HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2011 March 09.

Published in final edited form as:

Nat Immunol. 2010 August ; 11(8): 709–716. doi:10.1038/ni.1898.

PLZF$^+$ T cells regulate memory-like CD8$^+$ T cell development

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Abstract

Several gene deficiency models promote the development of "innate CD8$^+$ T cells" that have diverse TCRs, but display a memory phenotype and rapidly produce cytokines. We here demonstrate that similar cells develop in Kruppel-Like Factor 2 (KLF2) deficient mice. However, this is not due to intrinsic deficiency in KLF2, but rather to interleukin 4 (IL-4) produced by an expanded population of T cells expressing the PLZF transcription factor. The development of innate CD8$^+$ T cells in ITK and CBP transcription factor deficient mice is also attributable to this IL-4-dependent mechanism. Finally, we show that the same mechanism drives innate CD8$^+$ T cell differentiation in BALB/c mice. These findings reveal a novel mechanism of regulation of CD8$^+$ T cells via PLZF$^+$ T cell production of IL-4.

T cells expressing a diverse repertoire of T cell receptor (TCR) specificities develop in the thymus. Mature, naïve αβ T cells migrate from the thymus and through secondary lymphoid organs. During infection, pathogen specific T cells clonally expand, differentiate into effector cells, and migrate to infected tissues to clear the pathogen. After clearance, T cells contract, leaving a memory population that is again quiescent but differs from naïve T cells in phenotype (usually expressing higher levels of the hyaluronic acid receptor CD44 and cytokine receptors such as CD122) and having increased function when re-stimulated such as more rapid proliferation and production of cytokines such as interferon-γ (IFN-γ)[1].

Within the T cell population, there are other subsets of T cells that develop unique function and migration during development prior to infection. These subsets include regulatory T (T_{reg}) cells, natural killer T (NKT) cells, and mucosal associated invariant T (MAIT). Such cells can rapidly produce cytokines upon stimulation[2],[3] and have multiple and sometimes conflicting roles in protection from infection versus pathogenesis resulting from autoimmunity[4]. PLZF (promyelocytic leukemia zinc finger, Zbtb16) was recently identified as a key transcription factor involved in the development of NKT cells[5],[6]. Yet it remains unclear if and how such PLZF$^+$ populations affect the homeostasis of conventional T cells.

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AUTHOR CONTRIBUTIONS

M.A.W. designed and performed experiments, analyzed data, and wrote the manuscript. O.A.O. did analysis that inspired the direction of this research and provided input on interpretation. S.C.J. provided input to the research design and interpretation. K.A.H. conceptualized the research, directed the study, analyzed data, and edited the manuscript.

It has been proposed that the transcription factor Kruppel-Like Factor 2 (KLF2) might regulate multiple characteristics that differentiate T cell subsets. In mice with hematopoietic KLF2 deficiency, T cell development is normal, but the number of T cells present in peripheral organs is profoundly reduced[7]. This can be explained by the requirement of KLF2 for the expression of the sphingosine phospate receptor 1 ($S1P_1$) in T cells[8], as $S1P_1$ is necessary for T cell egress from the thymus and lymph nodes[9]. In addition KLF2 regulates naïve T cell migration by binding to the L-selectin promoter and inducing its expression[8],[10]. KLF2 deficient T cells express many inflammatory-type chemokine receptors, such as CXCR3[11]. This led to the idea that KLF2 repressed these chemokine receptors and in the absence of KLF2 T cells expressed these receptors and migrated to non-lymphoid tissues.

Using a *Cd4*-cre-*Klf2*$^{fl/fl}$ model it was recently shown that KLF2 does not repress CXCR3 transcription, but rather the upregulation CXCR3 was a cell-extrinsic effect[12]. Using unequal mixed bone marrow chimeras we found that when KLF2 deficient cells were the majority, both KLF2 deficient and wild-type (WT) T cells expressed CXCR3. However, in chimeras where WT cells were the majority, neither KLF2 deficient nor WT cells expressed CXCR3. T cells deficient in IL-4 receptor α (IL-4Rα), or the transcription factor eomesodermin (Eomes), did not upregulate CXCR3 in the majority KLF2 deficient environment. Thus, T cell KLF2 deficiency leads to a cell-intrinsic overproduction of IL-4, which causes a cell-extrinsic upregulation of CXCR3 on WT bystander cells[12].

The current study examines further the bystander effects caused by KLF2 deficiency and determine how KLF2 regulates IL-4 expression. We report that bystander $CD8^+$ T cells take on a memory-like phenotype ($CD44^{hi}$, $CD122^{hi}$) and have the ability to rapidly produce cytokines, much like the “innate $CD8^+$ T cells” generated in mice lacking IL-2-Inducible T-cell kinase (ITK) or CREB Binding Protein (CBP). We found that the IL-4 overproduction was a result of an expansion of cells expressing the transcription factor PLZF, including NKT and γδ NKT cells. When KLF2 deficient mice were also deficient in PLZF, $CD8^+$ T cells did not exhibit memory-like characteristics. We also found that a similar mechanism drives the $CD8^+$ phenotype in ITK and CBP deficient mice. Lastly, we found that in normal BALB/c mice the memory phenotype of $CD8^+$ T cells is dependent on IL-4 and the $PLZF^+$ population. Our findings support a model wherein $PLZF^+$ T cells, via IL-4 production, regulate the size of the memory phenotype $CD8^+$ T cell pool.

RESULTS

Characteristics of bystander $CD8^+$ T cells

It was previously reported that *Cd4*-cre-*Klf2*$^{fl/fl}$ thymocytes show increased expression of the chemokine receptor CXCR3. This upregulation was shown to be a cell-extrinsic effect through the use of unequal mixed bone marrow chimeras where *Cd4*-cre-*Klf2*$^{fl/fl}$ cells were the majority[12]. The unequal ratio in such chimeras creates an environment that is composed largely of gene deficient cells, yet allows one to determine if wild-type (WT) “bystander” cells sense a change in that environment. In *Klf2*$^{fl/fl}$ unequal mixed chimeras, bystander WT $CD8^+$ single positive (SP) thymocytes upregulated CXCR3. This effect was dependent on the IL-4Rα and the transcription factor Eomes[12], suggesting that KLF2 deficiency causes overproduction of the soluble factor IL-4, which upregulates CXCR3 through Eomes even in

WT bystander cells. Bystander effects were observed on CD4 T cells but to a lesser extent, so we focused on $CD8^+$ T cells in this report.

We sought to more completely characterize these cell-extrinsic effects using a similar mixed bone marrow chimera approach (Fig. 1a). Increased expression of CXCR3 is found in activated and memory T cells[13]. Thus we examined bystander $CD8^+$ T cells for other memory markers, and found that WT $CD8^+$ bystander cells in *Cd4*-cre-*Klf2*$^{fl/fl}$ mixed chimeras showed increased expression of CD44, CD122, and IL-4Rα, while β7 integrin was decreased. (Fig. 1b) However, the cells did not show increased CD69 or CD25, markers of recent activation (data not shown). Eomes and T-bet are Tbox transcription factors with overlapping but distinct roles in memory $CD8^+$ formation and function[14,15]. While most memory $CD8^+$ T cells express both Eomes and T-bet, bystander $CD8^+$ T cells express high levels of Eomes but not T-bet mRNA[12] and protein (Fig. 1c). In addition, we found that CD24 (heat stable antigen, HSA) expression was decreased on WT bystander $CD8^+$ SPs mixed with a majority of *Cd4*-cre-*Klf2*$^{fl/fl}$ compared to $CD8^+$ T cells from an all WT chimera (Fig. 1b). CD24 expression decreases progressively after positive selection, thus lower expression indicates more mature SP cells. A partial defect in thymocyte emigration is consistent with findings in transgenic mice that overexpress IL-4 in the thymus[16].

Next, to determine if bystander activated $CD8^+$ T cells functioned like memory cells, we stimulated thymocytes from mixed bone marrow chimeras *ex vivo* with phorbol myristate acetate (PMA) and ionomycin, then measured cytokine production by intracellular staining. A greater percentage of bystander $CD8^+$ T cells from a KLF2 deficient environment produced IFN-γ compared to $CD8^+$ SPs from an all WT thymus (Fig. 1d). Thus, we conclude that the KLF2 deficient thymic environment leads to the development of bystander $CD8^+$ SP that share functional and phenotypic characteristics of memory T cells. This phenotype included CD44 and CD122 expression and rapid cytokine production, but bystander $CD8^+$ T cells differed from memory $CD8^+$ T cells in that they expressed Eomes but not T-bet.

Increased function of bystander $CD8^+$ T cells

Since the *Cd4*-cre-*Klf2*$^{fl/fl}$ environment generates $CD8^+$ T cells that resemble memory cells, we asked if the bystander $CD8^+$ T cells had an increased functional capacity. One characteristic of antigen-specific memory $CD8^+$ T cells is their ability to make a more rapid secondary response. We generated chimeras with OT-I-Rag1$^{-/-}$ TCR transgenic T cells, which are major histocompatability (MHC) Class I-restricted and ovalbumin (OVA)-specific TCR transgenic, as the bystander cells. Since the KLF2 deficient mouse is lymphopenic, we used $S1P_1$ deficient bone marrow as a control because these mice are lymphopenic as well, but do not display the IL-4 dependent bystander effects (reference [9] and Supplementary Fig. 1). Fig. 2a diagrams the bone marrow chimera set-up, where OT-I and WT cells were mixed with the indicated majority population.

Bystander OT-I thymocytes had a similar memory-like phenotype to WT bystander cells when *Cd4*-cre-*Klf2*$^{fl/fl}$ cells were the majority (Supplementary Fig. 1) even though no ovalbumin antigen was ever present. The effects on OT-I bystander T cells and the low level of CD49d (α4 integrin) (data not shown) suggest that this is not an antigen-driven process[17].

While not as dramatically as in the thymus (Supplementary Fig. 1), it was apparent that splenic and lymph node derived OT-I cells from the KLF2 deficient environment were also affected and showed an increased percentage of CD44^{hi}, CD122^{hi} phenotype cells (Fig. 2b).

To test the bystander effects *in vivo*, we cotransferred OT-I cells from the spleen and lymph node of the chimeras with an equal number of naïve, CD44^{lo} OT-I T cells. We then infected the mice with an attenuated strain of *Listeria monocytogenes* expressing the ovalbumin protein. Bystander OT-I T cells from the KLF2-deficient environment outcompeted the competitor population of CD44^{lo} OT-I T cells to a greater extent than OT-I cells from the WT or S1P$_1$ deficient environment. This was apparent by the relative percentage in the blood and cell number in the spleen (Fig. 2c).

Memory CD8$^+$ T cells have been shown to produce IFN-γ antigen-independently as a result of inflammatory cytokines[17]. To test if bystander OT-I T cells also have this ability, we stimulated splenocytes with IL-2, 12, and 18 *in vitro* in the absence of TCR stimulation and measured IFN-γ production. A significantly greater proportion of peripheral OT-I T cells from the KLF2-deficient environment produced IFN-γ compared to those from WT chimeras (Fig. 2d). TCR-dependent cytokine production also appeared increased. Similar to our findings with thymocytes (Fig. 1d), we found that more spleen and lymph node polyclonal bystander CD8$^+$ T cells stimulated with PMA-ionomycin produced IFN-γ (data not shown). A similar trend was observed with OT-I T cells stimulated with OVA peptide (Fig. 2d). Together these results show that the KLF2-deficient environment leads to the expansion of a subset of CD8$^+$ T cells with increased function.

Bystander phenotype dependent on PLZF$^+$ T cells

Cd4-cre-*Klf2*$^{fl/fl}$ mice have increased production of IL-4 and the CD8$^+$ bystander phenotype is dependent on type I IL-4 receptor signaling[12]. We next sought to determine the cellular source of this IL-4. In the *Cd4*-cre-*Klf2*$^{fl/fl}$ model KLF2 deficiency is limited to T cells so T cells likely produce the excess IL-4. NKT cells are a subset of T cells that are known to rapidly produce cytokines, including IL-4. PLZF (promyelocytic leukemia zinc finger) is a transcription factor necessary for NKT development and function[5,6]. We found a dramatic increase in *Plzf* mRNA expression in the *Cd4*-cre-*Klf2*$^{fl/fl}$ thymus (Fig. 3a). PLZF was not increased on all thymocytes but rather an increased percentage of thymocytes expressed PLZF (Fig. 3b). In mixed bone marrow chimeras, *Plzf* expression was increased on *Cd4*-cre-*Klf2*$^{fl/fl}$ cells but not WT cells regardless of the chimera ratio (Fig. 3c). This demonstrates that PLZF$^+$ cell expansion is a cell intrinsic effect of KLF2 deficiency in T cells.

In normal mice, the PLZF$^+$ population is highly restricted in terms of TCR usage, being comprised primarily by invariant NKT cells (*i*NKT), recognized by their binding to CD1d-tetramers loaded with the glycolipid PBS-57, and γδ NKT cells[18,19]. Within the PLZF$^+$ populations, the proportion of these subsets is similar comparing WT and *Cd4*-cre-*Klf2*$^{fl/fl}$ (Table 1 **and** Supplementary Fig. 2a). KLF2 deficiency appears to expand the entire PLZF$^+$ population without a dramatic change in their composition. To better understand the mechanism of PLZF expansion with KLF2 deficiency, we crossed TCR transgenic mice that normally do not generate PLZF$^+$ cells to *Cd4*-cre-*Klf2*$^{fl/fl}$. KLF2 deficiency did not lead to PLZF expression in either CD4 TCR Tg (SMARTA) or CD8$^+$ TCR Tg (OT-I) T cells

(Supplementary Fig. 2b). These findings together with KLF2 expression being limited to the mature SP stage of thymocyte development[12] are consistent with KLF2 deficiency not affecting the selection of $PLZF^+$ cells but rather causing preferential survival or expansion of the normally selected $PLZF^+$ population.

To test if the expanded $PLZF^+$ population in *Cd4*-cre-*Klf2*$^{fl/fl}$ thymus are functional and produce IL-4, we stimulated thymocytes with PMA-ionomycin and measured IL-4 production by intracellular staining. We observed that *Cd4*-cre-*Klf2*$^{fl/fl}$ $PLZF^+$ cells did produce IL-4 and the majority of IL-4 production was in fact from $PLZF^+$ cells (Fig. 4a). Within the $PLZF^+$ subset, both $\gamma\delta^+$ and $CD1^d$ tetramer binding cells produced IL-4 (data not shown).

Since the bystander $CD8^+$ phenotype is dependent on IL-4, and $PLZF^+$ cells produce IL-4, we hypothesized that the $CD8^+$ phenotype is dependent on the expanded $PLZF^+$ population. To test this hypothesis, we bred *Cd4*-cre-*Klf2*$^{fl/fl}$ mice with mice with the luxoid mutation in PLZF (*Plzf*$^{lu/lu}$). The luxoid mutation was a spontaneously-arising nonsense mutation that results in a truncated PLZF protein that lacks the DNA-binding region and all zinc finger domains[20]. *Plzf*$^{lu/lu}$ and *Plzf*$^{-/-}$ mice have a similar phenotype and NKT defect[5,6]. *Cd4*-cre-*Klf2*$^{fl/fl}$-*Plzf*$^{lu/lu}$ have no detectable PLZF protein expression (data not shown). While *Cd4*-cre-*Klf2*$^{fl/fl}$ $CD8^+$ T cells had dysregulated IL-4Rα, CXCR3, CD44, CD122, and β_7 integrin, the additional deficiency of PLZF in *Cd4*-cre-*Klf2*$^{fl/fl}$-*Plzf*$^{lu/lu}$ mice restored to WT levels these bystander-affected molecules (Fig. 4c). The genetic removal of PLZF led to the loss of the memory-like $CD8^+$ phenotype, supporting the bystander effects being dependent on $PLZF^+$ cells.

We did not detect PLZF in the WT or *Cd4*-cre-*Klf2*$^{fl/fl}$ $CD8^+$ SP population (Supplementary Fig. 3a). In addition, WT bystander cells were not affected in a chimera where the majority of transferred BM cell were *Cd4*-cre-*Klf2*$^{fl/fl}$-*Plzf*$^{lu/lu}$ (Supplementary Fig. 3b). This indicates that PLZF is important for the induction but not the response to the bystander effects.

Evidence to date supports KLF2 controlling T cell migration by directly regulating the cell surface receptors $S1P_1$ and CD62L in T cells[8,10]. Both *Cd4*-cre-*Klf2*$^{fl/fl}$ and *Cd4*-cre-*Klf2*$^{fl/fl}$-*Plzf*$^{lu/lu}$ have an increased proportion of mature, $CD24^{lo}$ $CD8^+$ SP (Fig. 4c). This phenotype and high CD69 expression are consistent with KLF2 deficiency leading to a lack of $S1P_1$ and a thymic emigration defect[9]. In support of direct regulation, CD62L expression was lower and CD69 expression remained high on KLF2-deficient cells regardless of PLZF expression (data not shown). Thus, KLF2 has a cell intrinsic role in the regulation of CD62L, $S1P_1$, and PLZF and the bystander effects observed with KLF2 deficiency are dependent on the expansion of $PLZF^+$ cells.

$CD8^+$ T cell memory-like differentiation in other models

We were interested if these effects occur in other gene deficiency models. ITK is a Tec family kinase with a role in TCR signaling[21]. ITK-deficient $CD8^+$ SP thymocytes have been reported to have altered development, characterized by high CD44, CD122 and Eomes expression and low expression of CD24[22,23]. Such mice were recently reported to have an

expanded PLZF$^+$ γδ NKT population and showed signs of IL-4 overproduction *in vivo*, including hyper IgE [19,24]. Thus it seemed possible that the innate CD8$^+$ T cell phenotype was a bystander effect of IL-4 production and not an intrinsic effect of ITK gene deficiency. To test for extrinsic or intrinsic effects, we generated mixed bone marrow chimeras with *Itk*$^{-/-}$ and WT cells as diagrammed in Fig. 1a. In chimeras where *Itk*$^{-/-}$ cells were the majority, we observed a memory-like phenotype on WT bystander cells: increased IL-4Rα, CXCR3, CD44, CD122, and Eomes expression (Fig. 5a,b). In contrast, *Itk*$^{-/-}$ CD8$^+$ SPs that developed in a WT thymic environment had a normal phenotype (data not shown). These findings show that the CD8$^+$ changes are a cell-extrinsic effect of ITK deficiency.

In intact animals and mixed bone marrow chimeras *Itk*$^{-/-}$ thymocytes, like KLF2-deficient cells, had a statistically significant cell intrinsic increase in the proportion of PLZF$^+$ T cells (Table 1 and Fig. 5c). However, PLZF$^+$ population in *Itk*$^{-/-}$ mice differ from both WT and KLF2-deficient mice in that γδ NKTs are overrepresented (Table 1). This is consistent with reports that ITK deficiency results in impaired *i*NKT cell maturation and function[25,26] and enhanced γδ NKT cell development [19,24].

We wanted to determine if IL-4 production by PLZF$^+$ cells in *Itk*$^{-/-}$ mice causes an increase in memory-like CD8$^+$ T cells, as it does in KLF2-deficient mice. We bred *Itk*$^{-/-}$-*Il4ra*$^{-/-}$ and *Itk*$^{-/-}$-*Plzf*$^{Lu/Lu}$ double-deficient mice. This resulted in ITK-deficient T cells that could either not respond to IL-4 or lacked PLZF. CD8$^+$ T cells from both *Itk*$^{-/-}$-*Il4ra*$^{-/-}$ and *Itk*$^{-/-}$-*Plzf*$^{Lu/Lu}$ mice had normal expression of CXCR3, CD44, CD122, and Eomes (Fig. 6). This implies that the memory-like or innate CD8$^+$ phenotype is completely dependent on IL-4 produced by PLZF$^+$ T cells and not a result of altered thymic selection of ITK deficient cells as was originally inferred.

Recently, T cell deficiency in the histone acetyltransferase, CBP (gene name *Crebbp*), has been reported to have a similar CD8$^+$ phenotype as the ITK deficient mouse[27]. We again generated mixed bone marrow chimeras where Lck-cre-*Crebbp*$^{fl/fl}$ was the majority with WT bystander cells, to test for cell-extrinsic effects in this model. When CBP-deficient cells were the majority, we observed that both the Lck-cre-*Crebbp*$^{fl/fl}$ and WT bystander cells upregulate IL-4Rα, CXCR3, CD44, CD122 and Eomes (Supplementary Fig. 4). This phenotype was not apparent on either population when WT cells were the majority. These data are consistent with CBP deficiency leading to increased thymic IL-4 resulting in cell-extrinsic effects on CD8$^+$ SPs. In summary, we have shown that the generation of memory-like CD8$^+$ T cells occur by similar cell-extrinsic means in three apparently unrelated genetic mouse models.

A similar mechanism occurs in normal mice

While the PLZF-IL-4 mechanism for memory-like CD8$^+$ production is important in understanding the actual role of specific genes in deficiency models, we wanted to investigate if this mechanism might also play a role in the generation of memory-like cells in WT mice. It has been reported that inbred mouse strains vary in their number of αβ and γδ NKT cells, with BALB/c mice being high, and B6 being low[28-30]. Thus we investigated the phenotype of CD8$^+$ SP thymocytes in these two strains of mice. As expected we observed that PLZF$^+$ cells made up a larger percentage of thymocytes in BALB/c mice compared to

B6 (Fig. 7a). Interestingly, a higher percentage of BALB/c CD8$^+$ SP were memory phenotype (CD44^{hi}CD122^{hi}) compared to age-matched B6 (Fig. 7b). We also observed, in BALB/c mice, that the memory phenotype CD8$^+$ SP expressed high levels of Eomes but not T-bet (Fig. 7c). This is consistent with PLZF-IL-4 dependent memory-like CD8$^+$ T cell generation in BALB/c mice.

To test if BALB/c memory-like CD8$^+$ T cells were IL-4 dependent, we analyzed IL-4Rα deficient (*Il4rα*$^{-/-}$) mice on the BALB/c background. The percentage of CD44^{hi}, CD122^{hi} CD8$^+$ SP in the *Il4rα*$^{-/-}$ BALB/c thymus was lower than WT BALB/c (Fig. 7b). Strikingly, Eomes expression in *Il4rα*$^{-/-}$ BALB/c mice was reduced to levels comparable to WT B6 mice (Fig. 7c). We did not detect a difference between memory phenotype CD8$^+$s in WT and *Il4rα*$^{-/-}$ B6 (data not shown), likely because of the low percentage in the WT B6 thymus.

CD1d-restricted NKT cells are by far the most abundant thymic PLZF$^+$ population in BALB/c mice (Table 1). Thus to study the requirement for PLZF$^+$ T cells for the generation of memory-like CD8$^+$s in BALB/c mice, we analyzed *Cd1d*$^{-/-}$ BALB/c mice. As expected CD1d deficiency dramatically reduced the percentage of PLZF$^+$ T cells in the thymus (Fig. 7d). In the *Cd1d*$^{-/-}$ BALB/c thymus, we also found a decreased percentage of memory-like CD8$^+$ T cells (Fig. 7e,f). Consistent with our findings in the thymus, we observed a reduction memory-like CD8$^+$ T cells in the spleen of *Il4rα*$^{-/-}$ and *Cd1d*$^{-/-}$ BALB/c (Supplementary Fig. 5). These findings show that in WT mice there is an IL-4 and PLZF dependent memory phenotype CD8$^+$ T cell population. Thus, the novel mechanism for generation of memory-like CD8$^+$ T cells that we describe above in genetically deficient animals also occurs in WT animals.

DISCUSSION

Our findings support a model where IL-4 derived from PLZF$^+$ cells regulates the pool of memory-like CD8$^+$ T cells. Such cells are readily apparent in KLF2, ITK, and CBP gene deficiency models. They have also been observed in Id3-deficient mice, where they arise from a similar non-intrinsic mechanism (Barbara Kee, personal communication). The phenotype of ITK-deficient CD8$^+$ T cells, in particular, has been studied extensively [21,31]. Because of ITK's involvement in TCR signaling, it was proposed that ITK is necessary for thymic selection of conventional CD8$^+$ T cells. Thus in the absence of ITK, CD8$^+$ T cells were thought to be diverted into an innate-immune lineage. We show here that this CD8$^+$ phenotype is actually a cell extrinsic effect dependent on IL-4. Thus ITK, IL-4Rα and ITK, PLZF double-deficient mice will be a useful tool to study the direct effects of ITK deficiency on thymic selection.

We propose that the origin of the memory-like CD8$^+$ T cell phenotype in these models is the expanded PLZF$^+$ population. Mice lacking Id3 also have an expanded PLZF$^+$ population[32-35] similar to mice lacking KLF2, ITK, and CBP. Are the mechanism(s) resulting in PLZF expansion shared or distinct in each model? In ITK- and Id3-deficient mice, the PLZF$^+$ population is skewed toward increased γδ NKT cells[19,24]. ITK's positive role in TCR signaling has led to the proposition that γδ progenitors that lack ITK avoid

negative selection because of decreased signaling and instead become PLZF$^+$ γδ NKTs. CBP deficiency might lead to the same process since CBP-deficient T cells have defects in ITK dependent genes following TCR stimulation[27]. One way that ITK deficiency and KLF2 deficiency could be linked is if ITK deficiency led to decreased KLF2 expression in PLZF$^+$ cells. However when *Itk*$^{-/-}$ mice were crossed to KLF2-GFP reporter mice[12] no difference in KLF2 expression was observed in any PLZF$^+$ population (data not shown).

We believe the expansion of PLZF$^+$ with KLF2 deficiency is unlikely to be due to altered selection. KLF2 does not drastically alter the repertoire of PLZF$^+$ cells and KLF2 deficiency does not expand PLZF$^+$ cells when the TCR is fixed. Also, the expansion of PLZF$^+$ cells and subsequent bystander CD8$^+$ effects with KLF2 deficiency does not seem to be caused solely by altered trafficking. S1P$_1$ deficiency causes thymic retention of NKT cells, the major PLZF$^+$ population[36], yet we did not observe bystander effects with S1P$_1$ deficiency. We favor a model where KLF2 deficiency causes increased expansion or survival of PLZF$^+$ cells after selection.

PLZF deficiency leads to an overall reduction of NKT cells, yet it is interesting to consider that the CD1d tetramer-binding NKT that remain in PLZF-deficient mice express higher levels of CD62L and preferentially localize to lymph nodes instead of spleen and liver[5,6]. This phenotype is reminiscent of KLF2-expressing naïve T cells, and might suggest a mutual antagonism between PLZF and KLF2 in an NKT precursor. When we bred PLZF$^{Lu/Lu}$ mice to the KLF2-GFP reporter mouse[12] we found that PLZF-deficient NKT cells did indeed express higher levels of KLF2 (data not shown). In addition PLZF transgenic overexpression led to lower CD62L expression on T cells[6,37]. Further studies into the interactions between KLF2 and PLZF should be helpful in understanding the dichotomy between conventional and NKT cells.

NKT cells have diverse functions from autoimmunity to responses to pathogens and tumors[4]. The potential of NKT cells in therapeutics is also actively being investigated[38]. We report a novel role for NKTs cells in the development of a memory-like CD8$^+$ T cell population. We find evidence for this mechanism in both genetic deficiency models and "normal" inbred mouse strains. Variations in the number of CD1d-restricted NKTs and γδ NKT have been reported in inbred strains of mice[28-30]. In the BALB/c compared to B6 mice, we found an increase in the proportion of all PLZF-expressing T cells. Importantly, we show that this correlated with an IL-4 dependent, memory-like CD8$^+$ population in BALB/c mice. This effect is consistent with other studies showing that IL-4[39,40], including IL-4 from activated NKT[41], can directly promote CD8$^+$ T cell proliferation and differentiation. We were also able to show that CD1d deficient BALB/c mice had a decrease in memory-like CD8$^+$s. Thus, studying mice that are genetically deficient in NKTs might be complicated by the fact that those mice could also have an altered memory CD8$^+$ T cell population.

Since differences exist between inbred mouse strains, it seems likely that there could be variations in the PLZF$^+$ pool size within the genetically diverse human population. Indeed, humans might have more PLZF$^+$ cells because of a species-specific difference in how CD4 T cells are selected in the thymus. MHC class II is expressed on human but not mouse

thymocytes. Transgenic expression of MHC class II on mouse thymocytes allows selection of a unique T cell subset that shares many characteristics with NKT cells, including PLZF expression[42]. Recent evidence suggests that MHC class II thymocyte-thymocyte selection does lead to the generation of PLZF$^+$ cells with diverse TCR specificity in the human thymus[43], and humans have a higher proportion of PLZF$^+$ peripheral T cells than mice (Derek Sant'Angelo, personal communication). Thus it will be interesting to determine to what extent PLZF-derived IL-4 shapes the human memory CD8$^+$ T cell pool.

While we are not aware of any cases of human deficiency of IL-4 or PLZF, there has recently been a report of homozygous missense mutation in the *ITK* gene in a pair of sisters[44]. Both girls died from an Epstein-Barr Virus-associated lymphoproliferative disorder. Of interest to the current work, Eomes was upregulated in CD8$^+$ T cells of both patients. While this finding could certainly be complicated by the pathological situation in the patients, it is consistent with memory-like CD8$^+$ T cell formation in ITK-deficient mice.

The final consequence of the expanded PLZF$^+$ population and increased IL-4 is more memory-like CD8$^+$ T cells. Using bystander-effected OT-I transgenic T cells, we were able to demonstrate that such cells can subsequently promote both antigen-specific and non antigen-specific responses. Thus, the differentiation of memory-like CD8$^+$ T cells by IL-4 might contribute to more potent innate and adaptive immune responses.

One question that arises from these findings is why would IL-4 production by PLZF$^+$ T cells be coupled to memory-like CD8$^+$ T cells capable of making IFN-γ? One possibility is the need for multiple cell types to participate in innate immune production of IFN-γ. Thus, by linking differentiation of memory-like T cells to the rapid cytokine-producing PLZF$^+$ T cells ample IFN-γ-producing cells are generated. Innate IFN-γ production by memory CD8$^+$ T cells has been demonstrated in multiple infection models and have been reported to have an increased innate protective ability compared to NK cells[45-48]. PLZF-dependent, memory-like CD8$^+$ T cells might be particularly important neonatally before the immune system matures and infections have generated pathogen-specific memory.

METHODS

Mice

C57BL/6 (B6) and B6.SJL (CD45.1 congenic B6) mice were purchased from the National Cancer Institute; BALB/c, BALB/c *Il4rα*$^{-/-}$, and BALB/c *Cd1d*$^{-/-}$ mice from Jackson Labs. B6 *Il4rα*$^{-/-}$ mice were obtained from Fred Finkelman (University of Cincinnati). Cd4-cre and VavCre mice were obtained from Taconic farms and Dimitri Kioussis (National Institute for Medical Research, London), respectively. *Klf2*$^{fl/fl}$ mice were generated by Jerry Lingrel's laboratory at the University of Cincinnati and have been described previously[12]. *S1pr1*$^{fl/fl}$ mice were obtained from Richard Proia (National Institutes of Health NIDDK, Maryland). *Itk*$^{-/-}$ mice[23] were obtained from Yoji Shimizu (University of Minnesota). PLZF$^{Lu/Lu}$ mice were obtained from Robert Braun (Jackson Laboratory). LCMV-GP$_{61–80}$-specific SMARTA TCR Tg mice were obtained from David Masopust (University of Minnesota). All animal experimentation was conducted according to IACUC guidelines at the University of Minnesota.

Mixed bone marrow chimera

For chimeras with Lckcre-*Cbp*$^{fl/fl}$ bone marrow, femurs and tibias were shipped overnight on wet ice in RPMI $^{+}$ 10% FCS from Paul Brindle's laboratory (St. Jude Children's Research Hospital). Bone marrow was T cell depleted then experimental (CD45.2) cells were mixed with WT (CD45.1x2) and injecting I.V. into lethally irradiated B6.SJL (CD45.1) hosts. Chimeras were killed and analyzed 8-18 weeks post transplant.

Flow cytometry

Single cell suspensions from tissues were prepared. Biotinylated CD1^{d}-αGalCer monomers were obtained from the NIH tetramer facility. PLZF staining was done as previously described[6]. In brief, surface antibody staining was done then cells were fixed and using the FoxP3 buffer set (eBioscience). Permeabilized cells were incubated with anti-PLZF (D-9; Santa Cruz) followed by anti-mouse IgG_1 (A85-1; BD Biosciences) and anti-IL-4 (BVD6-24G2, eBioscience) when appropriate. Eomesodermin-APC and T-bet-PE (eBioscience) staining was done for 1 hour using the FoxP3 buffer set. CXCR3-PE antibody was purchased from R&D and used at a 1:50 dilution. H-2K^{b}–OVA tetramer was produced in house. All other antibodies were obtained commercially from eBioscience, BD or Biolegend and used at 1:200 dilutions and incubated for 30 minutes refrigerated. Cells were analyzed on Becton Dickinson a LSR II instrument and the data was processed using FlowJo (Tree Star) software.

Listeria monocytogenes infection

Spleen and lymph node cells were harvested from chimeras. Flow cytometry was used to assess the percentage of OT-I cells in each chimera. Naïve, CD44 low OT-I/Rag1$^{-/-}$ were purified using negative selection with the Macs magnetic bead system (Miltenyi). The equivalent of 3×10^5 OT-I cells were was mixed with an equal number of CD44 low OT-I and adoptively transferred to a CD45.1 host. The next day cells the mice were infected with 3×10^6 of an attenuated, ActA$^{-/-}$ strain of Listeria monocytogene expressing the ovalbumin protein. The ratio of OT-I cells derived from the chimeras compared to the naïve OT-Is was assessed by flow cytometry in the blood and spleen.

In vitro cytokine production

For PMA and ionomycin stimulation: cells were isolated and plated at 10^6/ml in RPMI $^{+}$ 10% FCS. Cells were incubated for five hours with 50 ng/ml PMA and 1.5 μM ionomycin (Sigma-Aldrich) with GolgiStop (BD Pharmingen) for the final three. For innate cytokine stimulation: 1.5×10^6 cells/mL were incubated with 5×10^2 U/mL human IL-2, 10 ng/mL murine IL-12, 10 ng/mL murine IL-18 for 24 hours with GolgiPlug (BD Pharmingen) added for the final 4 hours. For peptide stimulation splenocytes were stimulated for 4–5 h at 37 °C with OVA peptide (250 nM) of amino acids 257–264 (SIINFEKL) and GolgiPlug (BD Pharmingen). Cells were surface stained then intracellular staining was done with the BD fix/perm kit (BD Pharmingen) unless done concurrently with PLZF analysis described above.

Cell Sorting and Real-time RT-PCR

Fluorescence-activated cell sorting (FACS) was used to purify, CD25, NK1.1, TCRγδ, CD1d-tet negative CD4 SP. Each group was sorted in at least three independent experiments. Sorting was performed on a FACSAria (Becton Dickinson) and was reliably >90% of target population. The RNeasy kit, Qiagen (Valencia, CA) and the SuperScriptIII Platinum Two-Step qRT-PCR kit, Invitrogen (Carlsbad, CA) were used to isolate RNA and produce cDNA. Fast Start SYBR Green Master mix from Roche (Basel, Switzerland) and a SmartCycler, Cepheid (Sunnyvale, CA) were used for amplification and detection. HPRT was used to normalize samples. Primers: HPRT: CTTCCTCCTCAGACCGCTTT & ACCTGGTTCATCATCGCTAA, PLZF: GAGCAGTGCAGCGTGTGT & AACCGTTTTCCGCAGAGTT.

Statistical analysis

Statistical analysis was performed using Prism (Graphpad software). Unpaired, two-tailed t-tests were used for data analysis and generation of P values, with the exception of Fig. 3a where a paired t-test was used. Error bars indicate standard deviation (s.d.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors thank XiaoJie Ding, Jason Vevea, Juan Reyes and Carolina Mora-Solano for excellent technical assistance. We are grateful to Fred Finkelman, Leslie Berg, Pam Schwartzberg, and Paul Brindle for sharing mice or bone marrow and helpful discussion. Hope O'Donnell performed initial experiments on *Il4ra*$^{-/-}$ mice. This research was supported by RO1 AI35296 (to KAH), R37 AI38903 (to SCJ), T32 AI007313 and UMN doctoral dissertation fellowship (to MAW).

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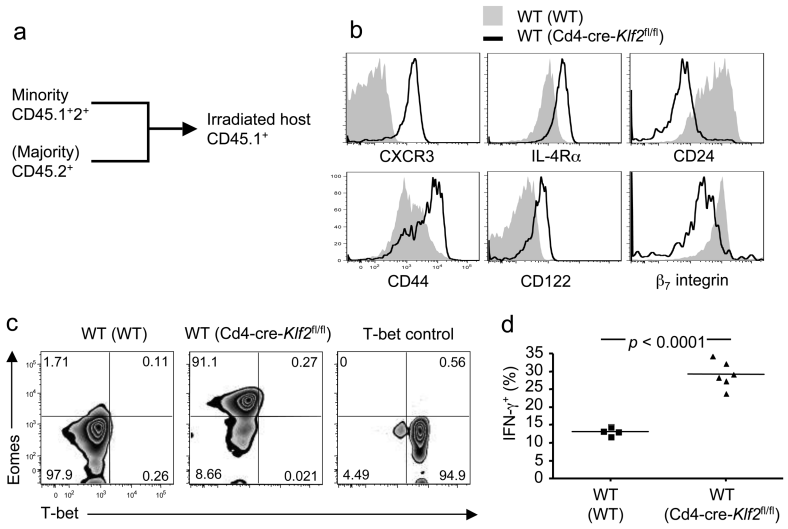

Figure 1.

KLF2-deficient T cells induce a memory-like phenotype on bystander CD8$^+$ thymocytes. (**a**) Schematic of the bone marrow chimera set-up used in the following experiments. The majority bone marrow is indicated with parentheses. (**b**) Analysis of the cell surface phenotype of wildtype (WT) bystander CD8$^+$ SP thymocytes. Black line WT CD8$^+$s from *Cd4*-cre-Klf2$^{fl/fl}$ majority, and filled gray all WT chimera. (**c**) Eomesodermin (Eomes) and T-bet expression on bystander CD8$^+$ thymocytes. T-bet staining control is CD1d-tet$^+$ cells from the thymus of the WT chimera. Results are representative of more than 5 experiments. (**d**) Thymocytes were stimulated with PMA and ionomycin and the percent of CD8$^+$ SP producing IFNγ was analyzed.

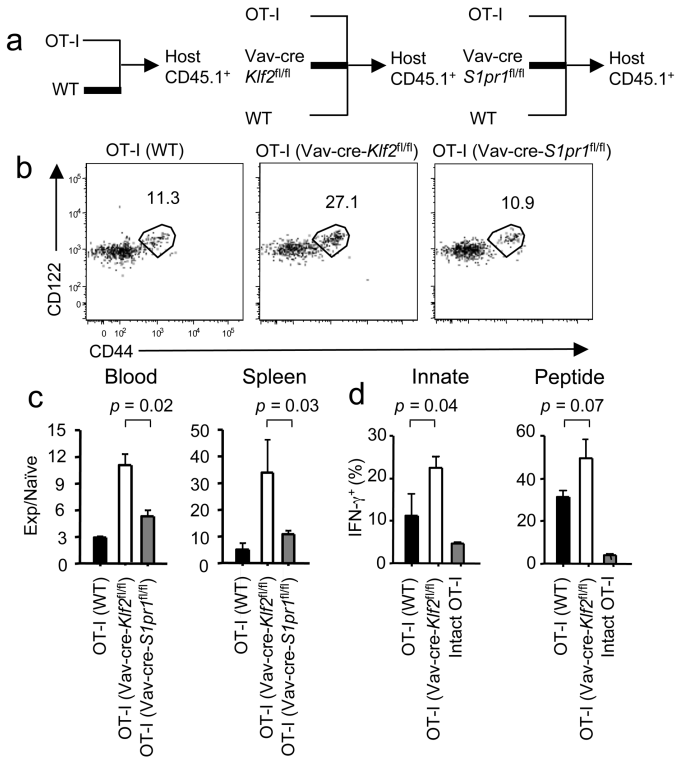

Figure 2.

Bystander CD8$^+$s have increased *in vivo* function. (**a**) Schematic of mixed bone marrow chimeras with OT-I-*Rag1*$^{-/-}$ CD8$^+$ T cells as minority bystander population. Thick line indicates majority population. (**b**) CD44 and CD122 expression of bystander OT-I CD8$^+$s from combined spleen and lymph node. (**c**) 3×10^5 OT-I T cells from indicated chimera was transferred with an equal number of naïve OT-I T cells. Mice were infected with Act$^{-/-}$ *Listeria monocytogenes* (LM)-OVA and ratio of OT-I from experimental (exp) chimera to naïve OT-I was analyzed. Blood (left panel) analyzed at day 4 and spleen (right panel) at day 7. n=3 per group. (**d**) IFN-γ production by OT-I T cells from indicated chimera or intact OT-I-Rag1$^{-/-}$ splenocytes following stimulation with IL-2, IL-12 and IL-18, left panel, or OVA peptide, right panel. Graphs represent at least 2 mice from 2 separate experiments. Error bars represent s.d.

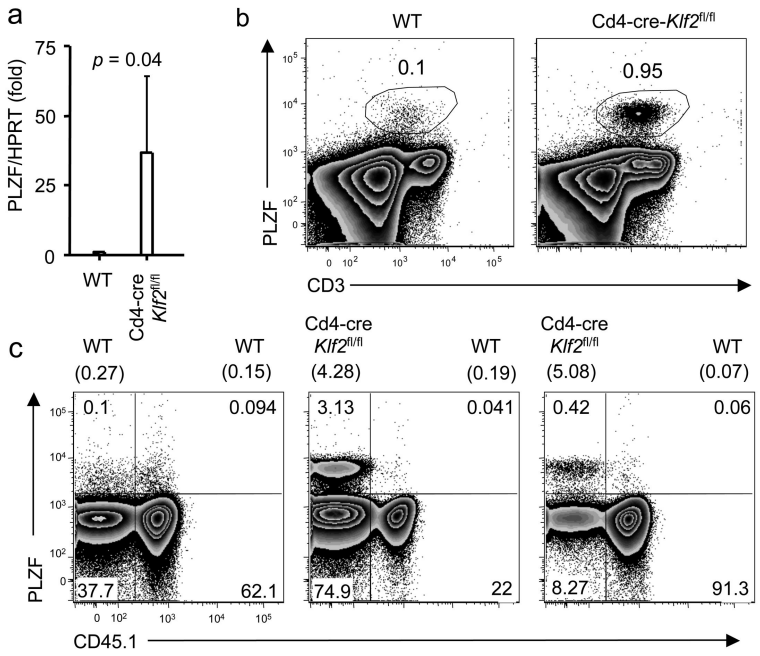

Figure 3.

KLF2 deficiency leads to an expansion of PLZF-expressing T cells. (**a**) PLZF mRNA expression on sorted CD4 SP thymocytes, quantified by real time PCR. n=5 (**b**) PLZF and CD3 expression on thymocytes from intact WT and *Cd4*-cre-*Klf2*$^{fl/fl}$ mice. Results are representative of more than ten mice. (**c**) PLZF expression on donor thymocytes from mixed chimeras of WT and *Cd4*-cre/*Klf2*$^{fl/fl}$ bone marrow. Percentage of PLZF^{+} cells within the CD45 congenic population is indicated in parentheses.

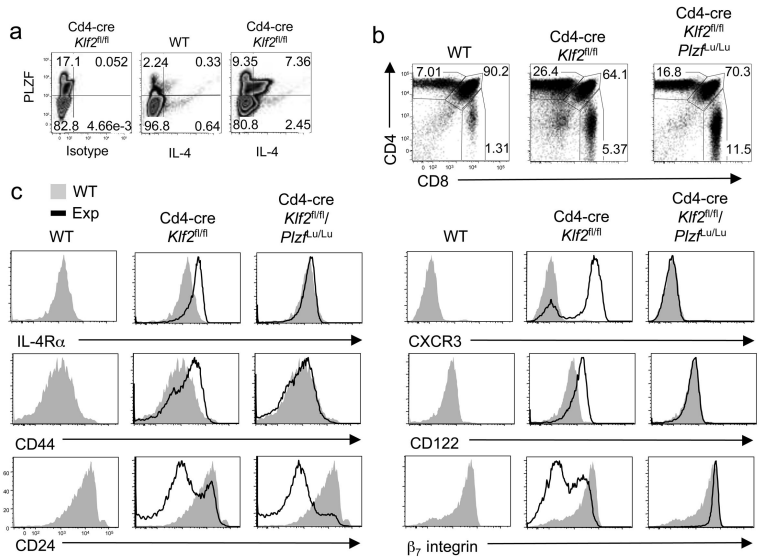

Figure 4.

PLZF^{+} cells are responsible for excess IL-4 production and CD8^{+} bystander effects in KLF2 deficient mice. (**a**) IL-4 and PLZF expression in thymocytes from intact WT and *Cd4*-cre-*Klf2*$^{fl/fl}$ mice. Thymocytes were isolated and stimulated with PMA and ionomycin for 5 hours then intracellular stained for IL-4 and PLZF. (**b**) Thymocyte expression of CD4 and CD8^{+} and (**c**) CD8^{+} SP phenotype from WT, *Cd4*-cre-*Klf2*$^{fl/fl}$ and *Cd4*-cre-*Klf2*$^{fl/fl}$-*Plzf*$^{Lu/Lu}$ mice.

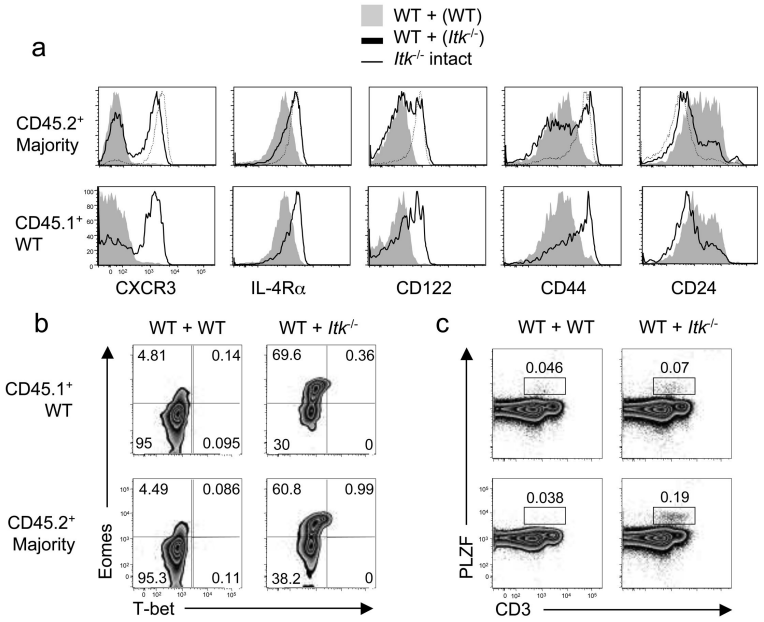

Figure 5.

Itk$^{-/-}$ mice have a cell-extrinsic CD8^{+} phenotype and expanded PLZF^{+} population. (**a**) CD8^{+} SP phenotype from intact *Itk*$^{-/-}$ mice (thin line), mixed chimera with *Itk*$^{-/-}$ majority (thick line) and an all WT chimera (shaded). Top row shows the majority population and bottom row shows the WT bystander population. (**c**) Eomesodermin and T-bet expression on the CD8^{+} SP population and (**d**) PLZF by CD3 expression on all thymocytes from mixed bone marrow chimeras. Results are representative of at least 4 mice.

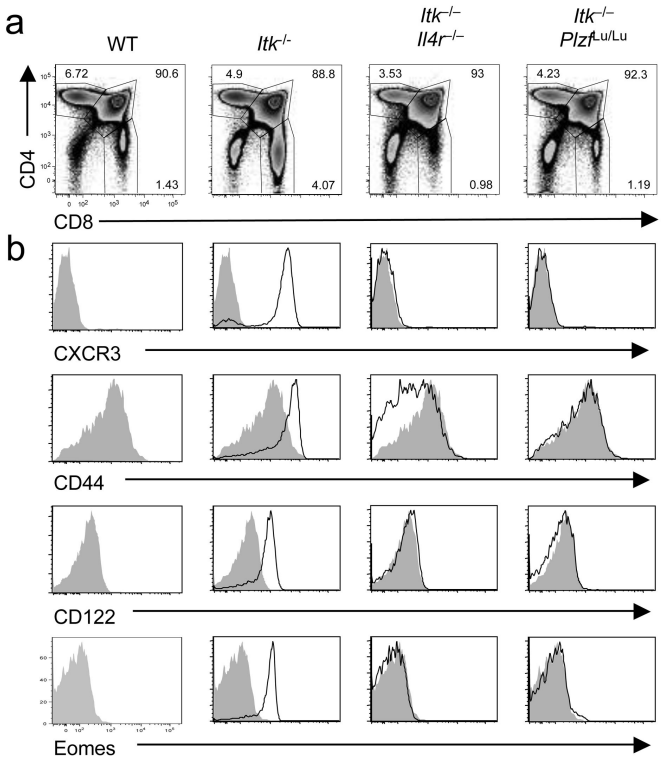

Figure 6.

The memory-like CD8$^+$ phenotype in *Itk*$^{-/-}$ mice is dependent on IL-4 and PLZF. Thymocyte analysis from WT, *Itk*$^{-/-}$, *Itk*$^{-/-}$-*Il4ra*$^{-/-}$, and *Itk*$^{-/-}$-*PLZF*$^{Lu/Lu}$ mice. (**a**) CD4 by CD8$^+$ expression. (**b**) Histogram overlays of CD8$^+$ SP phenotype. Gray shaded = WT and black lines from left to right represent *Itk*$^{-/-}$, *Itk*$^{-/-}$-*Il4ra*$^{-/-}$, and *Itk*$^{-/-}$-*PLZF*$^{Lu/Lu}$ mice. Graphs represent at least 2 mice from 2 separate experiments.

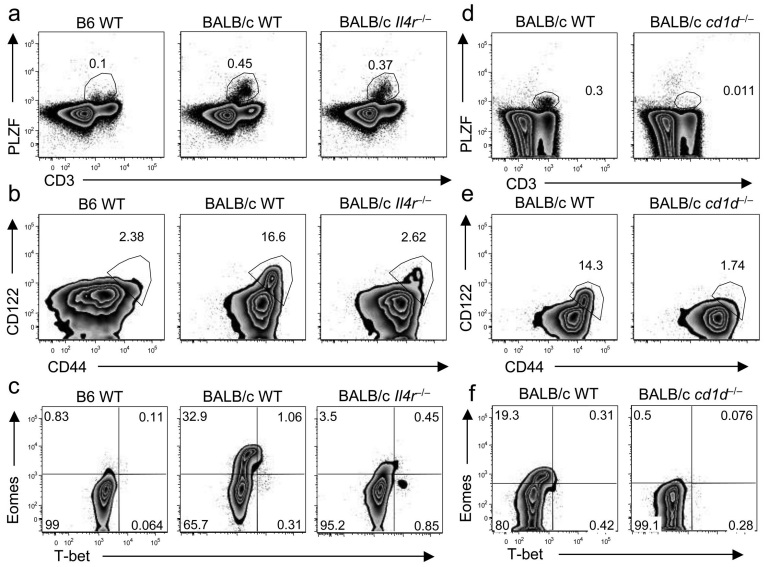

Figure 7.

An expanded PLZF^{+} population and an IL-4 and NKT dependent memory-like population in BALB/c mice. (**a**) PLZF and CD3 expression of thymocytes from B6, BALB/c and *Il4ra*$^{-/-}$ on BALB/c background and (**d**) BALB/c and *Cd1d*$^{-/-}$ on BALB/c background. (**b, e**) CD44 by CD122 and (**c, f**) Eomesodermin (Eomes) by T-bet expression on the CD8^{+} SP thymocyte populations. Results are representative of a minimum of 3 mice per experimental group.

Table 1

Characteristics of PLZF$^+$ populations in mouse models used. Row 1: Percentage PLZF$^+$ cells of total thymocytes. Row 2: Percentage of PLZF$^+$ thymocytes identified by CD1d-tetramers loaded with the glycolipid PBS-57. Row 3: Percentage of PLZF$^+$ thymocytes that are γδ TCR positive. Row 4: Percentage of PLZF$^+$ thymocytes that are CD4 positive. At least 5 animals were analyzed in each group.

	B6	Cd4-cre-*Klf2*$^{fl/fl}$	*Itk* $^{-/-}$	BALB/c
% PLZF$^+$ of total thymus	**0.09**	**0.82***	**0.35***	**0.27***
% CD1d$^+$ of PLZF$^+$	**52.5**	**35.7***	**14.8***	**79.1***
% γδ TCR$^+$ of PLZF$^+$	**10.2**	**11.3**	**37.7***	**2.2***
% CD4$^+$ of PLZF$^+$	**64.5**	**70.8**	**79.0**	**38.2***

* $p<0.05$ for difference compared to B6 using student's t-test