells was performed by DAPI staining. Cell doublets were removed by monitoring the pulse width channel. Data were analyzed using FlowJo software (TreeStar, Ashland, OR). Fixation and permeabilization for intracellular staining with PLZF and FoxP3 was performed using the FoxP3 eBioscience (San Diego, CA) kit. Intracellular staining for the analysis of cytokine secretion was performed using the BD Biosciences intracellular kit (San Jose, CA).

Analysis of CDR3 lengths

Naive (CD44<CD62L<6) and memory (CD44<CD62L<4) CD4 and CD8 lymphocytes were purified by cell sorting by the MSKCC FACS facility. mRNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was obtained using the reverse transcriptase Superscript-III 1st strand kit (Invitrogen). Amplification of CDR3 lengths from the variable Vβ, β, and γ regions was performed with a biotinylated primer and β, γ, and α5.1/5.2 (MR9-4), β6 (VR6-7), TR (TR310), β6 (F2.31), Vβ9 (MR10-2), Vβ10 (B21.5), Vβ12 (MR11-1), Vβ13 (MR12-3), Vβ14 (MR14-2), Vα2 (B20.1), Vα3.2b.c (RR3-16), Vα8 (B21.14 and Vα11.11.2h.d (RR8-1). For the analysis of intracellular cytoklines: anti-IFN-γ (XM1G2), anti–IL-4 (11B11), anti-GMCSF (MP1-22E9), anti-IL-17 (eBio17B7), anti–IL-2 (JES5-544). Abs were used with different fluorochrome conjugations: FITC-, PE-, PECy5.5-, PE-Cy7-, APC- and APC-Cy7.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (La Jolla, CA) software. All samples were analyzed using unpaired, two-tailed Student t tests.

Results

Normal NKT cell and T cell differentiation in Lck-PLZF transgenic mice

To test the effects of ectopic PLZF expression on T cell function, we generated PLZF transgenic mice in which expression of PLZF was restricted to T cells by the control of the proximal Lck
promoter. Four original founders were produced that expressed the transgene at different levels, as observed by intranuclear staining and FACS analysis (data not shown). The two founders expressing the highest levels of PLZF were used for these studies. Expression of PLZF in transgenic mice was restricted to T cells (Fig. 1A). Overall T cell development appeared to be normal (Fig. 1A of PLZF in transgenic mice was restricted to T cells (Fig. 1A). The ratio in the thymus in favor of CD8+ T cells. In contrast, the CD4/CD8 ratio was reversed with more CD4+ than CD8+ T cells in the spleen and lymph nodes (Fig. 1D). A similar skewing was seen in mice with ectopic expression of MHCII on thymocytes as a consequence of T cell-specific ectopic expression of CIITA (25). Interestingly, a sizable percentage of the CD4 T cells in these CIITA transgenic mice express PLZF (26). In the CIITA transgenic mice, the increase of CD8SP in these mice was shown to be due to increased IL-4 signaling, presumably produced by the PLZF-expressing T cells (25). The percentage of CD4 T cells was largely similar in tissues from WT and PLZF transgenic mice, with a significant difference found only in the spleen (Fig. 1E). The percentage of PLZF transgenic CD8 T cells, however, was significantly increased in the thymus and reduced in the lymph nodes and spleen (Fig. 1F). Finally, FoxP3+ regulatory T cells were present at WT levels in the spleen (Fig. 1G).

Interestingly, NKT cell percentages were not increased in the thymus, lymph nodes, spleen, or liver of PLZF transgenic mice (Fig. 2A). Furthermore, the capacity of NKT cells from PLZF transgenic mice to cosecrete IL-4 and IFN-γ upon ex vivo stimulation with PMA/ionomycin was not altered (Fig. 2B). Finally, the frequency of NKT cells in various tissues was not significantly altered in PLZF transgenic mice (Fig. 2C). Therefore, ectopic expression of PLZF in addition to expression from the endogenous locus skews T cell development, but does not appear to alter NKT cell development or cytokine production.

**PLZF transgenic T cells have a memory phenotype but do not express NK markers**

Consistent with previous reports (17, 22), transgenic PLZF expression in both CD4 and CD8 T cells led to upregulation of CD44 and downregulation of CD62L, a phenotype characteristic of NKT cells, innate-like T cells, and memory T cells. TCRβ+ T cells with the activated phenotype were clearly more prominent in peripheral tissues compared with the thymus. In particular, TCRβ+ T cells in the liver were nearly all CD62Llo and CD44hi, consistent with selective enrichment in nonlymphoid organs (Fig. 3A). CD27, a member of the TNFR family, is downregulated on activated T cells (27). PLZF transgenic T cells, however, expressed CD27 similarly to WT T cells (Fig. 3B). KLRG1, a killer cell lectin-like receptor, has been shown to be expressed on Ag-experienced T cells (28). Again, expression of this marker on transgenic PLZF T cells was essentially identical to expression on T cells from WT littermates (Fig. 3C). Interestingly, we found that IL-7R (CD127) expression was markedly increased on both CD4+ and CD8+ PLZF transgenic T cells compared with T cells from WT littermates (Fig. 3C–E). Upregulation of IL-7R occurs

**FIGURE 1.** A, FACS analysis showing intranuclear PLZF expression in lymphocytes from WT and PLZF transgenic (TG) spleens. B, FACS analysis for CD4 and CD8 expression from WT and PLZF TG mice in the indicated tissues. C, Total TCRβ+ cells in the thymus (THY), lymph nodes (LNs), spleen (SPL), and liver (LIV) from WT (white) or PLZF TG (black) mice. D, The ratio of CD4 to CD8 single positive thymocytes in the thymus THY or CD4+ to CD8+ T cells in the LNs, SPL, or LIV in WT (white) or PLZF TG (black) mice. E, Percentage of TCRβhi CD4SP or TCRβlo CD4+ T cells or (F) TCRβhi CD8SP or CD8+ T cells in WT and transgenic mice in the indicated tissues. Percentages exclude NKT cells. G, Intranuclear FACS analysis for FoxP3 expression within TCRβ+ spleen T cells from WT and PLZF TG mice. The p values for C–F were calculated using two-tailed Student t tests. In all panels, the numbers within the graphs represent the percentage of events within the gates. Data are representative of four or more independent experiments.
on long-lived memory T cells (29, 30). IL-7 is also important for the expansion and survival of NKT cells (31, 32). Overall, these results demonstrated that ectopic expression of PLZF results in the upregulation of some, but not all, markers associated with memory/effector T cells.

Other markers associated with a memory T cell phenotype were differentially upregulated on CD8 or CD4 transgenic T cells. Ly6C levels, for example, were elevated on CD4 SP thymocytes and both CD4 and CD8 T cells in the spleen (Fig. 4A). A subset of CD8 T cells in the spleen upregulated CD122 (IL-2Rβ-chain), which is normally expressed on NK cells and some activated T cells. Nearly all PLZF transgenic CD8 T cells were also found to express CXCR3. This chemokine receptor is thought to be involved in directing T cells to sites of inflammation (33). Interestingly, CXCR3 was also recently shown to retain NKT cells in the thymus (34). PLZF-expressing T cells did not show upregulation of NK/NKT cell-associated markers, such as NKG2D, NK1.1, CD94, DX5, and 2B4. This finding suggests that ectopic expression of PLZF was insufficient to convert conventional T cells into bona fide NKT cells (Fig. 4B).

PLZF-expressing memory T cells are not proliferating and have a variable repertoire

Conventional T cells generally acquire a memory phenotype after undergoing Ag-induced proliferation. We next tested whether the acquisition of the memory phenotype by transgenic PLZF expression involved a similar process. For this purpose we analyzed the proliferation of T cells in vivo by BrdU incorporation. Eighteen hours after a single injection of BrdU, the thymus and spleen were isolated and incorporation of BrdU in T cells was analyzed by FACS. As a positive control for the experiment, BrdU incorporation in highly proliferating double negative thymocytes was analyzed. Our results showed that the acquisition of the memory phenotype in T cells was not related to increased proliferation in PLZF transgenic mice (Fig. 5A).

Although PLZF transgenic T cells did not seem to be actively proliferating in vivo, it was still possible that the memory phenotype was the consequence of a selective expansion of certain T cell clones. This expansion would lead to a restricted variability of the T cell repertoire. To evaluate this possibility, we analyzed the distributions of the CDR3 lengths (spectratyping) of Vβ3 and Vβ5 TCRβ subunits from naive and memory WT and PLZF transgenic CD4 T cells. Our analysis showed that the distribution of the CDR3 lengths in PLZF transgenic CD4 T cells followed a Gaussian curve as in WT naive T cells, indicating a high variability of the TCR (Fig. 5B). A similar result was observed for CD8 lymphocytes (data not shown). Clonal expansion was also examined by comparing TCRβ and TCRα usage on CD4+ T cells from the spleens of WT and PLZF transgenic mice. No significant differences were seen for CD4 T cells (Fig. 5C) or for CD8 T cells (data not shown).
To assess the functional effects of ectopic PLZF expression on T cells, we next evaluated the cytokine secretion patterns following PMA/ionomycin stimulation. Our first observation was that the effect of PLZF expression in CD4 T cells was different from that in CD8 T cells. For example, PLZF expression conferred the ability to secrete IL-4 and IFN-\(\gamma\) by both WT naive and memory T cells. PLZF transgenic T cells were functionally equivalent to WT memory cells, with respect to the dependency for costimulation. Moreover, levels of IL-4, IL-2, GM-CSF, IFN-\(\gamma\), and IL-3 production were similar between these two populations. The notable exception was IL-17, which was substantially increased in PLZF-expressing CD8 lymphocytes relative to the other groups, suggesting a direct role for PLZF in the acquisition of the IL-17-secreting phenotype (Fig. 6B).

Finally, we explored the mechanistic basis for the increased capacity of PLZF transgenic CD4 T cells to produce IL-4 upon primary activation. NKT cells are able to rapidly produce IL-4, in part because of the constitutive transcription of the IL-4 gene. Indeed, IL-4 mRNA is easily detected in NKT cells, although IL-4 protein is produced only after activation (37). The presence of preformed IL-4 mRNA is dependent on the expression of PLZF (16). To test for the presence of IL-4 mRNA, we crossed the LCK-PLZF to the “4get” IL-4 GFP reporter mice (38). NKT cells in these mice have been shown to constitutively express GFP (39). Only a small increase of GFP-expressing CD4 T cells was found in 4get x PLZF transgenic mice (Fig. 6C). Therefore, it is possible that PLZF-induced constitutive activity of the IL-4 gene may account for some of the increased production of IL-4, but it certainly does not account for the entire increase.

**PLZF transgenic T cells are more responsive to stimulation**

Memory T cells are more responsive to TCR stimulation than are naive T cells (40, 41). To test how T cells were affected by transgenic expression of PLZF, we analyzed the proliferation of T cells after in vitro stimulation with variable doses of plate-bound anti-CD3 (Fig. 7). T cells were magnetically purified according to Thy1.2 expression, stained with CFSE, and stimulated with different concentrations of plate-bound anti-CD3 Abs in the presence or absence of soluble anti-CD28 for 72 h. Proliferation was analyzed by dilution of CFSE staining. We observed that at the highest anti-CD3 concentrations (2.5 and 0.5 \(\mu\)g/ml), proliferation was similar between WT and PLZF transgenic CD4 and CD8 T cells. In these conditions, costimulation with abs against CD28 slightly increased the proliferation of T cells. This effect was also similarly observed in WT and PLZF transgenic T cells (Fig. 7). Under suboptimal conditions of stimulation (0.1 \(\mu\)g/ml), proliferation was increased by PLZF expression in CD4 T cells but not in CD8 T cells. Furthermore, in contrast to WT NKT cells, only a small fraction of transgenic CD4 lymphocytes were able to produce both IL-4 and IFN-\(\gamma\). More than half of the CD8 T cells from PLZF transgenic mice produced IFN-\(\gamma\) secretion; however, the frequency of IFN-\(\gamma\) producing CD4 T cells did not increase. PLZF expression in CD4 and CD8 lymphocytes led to a reduction of IL-2 secretion, but no change in the percentage of IFN-\(\gamma\)/IL-2 double producers. PLZF expression led to increased GM-CSF secretion in CD4 and CD8 lymphocytes and to an increased proportion of IL-17-secreting CD8 lymphocytes (Fig. 6A).

Memory T cells are less dependent on costimulation for activation than are naive T cells (35, 36). To analyze the dependency of costimulation for cytokine secretion in PLZF transgenic T cells, we sorted WT naive, WT memory, and PLZF transgenic CD4 and CD8 lymphocytes and analyzed their cytokine secretion following stimulation with plate-bound anti-CD3 Abs in the presence or absence of soluble anti-CD28 Abs (Fig. 6B). This analysis allowed the direct comparison of the functionality of PLZF transgenic T cells to WT naive and memory T cells. PLZF transgenic T cells were functionally equivalent to WT memory cells, with respect to the dependency for costimulation. Moreover, levels of IL-4, IL-2, GM-CSF, IFN-\(\gamma\), and IL-3 production were similar between these two populations. The notable exception was IL-17, which was substantially increased in PLZF-expressing CD8 lymphocytes relative to the other groups, suggesting a direct role for PLZF in the acquisition of the IL-17-secreting phenotype (Fig. 6B).

**Altered cytokine secretion pattern in PLZF transgenic T cells**

To test how T cells were affected by transgenic expression of PLZF, we analyzed the proliferation of T cells after in vitro stimulation with variable doses of plate-bound anti-CD3 (Fig. 7). T cells were magnetically purified according to Thy1.2 expression, stained with CFSE, and stimulated with different concentrations of plate-bound anti-CD3 Abs in the presence or absence of soluble anti-CD28 for 72 h. Proliferation was analyzed by dilution of CFSE staining. We observed that at the highest anti-CD3 concentrations (2.5 and 0.5 \(\mu\)g/ml), proliferation was similar between WT and PLZF transgenic CD4 and CD8 T cells. In these conditions, costimulation with abs against CD28 slightly increased the proliferation of T cells. This effect was also similarly observed in WT and PLZF transgenic T cells (Fig. 7). Under suboptimal conditions of stimulation (0.1 \(\mu\)g/ml), proliferation was increased by PLZF expression in CD4 T cells but not in CD8 T cells. Furthermore, in contrast to WT NKT cells, only a small fraction of transgenic CD4 lymphocytes were able to produce both IL-4 and IFN-\(\gamma\). More than half of the CD8 T cells from PLZF transgenic mice produced IFN-\(\gamma\) secretion; however, the frequency of IFN-\(\gamma\) producing CD4 T cells did not increase. PLZF expression in CD4 and CD8 lymphocytes led to a reduction of IL-2 secretion, but no change in the percentage of IFN-\(\gamma\)/IL-2 double producers. PLZF expression led to increased GM-CSF secretion in CD4 and CD8 lymphocytes and to an increased proportion of IL-17-secreting CD8 lymphocytes (Fig. 6A).

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Finally, we explored the mechanistic basis for the increased capacity of PLZF transgenic CD4 T cells to produce IL-4 upon primary activation. NKT cells are able to rapidly produce IL-4, in part because of the constitutive transcription of the IL-4 gene. Indeed, IL-4 mRNA is easily detected in NKT cells, although IL-4 protein is produced only after activation (37). The presence of preformed IL-4 mRNA is dependent on the expression of PLZF (16). To test for the presence of IL-4 mRNA, we crossed the LCK-PLZF to the “4get” IL-4 GFP reporter mice (38). NKT cells in these mice have been shown to constitutively express GFP (39). Only a small increase of GFP-expressing CD4 T cells was found in 4get x PLZF transgenic mice (Fig. 6C). Therefore, it is possible that PLZF-induced constitutive activity of the IL-4 gene may account for some of the increased production of IL-4, but it certainly does not account for the entire increase.
differentiation of NKT cells, it is not clear whether PLZF acts downstream of the Slam/Sap/Fyn pathway. We previously showed that the few NKT lymphocytes that differentiate in Fyn-deficient mice have PLZF expression levels equivalent to WT NKT cells (16). However, these NKT cells may represent “escapes” that differentiated through the Slam/Sap pathway, but in a manner independent of Fyn. It is still therefore possible that PLZF expression and function lie downstream of these signaling cascades.

To test whether PLZF functions are dependent on Fyn or Sap activity, we generated PLZF transgenic mice that are Fyn- or Sap-deficient by breeding, and we analyzed whether PLZF expression could rescue the defects in NKT cell differentiation. As a control, we introduced the LCK-PLZF transgene into PLZF-deficient mice by breeding. Transgenic expression of PLZF in PLZF knockout (KO) mice did not fully restore NKT cell development (Fig. 8A). There was, however, a consistent increase in the frequency

FIGURE 6. A, Spleen cells from WT and PLZF TG mice were activated with PMA and ionomycin for a total of 5 h. Brefeldin A was added to the culture for the last 4 h. Cells were harvested stained with Abs against the TCR, CD4, and CD8, and then made permeable followed by staining with Abs against GM-CSF, IL-4, and IFN-γ or IL-2, IL-17, and IFN-γ. Numbers indicate the percentage of cells in each quadrant. Data are representative of four independent experiments. B, Cytokine secretion after in vitro stimulation of sorted WT naive (N) CD62L~CD44~, WT memory (M) CD62L~CD44+ or PLZF TG CD4 and CD8 lymphocytes with plate-bound anti-CD3 in the presence or absence of soluble anti-CD28. All data points correspond to n $\geq$ 6. C, FACS analysis of CD4+ T cells from WT, NKT cells from “4get” IL-4 GFP reporter mice (4get NKT), CD4+ T cells from 4get mice (4get CD4), and CD4+ T cells from 4get/PLZF transgenic mice (4get PLZF TG). Percentage of cells in each quadrant is shown. Two independent experiments gave similar results. M, memory; N, naive.

FIGURE 7. Analysis of CFSE dilution after ex vivo stimulation of magnetically purified T cells from WT or PLZF TG mice. Proliferation was analyzed at 72 h after stimulation with different concentrations of plate-bound anti-CD3 in the presence or absence of soluble anti-CD28 Abs. Analysis is shown on electronically gated CD4 and CD8 lymphocytes. Data are representative of three independent experiments.
of CD44hiCD62Llo NKT cells in the thymus of the PLZF-deficient, PLZF transgenic mice compared with PLZF-deficient mice without the transgene (Fig. 8A). The partial reconstitution was more clearly seen in the liver (Fig. 8B). Whereas liver NKT cells in the PLZF-deficient, PLZF transgenic mice acquired a mature CD44hiCD69hi phenotype and accumulated at a significantly higher frequency (Fig. 7C) compared with PLZF-deficient mice without the transgene, they were not reconstituted to the levels seen in WT mice. The partial rescue is likely related to the level of PLZF expression from the transgene. PLZF expression from the transgene is essentially equivalent to WT levels in mature NKT cells in the liver (Fig. 7D). Immature stage 1 WT NKT cells, however, express substantially higher levels than thymocytes from the PLZF-deficient, PLZF transgenic mice. Consistent with a requirement for high levels of PLZF specifically during NKT cell development, mice carrying one WT and one gene-targeted allele of PLZF mice also exhibit a partial reduction in the number of NKT cells (data not shown).

Fyn-deficient mice have a severe block in NKT cell development (6, 7, 42). Some NKT cells, however, mature and accumulate in the liver (Fig. 8E). Introducing the PLZF transgene into Fyn-deficient mice, however, had no effect on the development of NKT cells (Fig. 8E, 8F). The absence of SAP has an even more profound effect on the development of NKT cells. Although a few immature NKT cells can be detected in the thymus, there are none in the liver. Like in the Fyn-deficient mice, the PLZF transgene had no effect on NKT cell development in the absence of SAP (Fig. 8G).

**FIGURE 8.** NKT cells in the (A) thymus and (B) liver from PLZF-deficient mice (PLZF KO) or PLZF-deficient mice carrying the Lck-PLZF transgene (PLZF KO/PLZF TG) were identified with CD1d tetramer and anti-TCRβ Ab. Number indicates the percentage of NKT cells in each tissue. CD44 and CD69 expression on the NKT cells in each tissue is shown in the bottom panels of (A) and (B). The numbers indicate the percentage of cells in each quadrant. C, A statistically significant (p = 0.0029) increase in the percentage of NKT cells was observed in PLZF-deficient mice carrying the PLZF transgene. n = 3 Fyn-deficient, 3 SAP-deficient, 5 Fyn KO x PLZF TG, 5 SAP KO x PLZF TG. D, PLZF levels for WT (dotted line) and PLZF-deficient mice carrying the PLZF transgene (solid line) in stage 1 thymic NKT cells (CD44hi) and liver NKT cells. E, FACS analysis showing the percentage and phenotype of NKT cells in the livers of Fyn-deficient mice and Fyn-deficient mice carrying the PLZF transgene. F, The percentage of NKT cells in the livers of Fyn-deficient (TG−) and Fyn-deficient PLZF transgenic (TG+) mice. n = 4. G, FACS analysis showing the percentage and phenotype of NKT cells in the livers of SAP-deficient mice and SAP-deficient mice carrying the PLZF transgene. Numbers indicate the percentage in each region. ND, not detected.

**Memory/effector functions in PLZF transgenic T cells are induced independently of Fyn and SAP**

Transgenic expression of PLZF failed to rescue NKT cell differentiation in the Fyn- and SAP-deficient mice. Therefore, the activity of PLZF in NKT cells cannot supersede the requirement for signaling via these two molecules. We next asked whether the spontaneous acquisition of effector/memory phenotype and function as a consequence of ectopic expression of PLZF in conventional T cells was also dependent on Fyn and/or SAP signaling pathways as previously shown for other innate-like T cells, such as NKT cells (5–7) and Vγ1Vδ6.3 γδ T cells (20). T cells from the spleens and livers of Fyn-deficient, PLZF-transgenic mice were predominately CD44hiCD62Llo compared with transgene-negative, Fyn-deficient littermates (Fig. 9A). T cells from SAP-deficient, PLZF-transgenic mice also exhibited an activated phenotype compared with PLZF transgene-negative littermates (Fig. 9B).

Transgenic PLZF expression also led to alteration of the cytokine secretion pattern in T cells from these strains. In particular, there was a clear increase in the percentage of IFN-γ expressing CD8 T cells from the PLZF transgenic both in the absence of Fyn (Fig. 9C) and in the absence of SAP (Fig. 9D). There also was a slight increase in the percentage of IL-4 producing CD4 T cells in the absence of these two signaling molecules (Fig. 9C, 9D). Notably, even the small increase in the frequency of IL-4 and IFN-γ double secretors was still observed in both Fyn-deficient and SAP-deficient PLZF transgenic CD4 T cells. These data indicate that, in contrast to NKT cells, PLZF-mediated phenotypic changes of conventional T cells are not dependent on Fyn or SAP.

**Discussion**

The transcription factor PLZF is necessary for the development of NKT cell effector functions and the characteristic activated phenotype of these innate-like T cells (16, 17). Furthermore, ectopic expression of PLZF in conventional T cells has been shown to induce a memory-like phenotype similar to NKT cells (17, 22). Using a transgene to drive T cell-specific ectopic expression of PLZF, we further explore how PLZF controls the development of NKT cells and also the effects of PLZF expression on CD4 and CD8 T cell function.

Consistent with previous reports using different PLZF transgenic models (17, 22), we observed that transgenic expression of PLZF is sufficient to confer an effector/memory phenotype to T cells. We observed that T cells with this activated phenotype accumulated in the spleen and liver, but were almost undetectable in the
thymus. We also found that a large percentage of CD8 T cells from spleens or livers from (CD44 versus CD62L expression of electronically gated CD3+ T cells. As measured by the distribution of the CDR3 lengths of TCR T cells, as indicated strains. The numbers in the graphs indicate the frequency of events within the respective gates. Data are representative of four independent experiments.

Previous reports suggested that PLZF overexpression was sufficient to confer innate functions to T cells (22), providing characteristics that are unique to NKT cells, such as the double production of IFN-γ and IL-4 cytokines (17). In this study, we performed a direct comparison between WT memory T cells and PLZF transgenic T cells. These experiments showed that, overall, the function of T cells overexpressing PLZF was similar to WT T cells, as measured by the distribution of the CDR3 lengths of TCRβ molecules. Furthermore, there was no obvious increase in proliferation of PLZF transgenic T cells in vivo. Acquisition of the effector memory phenotype and functions of PLZF transgenic T cells, therefore, appears to be spontaneous and not the consequence of amplification of certain clones or immune activation.

![Figure 9](image)

**FIGURE 9.** CD44 versus CD62L expression of electronically gated CD3+ T cells from spleens or livers from (A) Fyn-deficient or Fyn-deficient PLZF TG mice and (B) SAP-deficient or SAP-deficient PLZF TG mice. C and D, Intracellular staining for IFN-γ versus IL-4 after 5 h stimulation with PMA and ionomycin in electronically gated CD4 and CD8 T cells from the indicated strains. The numbers in the graphs indicate the frequency of events within the respective gates. Data are representative of four independent experiments.

Recent data have shown that loss of RORγt (49, 50) or the E protein HEB (51) results in a near complete loss of NKT cells. The failure to rearrange the invariant TCR, and development was driven expression of PLZF only partially restored NKT cell development in PLZF-deficient mice. The failure to restore normal NKT cell development was likely due to lower levels of PLZF expression from the transgene compared with the endogenous gene. This finding is consistent with the finding that haploinsufficiency of PLZF results in a significant defect in NKT cell development (E.S. Alonzo and D.B. Sant’Angelo, unpublished observations). Therefore, extremely high levels of PLZF expression at the most immature stage of NKT cell development appears to be necessary to establish the programming required for the innate T cell lineage.

The mechanisms involved in the acquisition of a memory phenotype by PLZF expression in T cells without Ag stimulation are not known. Spontaneous acquisition of memory has been identified in WT CD8 lymphocytes, because memory cells can be detected in germ-free mice. This phenotype might be related to homeostatic proliferation and maintenance of memory features (44). It is possible, therefore, that PLZF expression drives a similar process in NKT cells, however we did not detect increased proliferation of lymphocytes as a result of PLZF expression. Furthermore, NKT cells are not dependent on PLZF to undergo proliferation, as has been shown in PLZF-deficient mice (16, 17). Indeed, PLZF has been reported to inhibit cell cycle progression (45, 46) and to cause cell quiescence (47) and suppression of cell growth (48). Therefore, it is possible that the NKT cell proliferative burst is actually dependent on the rapid downregulation of PLZF that we reported (16).

We and others have shown that residual NKT cells that appear in Fyn- and SAP-deficient mice express PLZF (16, 17). This finding suggests that PLZF expression is not sufficient to drive the full differentiation of NKT cells. To more directly test this important aspect of NKT cell development, we introduced a T cell-specific PLZF transgene into both Fyn- and SAP-deficient mice. Data from these mice clearly showed that expression of PLZF did not rescue the development of NKT cells in the absence of Fyn- or SAP-mediated signaling.

In contrast, in the absence of Fyn or SAP, PLZF transgenic T cells still acquired an activated/memory phenotype as seen by the upregulation of CD44 and the loss of CD62L. Furthermore, the transgenic cells retained the increased capacity to produce cytokines upon primary activation. Therefore, the effects of ectopic expression of PLZF on conventional T cells was independent of signalling mediated by SAP and Fyn. These data indicate that the function of PLZF is independent of SAP and Fyn signals.

Recent data have shown that loss of RORγt (49, 50) or the E protein HEB (51) results in a near complete loss of NKT cells. The loss of NKT cells in these mutant mice, however, was due to a failure to rearrange the invariant TCR, and development was fully restored by introducing a Vα14 TCR transgene (50, 51).
is also dispensable for the development of NKT cells (51); this was surprising, considering the role that Id3 and E2A appear to play in the development of PLZF-expressing γδ T cells (20, 52). High TCR avidity for self ligands has long been proposed as a critical factor for the development of NKT cells (53). Consistent with this hypothesis was the finding that ITK-deficient and SLP76γδ145F mutant mice, which have reduced TCR signaling capacities, have significant defects in NKT cell development (54, 55). Furthermore, PMA plus ionomycin stimulation of DP thymocytes followed by 5 d in culture with OP9-dl1 cells was shown to induce PLZF in γδ T cells (18). Both ITK-deficient and SLP76γδ145F mutant mice, however, actually have a massive increase of PLZF-expressing γδ T cells (19, 20). It is therefore difficult to reconcile a strength of TCR signaling model with the induction of PLZF. Combined with our new data showing that PLZF functions independently of Fyn and SAP, it seems likely that the factors that control the cell-specific expression of PLZF may require the identification of novel signaling pathways that are potentially unique to innate T cells.

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Disclosures

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References


