The Kruppel, the Like, the Factor, the 2, and the Ology

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Dedication

This thesis is dedicated to my parents, Colleen and Hugh Weinreich. I never would have accomplished this without their outstanding examples and hardwork.

Abstract

This thesis project began with testing the role of IL-7 and Erk5 in regulation of KLF2 expression and SP maturation. We provide evidence that SP maturation occurs independently of both IL-7 and Erk5. Next we examined the direct effects of KLF2 deficiency in T cells. Here we made the surprising discovery that KLF2 deficiency causes cell extrinsic effects on wild type (WT) thymocytes using mixed bone marrow chimeras. In the third chapter we show that the cell extrinsic effects result in expression of the chemokine receptor CXCR3 and are dependent on IL-4 receptor signaling acting through the transcription factor eomesodermin. The cell extrinsic effects also lead to delayed thymocyte emigration and we investigate the mechanism for this emigration defect in chapter 4. This leads to the novel finding that CXCR3 is necessary for the maintenance of returning memory CD8 T cells in the thymus. In the fifth chapter, we identify a cell intrinsic expansion of PLZF+ T cells as the source of IL-4 in the KLF2 deficient thymus. We also show that the cell extrinsic effects lead to memory phenotype and function on bystander CD8 T cells. We show that this mechanism occurs in other gene deficiency models and in WT BALB/c mice.

The research presented in this thesis provides an advance in our understanding of the role of the transcription factor KLF2 in T cells. More broadly this work provides insight into how cell extrinsic effects may complicate our interpretation of gene deficient mouse models. Intriguingly, we discover a novel mechanism for the generation of more potent, memory-like T cells, which occurs in normal mice and possibly humans.

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Chapter 1

Introduction

Innate and adaptive immunity

Many organisms have evolved to take advantage of the success of other organisms. For example in an area with a lot of ants, it is easy to imagine that an animal that was adapted to eating ants, such as an anteater, might flourish. Being eaten is not in the best interest for survival of the ants so they developed protective mechanisms to not be eaten. Some ants are specialized to protect the colony by stinging and biting an intruder. The molecular basis for the behavioral differences in ants are being understood(1).

Humans are currently a relatively successful organism so many bacteria and viruses, which we will refer to as pathogens, have evolved to take advantage of our success. As with the anteater eating the ants, the success of these pathogens is often not to the advantage of the human. Humans have an intricate, well-developed immune system for protection from pathogens. Humans are not the first or only organism to have to deal with the problem of pathogens. There is evidence that the first innate defense mechanisms evolved 550 million years ago in cnidarians, like coral(2). Innate immunity is characterized by genetically encoded mechanisms that recognize patterns and distinguish self from non-self and respond to the non-self signals. While innate immunity remains vital for the rapid response to a pathogen, this system is limited in its ability to adapt to novel pathogens. Higher mammals have a lower rate of mutation and longer lifetime than pathogens, so evolution and adaptation between generations of mammals is to the advantage of the pathogen. The adaptive immune system allows adaptation within an organism on a much shorter time scale.

The adaptive immune systems of jawed and jawless vertebrates have evolved separately through convergent evolution(3, 4). The adaptive immune system arising separately twice indicates that adaptive immunity provides an evolutionary advantage. We have chosen to study the adaptive immune system of jawed vertebrates since humans have jaws. This makes it more likely that our findings may be applicable to the human immune system. Adaptive immunity allows organisms an enormous repertoire of receptors to recognize pathogens. This not only provides a way for an organism to respond to a pathogen that the organism itself has never encountered but also respond to a pathogen that has never been seen by any organism. In addition, this system is set up in a way that immunological memory is generated. Immunological memory allows a better functional response and rapid elimination of subsequent infection from the same pathogen.

The two main cell types in the adaptive immune system are B cells and T cells. The role of B cells is to make antibody and provide humoral immune protection. B cell and humoral immunity is specialized against extracellular pathogens, such as most bacteria. T cells can be divided into two main subsets, CD4 expressing T cells and CD8 expressing T cells. The development of other T cell subsets will be discussed later. CD8 T cells or cytotoxic T cells provide the cell-mediated immune response necessary for protection from intracellular pathogens, such as viruses. CD4 T cells or helper T cells are vital for the full activation and response of both B cells and CD8 T cells.

Conventional T cell selection

The connection between the thymus, T cells and the immune system is now obvious. However, the immunological importance of the thymus was not known until the 1960's. Using neonatal thymectomized mice; Jacques Miller was the first to show that without a thymus normal immune responses did not develop(5). T cells develop in the thymus in an ordered progression, which results in the production of T cells with a large repertoire of unique T cell receptors (TCRs).

All blood cells, including T cells, are derived from hematopoietic stem cells in the bone marrow. T cell progenitors enter the thymus and are double negative (DN) for both TCR coreceptors CD4 and CD8. DN thymocytes proliferate and develop into double positive (DP) thymocytes that express both CD4 and CD8. At the DP stage both chains of the TCR are expressed for the first time. The diverse repertoire of TCRs is generated by the activities of recombination activating gene (RAG) 1 and 2. These genes are only expressed in lymphocytes and are essential pieces of the complex that leads to the recombination of the DNA encoding the TCR. Diversification at the DNA level allows the TCR repertoire to be heritable when a T cell divides.

TCRs are only signaled when recognizing peptides in the context of the major histocompatibility complex (MHC) because CD4 and CD8 coreceptors limit the localization of the tyrosine kinase Lck(6). DPs expressing TCRs interact with cortical thymic epithelial cells (cTECs), which express self peptide-MHC complexes. DPs expressing a TCR that is unable to recognize the MHC die by neglect. Those that interact with a self peptide-MHC complex with high affinity also die by negative selection(7). Negative selection in the cortex is one mechanism that rids the organism of potentially dangerous, self-reactive T cells. Finally those DPs with TCRs that are

able to interact with a self peptide-MHC complex but not with high enough affinity to induce negative selection are signaled to survive. DPs that are positively selected upon MHC class I interactions are destined to become CD8 T cells and those selected on MHC class II become CD4s.

Unconventional T cell selection

Besides the conventional CD4 and CD8 T cells there are other smaller subsets of T cells that provide unique functions and may be selected in unique ways. It has been proposed that at least some of these subsets may be selected by avoiding negative selection when encountering agonist peptides. Thus may have T cell receptors (TCRs) with high affinity for self peptides(8). These cell types include CD4+, FoxP3+ regulatory T cells (Tregs). This is a suppressive cell type whose importance can be demonstrated in the human multi-organ autoimmune disease IPEX in which Tregs are defective(9-11). Multiple lines of evidence indicate that the TCR repertoire of Tregs may be of higher affinity compared to conventional CD4 T cells(12).

Natural Killer (NK) T cells are another thymic derived T cell subset that regulates immune responses through rapid production of cytokines. Originally named because of expression of NK cell markers, NKTs are most accurately defined as T cells that have highly restricted repertoires recognizing non-classical MHC molecules. Within the NKT population there are distinct subsets including CD1d-restricted invariant NKT, γδ NKTs, and mucosal NKT(13). As a result, NKTs have a restricted TCR repertoire. Despite their restricted repertoire NKTs add novel specificity because

conventional T cells recognize only peptides. NKTs are able to recognize glycolipids from limited pathogens(14). More often NKTs are indirectly activated during infections(15). NKT function seems to vary dependent on the situation(16). They can play an innate-like role and rapidly produce cytokine upon stimulation. In other situations NKTs appear to suppress the immune response.

Selection of NKT cells is also unique, rather than being selected by cTECs, NKT cells are selected on other DP thymocytes(17). Selection on DPs allows homotypic signaling lymphocytic activation molecule (SLAM) interactions during selection, which explains why SLAM associated protein (SAP) is required for NKT development but dispensable for conventional T cell development(18). Another example of the unique requirements for NKT development is the transcription factor PLZF. While dispensible for NKT selection, PLZF is necessary for NKT but not conventional T cell maturation and function (19, 20). Without PLZF mice have a NKT number is greatly decreased and those that do survive do not rapidly produce cytokine.

Also γδ T cells (γδ NKTs) have been identified. γδ NKTs have a conserved TCR, like αβ NKT cells, Vγ1.1+Vδ6.3+, and are SAP and PLZF dependent but the specificity of these cells is not known(21). γδ NKTs are overrepresented in mice deficient in IL-2 inducible T cell kinase (ITK), while αβ NKTs are diminished (22-24). Since ITK effects TCR signaling and these two subsets of NKT cells may differ in the selection process.

Thymic medulla and negative selection

After positive selection thymocytes upregulate the chemokine receptor CCR7 and migrate into the medulla, which will be discussed in a subsequent section(25). Instead of exiting directly after positive selection they spend 4-5 days in the thymic medulla(26). During their time in the medulla SP thymocytes undergo functional maturation. Phenotypic and functional changes mark SP maturation (Fig. 1). The hallmark of functional SP maturation is the change in response to agonist TCR signaling changes. Semi-mature SP apoptose from an agonist TCR signal. The more differentiated mature SPs proliferate, similar to mature T cells outside of the thymus(27). This means that SP thymocytes remain susceptible to negative selection during their time in the medulla.

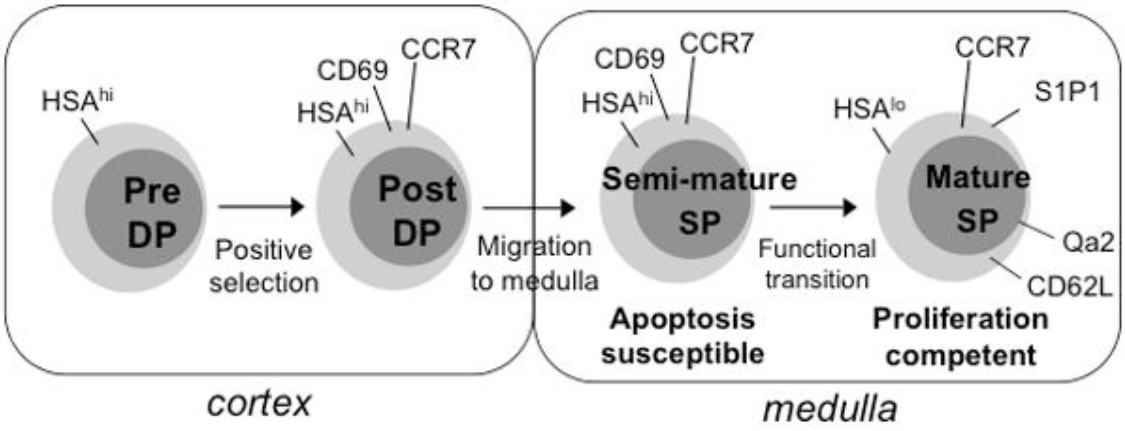

Figure 1-1. Developmental events that occur after positive selection in the thymus Prior to positive selection HSA^{hi} DP thymocytes reside in the cortex. Upon interaction with selecting MHC, they upregulate CD69 and CCR7 and migrate to the medulla, commensurate with becoming an SP. Semi-mature SP thymocytes remain susceptible to apoptosis, which is presumably critical for tolerance to tissue specific antigens displayed by APC in the medulla. Ultimately the progenitor undergoes final functional maturation, after which it is competent to proliferate when triggered through the TCR. At this stage it upregulates Qa2, CD62L, and S1P1, and emigrates.

Negative selection in the medulla is critical because tissue specific antigens (TSAs) are expressed in the thymus only by medullary thymic epithelial cells (mTECs). The autoimmune regulator (Aire) gene is only expressed by mTECs and is responsible for TSA expression. The dysfunction of this gene leads to autoimmunity in humans and mice (28, 29). Further proof of the importance of thymic medullary residency is in models where thymocytes do not migrate to the medulla (CCR7 deficiency) or exit the thymus early (S1P1 transgenic) and autoimmunity ensues(30, 31).

Thymocyte emigration

I coauthored a review of review on thymocyte emigration in 2008(32). For this introduction I will just highlight the major findings that are relevant to my thesis work. The description by Jason Cyster's laboratory of the S1P1 receptor as a necessary receptor for emigration of thymocytes from the thymus has been key to our understanding of this process(33). Our laboratory has contributed to the field by showing that the transcription factor KLF2 regulates the expression of S1P1(34). This will be discussed in detail in the next section. More recently the basic understanding of this field has been furthered by again by the Cyster laboratory showing that thymocytes in a healthy thymus emigrate via the blood but not the lymph(31).

The role of Kruppel like factor 2 in T cell biology

As discussed above, the transcription factor KLF2 is expressed during SP thymocyte maturation and is required for normal emigration of T cells. Kruppel like

factors are zinc finger transcription factors that bind similar CACCC elements in DNA. There are 15 human KLFs (35). KLF2 was originally called lung KLF because of its high expression in that tissue(36). KLF2 gene knock out mice revealed that KLF2 is necessary for vascular stability and embryos die between embryonic day 12.5 and 14.5 from these defects(37). Thus, hematopoietic or T cell specific deletion is necessary to study KLF2 function in T cells.

Jeffrey Leiden's group did the seminal studies of KLF2 function in T cells. The most striking result was that while T cell progenitors were found in the thymus at near normal numbers, the mice were extremely T cell lymphopenic outside of the thymus(38). In addition, they found that KLF2 was only expressed in the medulla by SP thymocytes. The KLF2 deficient T cells found in the spleen had an activated cell surface (CD44 high, CD69 high, CD62L low) phenotype. In addition Leiden's group showed that KLF2 overexpression led to decreased cell proliferation and a more quiescent phenotype in a T cell tumor(39). KLF2 was hypothesized to be a quiescence regulator and without KLF2 T cells were spontaneously activated and underwent cell death.

Consistent with a role for KLF2 in quiescence, naïve T cells express KLF2 but with activation of T cells KLF2 protein is rapidly degraded and transcription stops(38). After activation, KLF2 is reexpressed in memory T cells(40). It has been shown in vitro that following TCR stimulation and loss of KLF2, reexpression will occur after culture with cytokines IL-2, IL-4 and IL-7 at certain concentrations(41, 42). Thus, KLF2 loss after T cell activation is a transient process that may be regulated by cytokines.

Subsequently our laboratory showed that the lack of KLF2 deficient T cells in the periphery was due not to cell death, but to defective thymocyte emigration because KLF2 is required for S1P1 expression(34). It has also been shown that KLF2 can directly bind and transactivate to the promoters of S1P1 and CD62L(34, 41). In addition we observed the dysregulation of other cell surface molecules (β7 integrin, CCR7) in KLF2 deficient SP thymocytes.

Open questions relevant to this thesis are: 1) How is KLF2 expression regulated in SP thymocytes? 2) How or if KLF2 regulates other cells surface receptors? 3) What role does KLF2 play in the development of unconventional T cells subsets?

Chapter 2

Post-selection thymocyte maturation and emigration are independent of IL-7 and ERK5

Abstract

The transcription factor Kruppel-Like Factor 2 (KLF2) controls the emigration of conventional T cells from the thymus through its regulation of the cell surface receptor, S1P1. Prior to KLF2 expression, developing T cells require a positive selection signal through the T cell receptor. However, following positive selection there are time, spatial and maturational events that occur before KLF2 is upregulated and emigration occurs. We are interested in determining the signals that upregulate KLF2 and allow thymocytes to emigrate into circulation, and if they are linked to functional maturation. In endothelial cells KLF2 expression has been shown to be dependent on the MAP kinase ERK5. After positive selection, T cells become sensitive to IL-7 signaling. Furthermore, it has been reported that IL-7 signaling leads to the phosphorylation of ERK5. We hypothesized that post-positive selection IL-7 receptor signaling through ERK5 drives the expression of KLF2. In this chapter we provide evidence that this hypothesis is incorrect. We showed in vivo that T cells complete maturation and express KLF2 independently of both ERK5 and IL-7.

Introduction

T cells develop in ordered differentiation stages within the thymus. These stages can be differentiated by expression of T cell receptor (TCR) coreceptors, CD4 and CD8. The most immature progenitors, the double negative (DN) thymocytes, express neither CD4 nor CD8. During the DN stage of selection, expression of the β chain of the TCR occurs. At this stage the thymocytes proliferate and their survival is dependent on the cytokine IL-7. Thymocytes then express both CD4 and CD8, signifying the double positive (DP) stage.

Positive selection of DP thymocytes is marked by high levels of the TCR and upregulation of CD69 and CCR7 on the cell surface. Following positive selection, the DP thymocytes down-regulate one of their coreceptors in a manner that is dependent on the class of the selective MHC. Those selected on Class II MHC become CD4 single positive (SP) thymocytes and those selected on Class I become CD8 SPs. Thymocytes transitioning from the DP to SP stage migrate to the medulla, dependent on CCR7-mediated chemotaxis.

The maturation state of the SP population can be further differentiated using additional cell surface markers. Heat stable antigen (HSA) and CD69 are highly expressed post-selection and on semi-mature SPs. HSA and CD69 are down regulated with maturation. The opposite pattern is observed with Qa-2 and CD62L, as expression increases with maturation(32). (See Figure 1-1) The maturation is not only a superficial change in surface receptors but also functional. Kishimoto and Sprent demonstrated that TCR stimulation of semi-mature (HSA high) SPs induces death while mature

thymocytes respond by proliferating(27). In other words, semi-mature SPs remain susceptible to negative selection.

This time is important to allow interactions between semi-mature thymocytes and the unique medullary stroma. The Benoist-Mathis group demonstrated that some tissue specific antigens that are expressed only by medullary thymic epithelial cells are dependent on the transcription factor Aire (autoimmune regulator) (29). The Aire gene was originally discovered as mutated in human patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (28). Additionally, Takahama and colleagues have demonstrated that thymocytes from mice deficient in the chemokine receptor CCR7 do not travel to the thymic medulla and these thymocytes are reactive to self-antigens (25, 43). More recently the Cyster group found that forcing thymocytes to emigrate from the thymus early, with transgenic expression of S1P1, led to an increase in lymphoid infiltrates in tissues(31). All of these findings support an important role for allowing negative-selection susceptible thymocytes to survey the thymic medulla.

The transcription factor Kruppel-like factor 2 (KLF2) is required for T cells to emigrate from the thymus via its role in regulating the receptor S1P1(34). To better understand the mechanisms that control medullary residency of thymocytes we investigated the regulation of KLF2. Positive selection is an important checkpoint for thymocyte development prior to KLF2 expression. Since KLF2 is not expressed until a minimum of two days after positive selection and after migration from the thymic cortex to the medulla occurs(26), we felt that it was unlikely that positive selection directly induces KLF2 expression.

The cytokine IL-7 is necessary for thymocyte and T cell survival(44). Signaling through the IL-7 receptor is necessary for the survival of DNs(45). However at the DP stage the IL-7R is not expressed and DPs are refractory to cytokine signaling because of expression of the signaling suppressor SOCS1(46). Furthermore, the major source of IL-7 in the thymus is the medullary compartment. We speculated that if KLF2 is dependent on IL-7 signaling this would explain the delayed expression following positive selection, and would ensure that SP thymocytes did not emigrate from the organ until they spent at least some time surveying the medulla.

If our hypothesis was correct in implicating IL-7 in the regulation of KLF2 then what signaling pathway does this work through? Erk5 is an important regulator of KLF2 in other cell types but no role in thymocyte development has been established. Erk5 is a member of the mitogen-activated protein kinase (MAPK) family. Erk5 activates the transcription factor myocyte enhancer factor 2 (MEF2)(47). Mice deficient of either Erk5 or KLF2 die embryologically of angiogenic defects(48, 49). Winoto and colleagues demonstrated MEF2 can bind the KLF2 promoter and that knockdown of Erk5 reduces the expression of KLF2 in T cells(47). Importantly, this group reported that T cell stimulation with IL-7 led to the phosphorylation of ERK5(47).

In this chapter we sought to test the hypothesis that post-positive selection signaling by IL-7 through ERK5 leads to the expression of KLF2 and resulting emigration of thymocytes. We found no evidence for IL-7 regulation of KLF2 using genetically deficient cells and in vivo IL-7R blockade. We also found that genetically ERK5 deficient T cells underwent normal development and showed no deficiency in

KLF2. Our findings lead us to conclude that IL-7 and ERK5 do not control KLF2 expression or the semi-mature to mature SP thymocyte transition.

Results

KLF2 is expressed in IL-7 receptor deficient T cells

We set out to test the hypothesis that KLF2 expression in T cells was dependent on post-positive selection IL-7 signals. Consistent with published reports(26, 46) we show that both IL-7 receptor and KLF2 are not expressed in DP thymocytes. IL-7R is expressed directly after positive selection in the "youngest" SP thymocytes (as determined by GFP expression in RAG2GFP mice (26, 50) and continues to be expressed in all subsequent thymocyte subsets. In contrast KLF2 is only expressed in more mature SP subsets (Fig. 2-1). Since post-selection thymocytes migrate to the cytokine rich medulla, dependent on CCR7(25, 51), we conclude that developing thymocytes are likely to be receiving signals through the IL-7R prior to expression of KLF2.

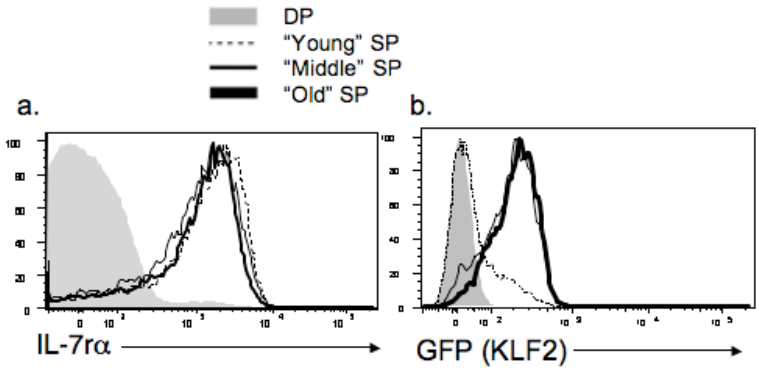

Figure 2-1: IL-7 receptor precedes KLF2 expression post-positive selection. a. Temporal expression of IL-7R in CD4 SP thymocytes with age was determined by GFP expression in Rag2p-GFP mouse. b. Expression of KLF2 (GFP) from *Klf2*^{GFP} reporter mice using CD69 and HSA expression to determine age.

Next, to help us understand the dependence on IL-7 for KLF2 expression we analyzed Il7r$^{-/-}$ mice. Il7r$^{-/-}$ mice have greatly reduced thymocyte cell number due the

role of IL-7 in survival of thymocytes at the DN stage(45, 52). However, as previously reported, we find a population of CD4 and CD8 SP thymocytes that develop in these mice (Fig. 2-2a). Because of the low cell numbers we focused our analysis on the more prevalent CD4 SP population but we observed similar trends in CD8 SP (data not shown). To attempt to eliminate error based on comparing altered subsets within the SP thymocyte population, we limited our analysis to conventional αβ T cells by excluding regulatory, γδ, and NK T cells by gating out CD25, γδ TCR, and NK1.1. Since KLF2 is necessary for emigration from the thymus, if IL-7R deficient SPs were deficient in KLF2 expression we would expect thymocyte retention. In the CD4 SPs from the $Il7r^{-/-}$ thymus appeared more mature (HSA low, Qa2 high) consistent with retention (Fig. 2-2b). CD69 and CD62L, which also change with SP maturation, were not as severely affected (Fig. 2-2b). This is possibly because KLF2 directly regulates CD62L and S1P1, which has a reciprocal relationship with CD69(34, 41, 53).

To directly measure KLF2 expression in IL-7R deficient mice, we pooled thymocytes from 15 $Il7r^{-/-}$ mice and sorted DP, CD4 SP and CD8 SP cells. Quantitative analysis of mRNA expression for these subsets compared to WT thymocytes showed similar levels of KLF2 expression (Fig. 2-2c and data not shown). These findings indicate that the few mature thymocytes that develop in IL-7R deficient mice are able express KLF2, arguing against an absolute requirement for IL-7R signals.

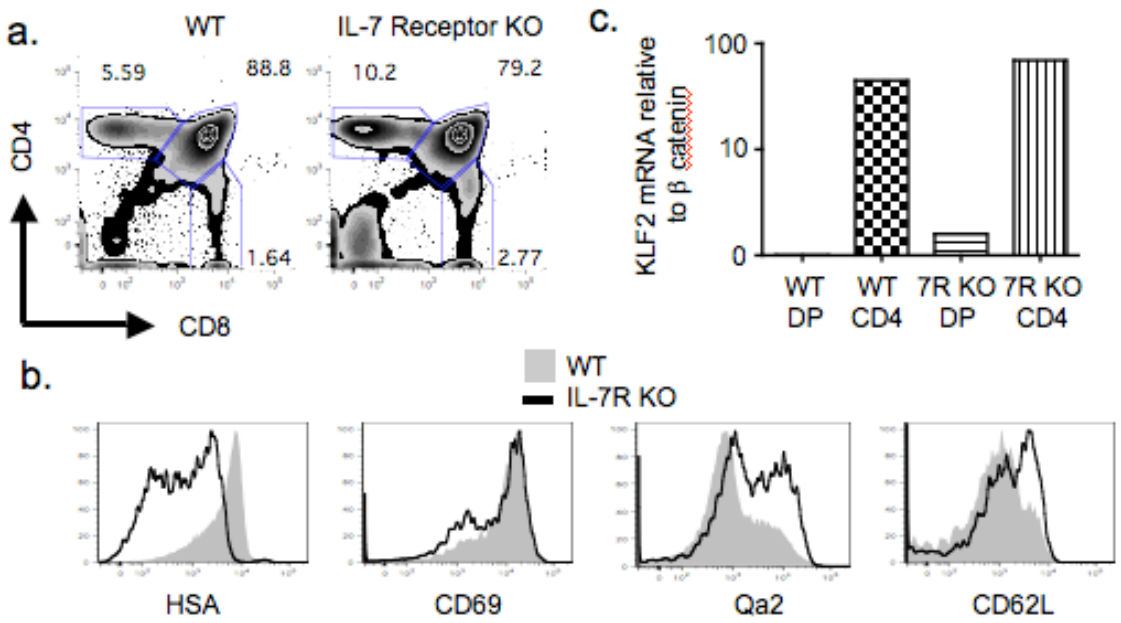

Figure 2-2: KLF2 is expressed in IL-7R KO thymocytes.
a. CD4 by CD8 profile of control and IL-7R KO thymi. b. CD69, CD62L, HSA, and Qa2 expression on Dump (CD25, γδ TCR, NK1.1) negative, CD4 SP thymocytes. c. KLF2 mRNA expression from indicated cell sorted thymus subsets relative to expression in control DP thymocytes. 1 WT B6 and 15 pooled IL-7R KO mice were used for the sort.

Thymocyte emigration does not depend on IL-7 receptor signals

Since development for the vast majority of thymocytes is dependent on IL-7, we considered the possibility that our findings with IL-7R deficient thymocytes were not representative of what occurs when thymocytes develop normally with IL-7 receptor signaling. To allow thymocytes to develop normally through the DN stage then block the IL-7R, we devised a short-term antibody blockade strategy, depicted in figure 2-3a. We limited the blockade to 9 days to minimize the complications from effects of the blockade on the DN population subsequently entering the SP populations. As an emigration blockade positive control, we treated mice with a S1P1 inhibitor.

First we evaluated if the in vivo blockade of IL-7R was effective. Consistent with effective blockade, we observed a profound reduction of developing B cells in the bone marrow, as B cells also require IL-7(52) (Fig. 2-3b). In the thymus we found no significant change in the DP to SP ratio with IL-7R blocking antibody treatment, though the total thymocyte number did decrease with IL-7R antibody treatment, indicating that the antibody effectively reached the thymus and blocked IL-7R signaling (Fig. 2-3d). While the S1P1 inhibitor increased the retention of SP as evidenced by the increased SP proportion (Fig. 2-3c, d), IL-7R blockade did not, suggesting that IL-7 signaling does not play an obligatory role in thymic emigration. We also used a BrdU pulse chase strategy to label a cohort of proliferating DN/DP thymocytes and allow them to develop with normal IL-7R signaling and then subsequently did the IL-7R or S1P1 blockade for 6 days (Fig. 2-3a). Again the S1P1 inhibitor led to an accumulation of labeled cells while IL-7R blockade did not have an effect (Fig. 2-3e). Analysis of IL-7R genetically deficient thymocytes and in vivo blockade of IL-7R do not support a role for IL-7R in KLF2 expression and thymic emigration. We also did not observe an alteration in the CD4 to CD8 SP ratio, suggesting that IL-7 does not play an obligatory role in the lineage commitment or survival of CD8 T cells.

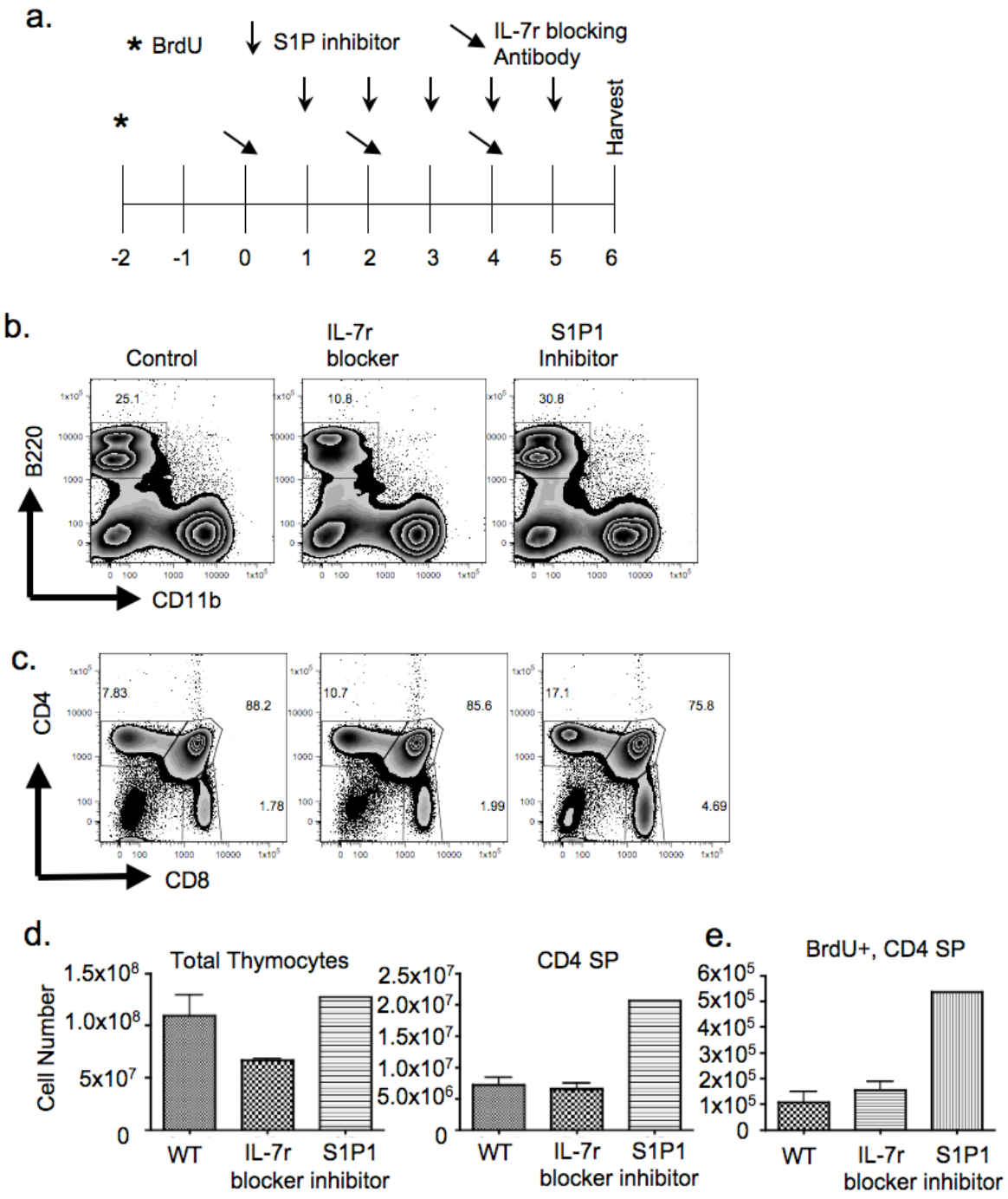

Figure 2-3: IL-7R blockage does not prevent thymocyte emigration.
a. Experimental design and dosing schedule for IL-7R receptor and S1P1 blockage. b. B220 and CD11b staining of bone marrow to examine the B cell and myeloid subsets. c. CD4 by CD8 thymus profile of representative treated and control mice. d. Total thymus cell number (left panel) and CD4 SP number (right panel). Control and IL-7R blockage: n=3 and S1P1 inhibitor n=1. e. Enumeration of BrdU-labeled cells in the dump-, CD4 SP gate.

Redundant function of ERK5 in T cell development

The ERK5 knockout mouse is embryonically lethal from similar endothelial defects as the KLF2 knockout mouse (49). In addition it has been reported that ERK5 is required for KLF2 expression in mouse embryonic fibroblasts cells and ERK5 knockdown in mature T cells decreased KLF2 expression (47). IL-7R signaling was shown to result in ERK5 phosphorylation in T cells (47). Although we did not observe an obligatory role for IL-7 in KLF2 expression in thymocytes, it is possible that redundant cytokines or other signals would activate ERK5 in thymocytes to induce KLF2 expression. To test the role of ERK5 in T cell development and expression of KLF2 in T cells, we obtained ERK5 floxed mice and bred them to CD4-cre mice to generate a T cell specific ERK5 deletion (54).

We found no significant change in CD4 or CD8 T cell number in the thymus, spleen, or lymph node (Fig. 2-4a and data not shown). Also we did not observe a difference in cell surface phenotype, including activation and migratory receptors, in the ERK5 deficient T cells (Fig. 2-4b). To investigate the relationship between ERK5 and KLF2, we found that the mRNA expression of KLF2 was similar in WT and ERK5 deficient T cells (Fig. 2-4c). Also we found that ERK5 deficient, like WT, T cells downregulated KLF2 after TCR stimulation and reexpressed KLF2 following culture with IL-7 (Fig. 2-4c). We also did not observe a difference in proliferation or memory cell surface phenotype, in intact or ERK5 deficient T cells in mixed bone marrow chimeras (data not shown). Together our findings do not provide evidence for a non-redundant role for ERK5 in T cell development.

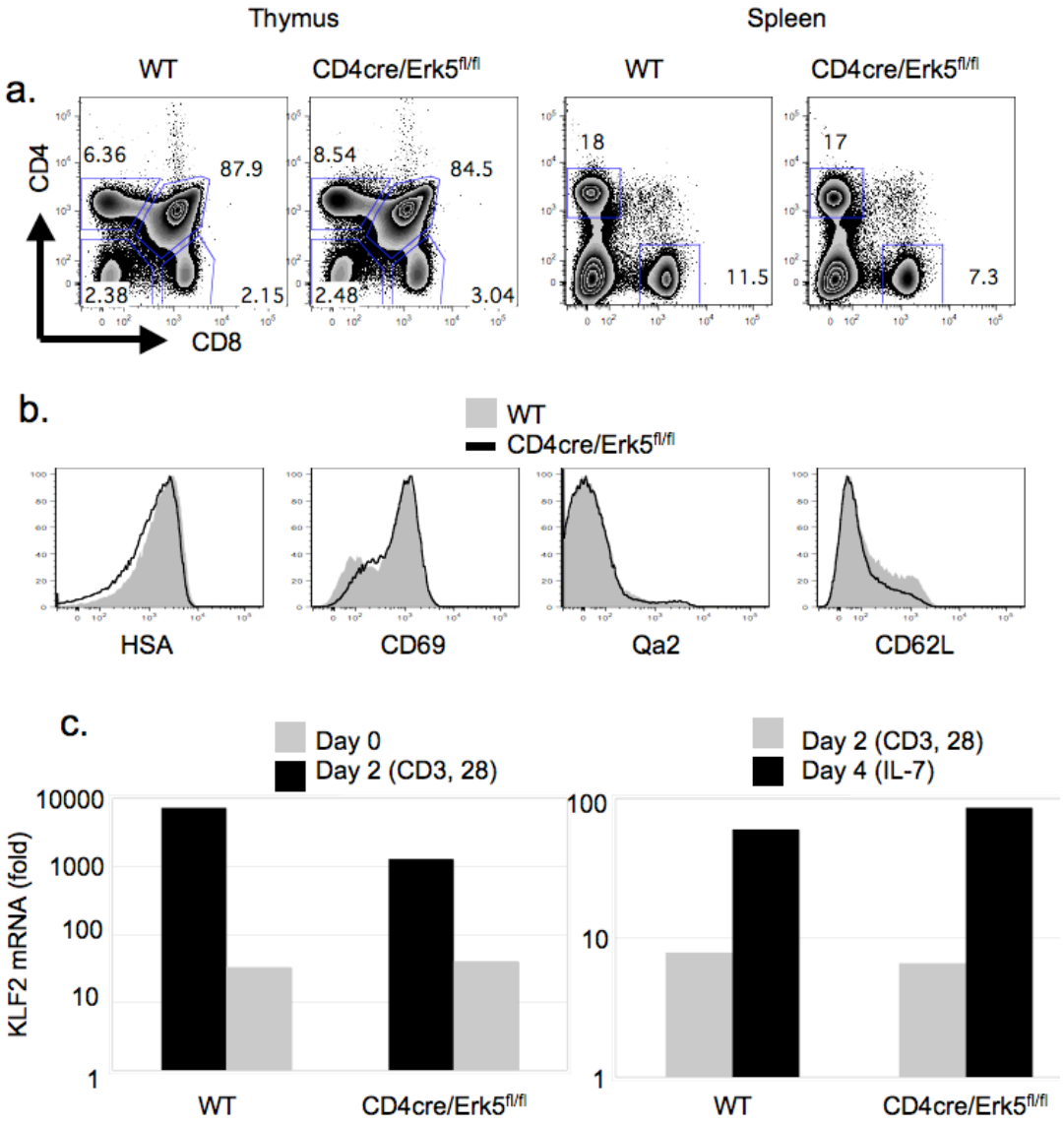

Figure 2-4: ERK5 deficient T cells develop and express KLF2 normally.
a. Representative CD4 by CD8 thymocyte profile, left two panels, and spleen, right, from CD4cre/Erk5$^{fl/fl}$ and littermate control mice. b. Cell surface phenotype of CD4 SP thymocytes, gray solid=littermate control and black line CD4cre/Erk5$^{fl/fl}$. c. Analysis of KLF2 mRNA relative to GapDH. CD8 T cells from the spleen were purified and stimulated in vitro with plate bound CD3 and CD28 for two days, left panel.
After 2 day CD3, CD28 stimulation, live cells were harvested and cultured with IL-7 for 2 additional days.

DISCUSSION

The findings presented here provide strong evidence against the hypothesis that KLF2 expression in T cells is regulated by IL-7R working through the ERK5 signaling pathway. After we generated this data, J. Simon C. Arthur's group published similar findings with ERK5 deficient T cells from conditionally deficient mice(55). They reported no defect in development for ERK5 deficient T cells and normal numbers of T cells in the spleen and lymph node. While no change was found in basal KLF2 levels in the periphery, the group did report lower KLF2 expression after TCR stimulation of ERK5 deficient T cells but this did not have any functional consequences with no differences in KLF2 target genes, such as S1P1 and CD62L. Taken together, ERK5 does not appear to play a critical, non-redundant role in T cell development.

Signaling through the IL-7 receptor on the other hand has been suggested to play a major role in the survival and function of T cells at multiple steps during development. However, using two independent loss of function approaches we did not find an obligatory role for IL-7R in regulation of KLF2 expression. We also did not observe a reduction in the proportion or number of CD8 SP thymocytes, as predicted by recent work suggesting that IL-7 signals are sufficient to carry out positive selection of CD8 T cells (56). However, that study employed a gain of function approach to suggest that IL-7 signals drive CD8 T cell development, and did not test the hypothesis using loss of function approaches, such as we used here. Interestingly, they used a loss of function approach to show that γc cytokines (of which IL-7 is one) are required for CD8 survival and lineage commitment. Thus it remains possible that IL-7 can contribute to

CD8 survival and lineage commitment, but in its absence, other γc cytokines, such as IL-4, can substitute.

It is worth noting here that the termination of RAG, and induction of migration to the medulla, which are key initial steps in positive selection initiated by the TCR signal, are not replaceable by either IL-7 or other γc cytokine signals. Thus we would argue that positive selection is best understood as a multistep process consisting of: 1) initial survival, receptor specificity fixation, and migration activities in DP cells, 2) that are followed immediately or concurrently by signals (through cytokines in the case of CD8 T cells) that fix cytotoxic/helper lineage in emerging CD4/8 SP cells, and 3) finalized by a biochemical realignment of the TCR signaling apparatus to achieve proliferation competence, that happens as cells transition from semi-mature SP to mature SP. It is this final stage where KLF2 is upregulated and which is still largely not understood in genetic/biochemical terms. Since large changes are occurring at this stage both in the functional response to TCR stimulation and migration pattern of these T cells, it is interesting to speculate that genome wide changes in chromatin structure underlie the biological effects. Another possibility would be altered microRNA expression, which can alter the expression of many genes simultaneously. The transition from semimature to functionally competent T cells is an important developmental step that is regulated by a yet undefined mechanism.

MATERIALS AND METHODS

Mice. Rag2p-GFP mice have been described (57) and provided by P. Fink (University of Washington, Seattle, WA) and IL-7 receptor KO mice have been described (52) and provided by M. Farrar (University of Minnesota, Minneapolis, MN). KLF2-GFP reporter mouse was generated for our laboratory and described previously (58). All mice were treated in accordance to federal guidelines approved by the University of Minnesota Institutional Animal Care Committee.

In vivo IL-7R and S1P1 blockage. Anti-IL-7R (A7R34) was purified in our laboratory from a hybridoma. The antibody was injected at 1 mg/mouse/day intraperitoneally (I.P.) every two days. The S1P receptor agonist was generated by Merck (Rahway, NJ) and provided to us by Marc Jenkins, University of Minnesota. This was injected 1 mg/kg/day daily I.P.

Flow cytometry. All antibodies were purchased from BD Bioscience, eBioscience, Invitrogen, or BioLegend. For surface staining, cells were incubated with antibodies in FACS buffer (PBS, 1% FCS, and 0.02% azide) for 30 minutes on ice. Data was collected on a LSR-II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.).

BrdU labeling. BrdU labeling and staining was performed as previously described (26). 100 μL of 10 mg/mL BrdU (Sigma-Aldrich; B5002) was injected I.P. twice at 4-hour intervals. Mice were harvested and analyzed 8 days later. Thymus and lymph

node cells were cell surface stained as described. Then intracellular staining was done using the APC BrdU flow kit (BD Biosciences), substituting the anti-BrdU antibody (PRB-1) from Phoenix Flow Systems.

Cell Sorting. Conventional SP thymocytes were purified by on a FACSVantage (Becton Dickinson) by gating on CD25, GL3, NK1.1 negative cells then selecting the CD4 and CD8 SP populations. 1 WT and 15 IL-7R KO thymi were pooled for this experiment.

RNA purification and analysis. RNA was purified using the RNeasy kit (Quigen). cDNA was generated using SuperScriptII Platinum Two-Step qRT-PCR kit (Invitrogen). qPCR was performed on a SmartCycler real-time PCR machine (Cepheid) using FastStart SYBR Green Master Mix kit (Roche). Analysis was done using β-catenin as the reference gene.

Chapter 3

KLF2 deficiency in T cells results in unrestrained cytokine production and bystander chemokine receptor upregulation

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Abstract

The transcription factor KLF2 regulates T cell trafficking by promoting expression of the lipid binding receptor, $S1P_1$, and the selectin, CD62L. Recently, it was proposed that KLF2 also represses the expression of chemokine receptors. We confirm the upregulation of the chemokine receptor CXCR3 on KLF2 deficient T cells. However, we show that this is a cell nonautonomous effect, as revealed by CXCR3 upregulation on WT bystander cells in mixed bone marrow chimeras with KLF2 deficient cells. Furthermore, we show that KLF2 deficient T cells overproduce IL-4, leading to the upregulation of CXCR3 through an IL-4 receptor and eomesodermin dependent pathway. Consistent with the increased IL-4 production, we find high levels of serum IgE in mice with T cell specific KLF2 deficiency. Our findings support a model where KLF2 regulates T cell trafficking by direct regulation of $S1P_1$ and CD62L, and restrains spontaneous cytokine production in naive T cells.

Introduction

Kruppel-Like factors (KLFs) are a family of zinc-finger transcription factors that are expressed in a broad range of tissues and at various times in ontogeny (59). Germline knockout of one of these factors, KLF2, is not compatible with life because of vascular defects (37, 60). Studies done with KLF2 deficiency limited to only hematopoietic cells reported a striking loss of T cells from the blood, lymph node and spleen with thymic development appearing grossly normal (38).

Our laboratory previously reported an increase of mature CD4 and CD8 single positive (SP) cells in the KLF2 deficient thymus (34). KLF2 deficient SP thymocytes survived *in vitro* and *in vivo* so that the lack of peripheral T cells is seemingly not a result of cell death (34, 61). Thus, the accumulation of mature SP cells in the thymus implied an emigration defect. Consistent with this, KLF2 deficient T cells showed severely reduced $S1P_1$ expression (34). $S1P_1$ is a cell surface receptor for the phospholipid sphingosine-1-phosphate (S1P) and is required for thymic emigration (33, 62). KLF2 directly binds to the $S1P_1$ promoter and induces $S1P_1$ transcription (34, 41). KLF2 also regulates T cell expression of L-selectin (CD62L) (34, 41, 61, 63). Although CD62L is not required for thymic emigration, it is required for entry into lymph nodes (64), and $S1P_1$ is required for egress from lymph nodes (33). Thus, KLF2 acts as a single transcription factor controlling two key molecules—$S1P_1$ and CD62L—required for naïve T cell trafficking through secondary lymphoid organs (SLO).

A recent report found that CD4 positive T cells from KLF2 deficient mice expressed multiple inflammatory chemokine receptors, suggesting that loss of KLF2

leads to redirection of naïve T cells to non-lymphoid sites (61). Together these findings leave us with the appealing idea that KLF2 acts as a master regulator of naïve T cell trafficking. KLF2 would direct naïve T cells through SLOs by positively regulating CD62L and $S1P_1$, and would negatively regulate inflammatory chemokine receptors to prevent naïve T cells entering nonlymphoid tissues. However, in this report we demonstrate that expression of the chemokine receptor CXCR3 in KLF2 deficient T cells is regulated via a cell-nonautonomous pathway. We find that KLF2-knockout T cells exhibit dysregulated IL-4 production, which can act on bystander wild type T cells to induce aberrant expression of CXCR3. These data suggest KLF2 enforces naïve T cell trafficking by both autonomous and nonautonomous mechanisms. Furthermore, it suggests that KLF2 also maintains naïve T cell identity in terms of cytokine production, as KLF2 deficient T cells rapidly produce IL-4, a property usually associated with innate-immune and memory T cells.

Results

CXCR3 is indirectly regulated in KLF2 deficient T cells

To further study how KLF2 regulates chemokine receptor expression, we employed mice with a T cell specific deficiency in KLF2. We used CD4-cre mice crossed to mice with KLF2 flanked by loxP sites ($KLF2^{fl}$) (58). In this model, the KLF2 gene is excised at the DN4/DP stage of thymocyte development prior to the SP stage where KLF2 is normally first expressed. Such mice have a similar T cell phenotype to KLF2 deficient fetal liver chimeras (34) and to Vav-Cre/$KLF2^{fl/fl}$ mice (61). This includes severe peripheral T cell lymphopenia and a two-fold accumulation of mature SP thymocytes.

We compared chemokine receptors on SP thymocytes purified from WT versus CD4Cre/$KLF2^{fl/fl}$ mice (here on referred to as "KLF2 KO"). Although most SP thymocytes are conventional αβ T cell precursors, this population normally also includes low numbers of NKT, γδT cells, $T_{reg,}$ and recirculating memory T cells. Our lab previously showed that such nonconventional T cells can complicate the analysis of SP thymocytes (26). To focus our analysis on conventional αβ T cells, we used a "dump strategy" to exclude cells that expressed CD25, TCRγ, NK1.1, or were capable of binding $CD1^{d}$/αGal-cer tetramers. We observed a striking overexpression of CXCR3 mRNA in KLF2 deficient conventional thymocytes (Figure 3-1A), ten-fold for CD4SP and 40-fold for CD8SP compared to WT. CXCR3 protein expression was confirmed by flow cytometry and was observed both on thymocytes (Figure 3-1B) and peripheral T cells (data not shown). CXCR3 is typically not expressed on naïve cells, but can be

upregulated on effector and memory T cells. It has three ligands: CXCL9, CXCL10, and CXCL11, which are produced at sites of inflammation. In contrast to a previous publication (61), we did not observe upregulation of any other inflammatory chemokine receptor mRNA, nor could we confirm protein upregulation for CCR3, CCR5, or CCR6 (Figure 3-1B and data not shown). While we cannot fully explain the discrepancies at this time, there are two key differences between the studies. First, Sebzda et al. used vav-cre while we used CD4-cre to delete KLF2. The other is our use of the dump strategy to exclude non-conventional T cells, as such cells are dysregulated in KLF2 deficient mice (Figure 3-2). Nonetheless, CXCR3 protein was clearly upregulated on conventional αβ T cells from CD4Cre/KLF2$^{fl/fl}$ mice.

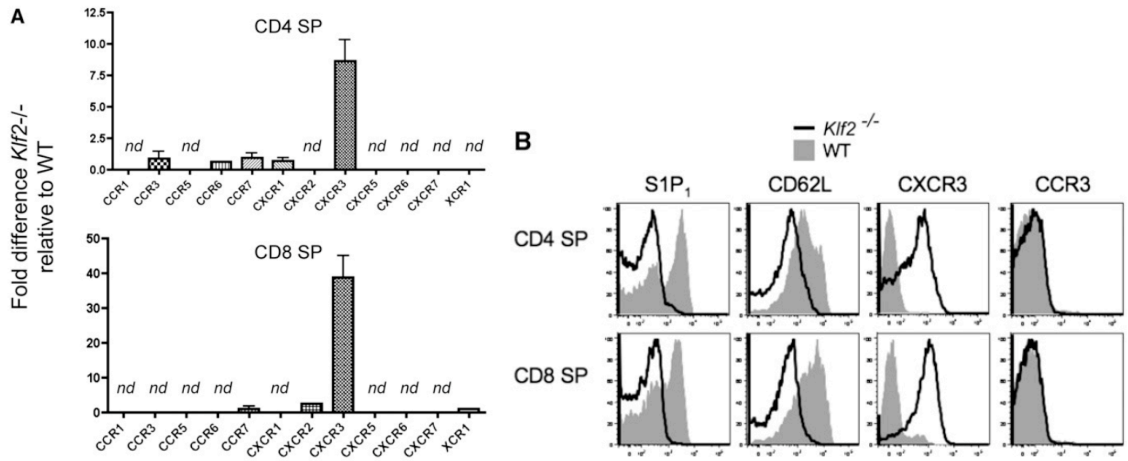


Figure 3-1. CXCR3 is upregulated on KLF2 deficient SP thymocytes. A) Relative chemokine receptor gene expression from sorted WT and CD4Cre/KLF2$^{fl/fl}$ (KLF2KO) thymocytes. CD4 or CD8 SP, TCRβ+ cells were sorted using a "dump strategy" to exclude CD25, TCRγ, NK1.1, CD1^{d}/αGal-cer tetramer binding cells. RNA was prepared from cells of 2 independent sorts from 2 animals and 2 littermate controls. Two independent cDNA preparations were made from each RNA sample. B) Flow cytometry histograms of dump negative CD4 or CD8 SP thymocytes. Black line indicates KLF2 KO and gray shaded histogram indicates the WT. In $S1P_1$ histograms, the dark gray, broken line represents staining with a control, anti-GST antibody. "*nd*" indicates not detected. Data in B are representative of independent analysis from at least 3 animals of each genotype.

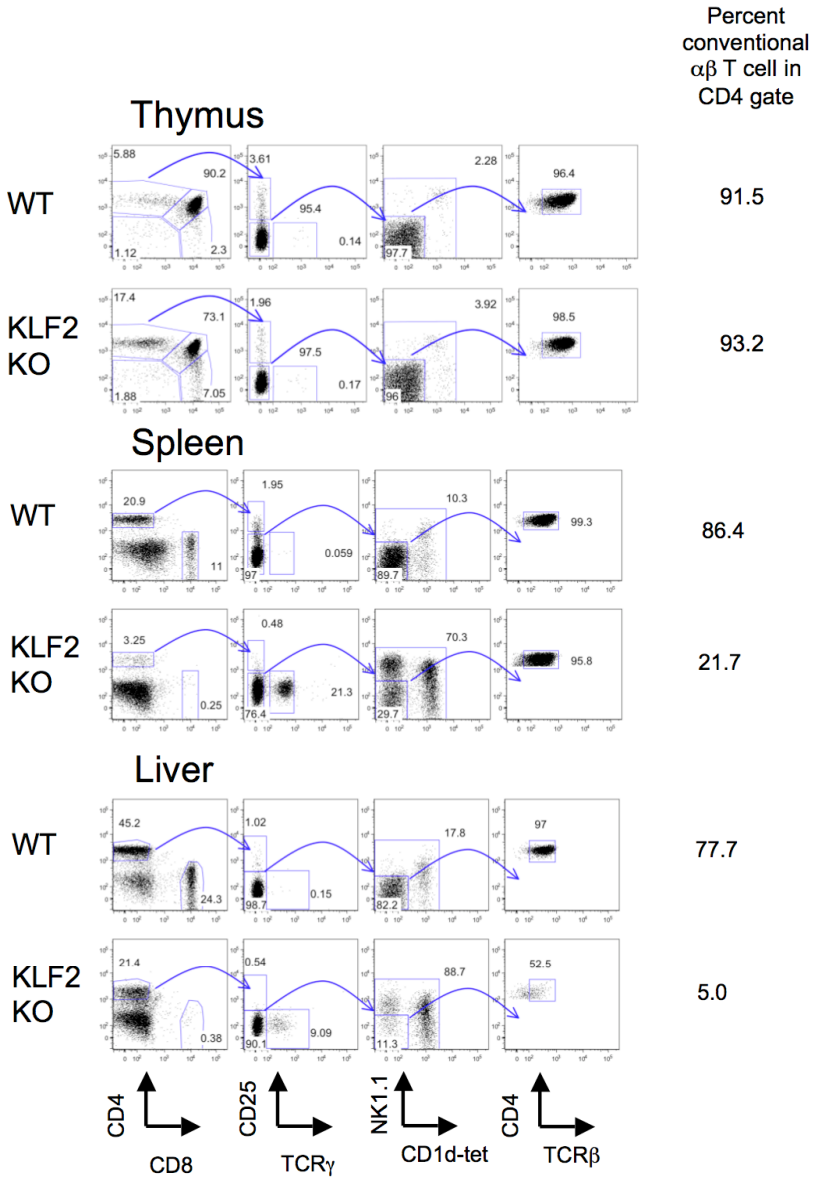


Figure 3-2. Nonconventional T cells are overrepresented in the CD4 gate of *Klf2*-/- mice. Iterative gating strategy of thymus, spleen, and liver from left to right: all lymphocytes, CD4 single positive, CD25- and TCRγ-negative, NK1.1- and CD1d/αGal- cer tetramer-negative. Numbers indicate the percentage of relevant gate. Numbers to the right indicate percentage of the respective CD4 SP gate that is TCRβ+, and CD25-, TCRγ-, NK1.1-, CD1d-tet-. The increase in proportion of nonconventional T cells in periphery is a result of the dramatic decrease in conventional T cells not an increase in nonconventional T cells. For example, in the spleen there was a 25-fold decrease in conventional T cell number and a 1.4-fold decrease in nonconventional T cell number comparing *Klf2*-/- to WT mice. Results are representative of 4 mice in each group.

During our study of CXCR3 on KLF2 deficient T cells, we noticed variation in the expression of CXCR3. In particular, CD8 T cells often expressed more CXCR3 than CD4 T cells, and the percentage of KLF2 deficient T cells expressing CXCR3 increased with age. In contrast, direct KLF2 targets—CD62L and $S1P_1$—were consistently downregulated in KLF2 deficient T cells, independent of age (data not shown). We hypothesized that an altered thymic environment induced by KLF2 deficiency might lead to the increased CXCR3. To investigate the possibility of a bystander effect caused by KLF2 deficient cells, we set up mixed bone marrow chimeras where KLF2 deficient progenitors were a majority and WT cells were a minority (Figure 3-3A). Allelic markers, Thy1.1 and CD45.2, were used to distinguish donor populations from each other, and from host cells. Gating on CD4SP thymocytes from such chimeras, CD62L expression was reduced only in KLF2 deficient cells (upper left panel figure 3-2B), and not in WT progenitors (upper right panel figure 3-3B), confirming direct regulation by KLF2. As expected, CXCR3 was upregulated on KLF2 deficient thymocytes, and in chimeras of this age the CXCR3 upregulation was more striking on CD8SP than CD4SP (lower left panels figure 3-3B). Clearly, CXCR3 was also expressed on WT thymocytes in the predominantly KLF2 deficient thymus (lower right panels figure 3-3B), again more so on CD8SP than CD4SP. The expression of CXCR3 on WT cells indicates that KLF2 deficiency in T cells leads to cell extrinsic upregulation of CXCR3 on bystander cells.

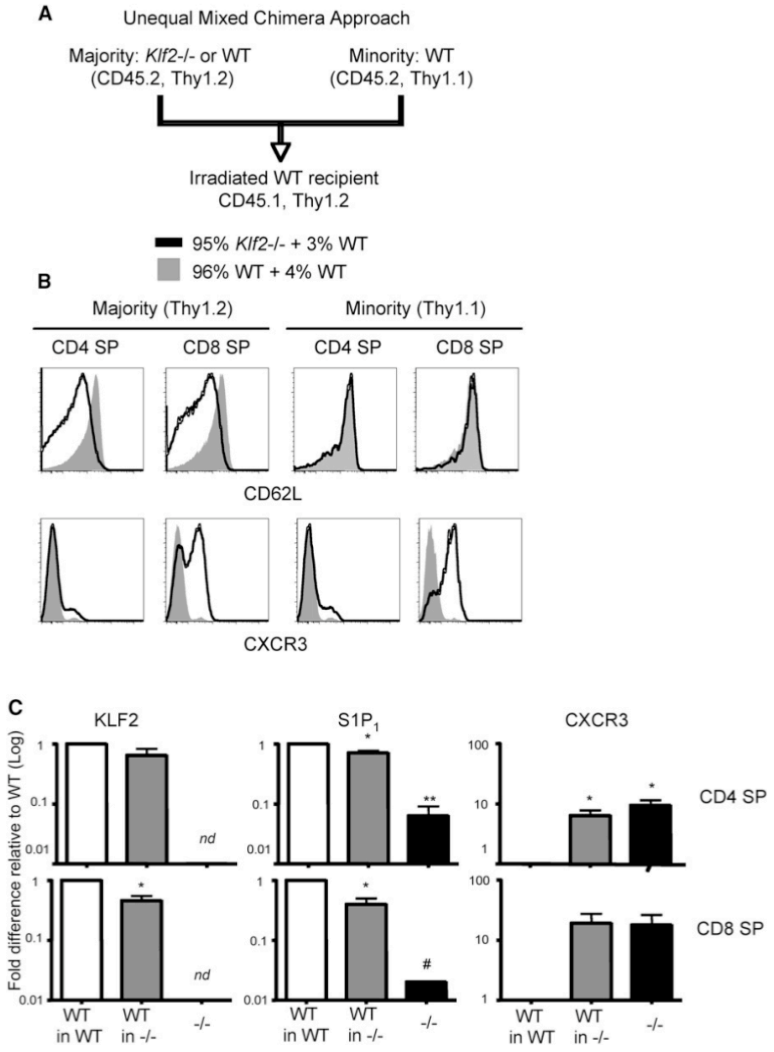


Figure 3-3. CXCR3 is expressed on wild type thymocytes in KLF2 deficient environment. Mixed bone marrow chimeras were created by mixing bone marrow from Thy1.1+/CD45.2+ WT mice with that from Thy1.2+/CD45.2+ KLF2 KO mice at unequal ratios and reconstituting CD45.1+ lethally irradiated recipients. A) Schematic for unequal mixed bone marrow chimera set up. B) Flow cytometry for surface expression of CD62L and CXCR3 on "dump negative" CD4 or CD8 SP thymocytes, as defined in figure 1 and gated as indicated. The observed chimerism ratio in SP thymocytes for these particular 8 week post-transplant animals is shown. Similar results were observed in over 10 chimeric animals in multiple experiments. (C) Single positive thymocytes were sorted from wild-type (Thy1.1+) cells from a wild-type thymus (left bar), wild-type (Thy1.1+) cells from a KLF2- deficient majority mixed-chimera thymus (middle bar), or KLF2-deficient cells from a KLF2-deficient thymus (right bar). All values are shown relative to the value from wild-type cells sorted on the same day. Graph is of the mean ± SD of four PCR experiments and two separate sorts. y axis is on a logarithmic scale. ''nd'' indicates not detected. # indicates detection in only one PCR reaction. *$p < 0.05$, **$p < 0.0001$.

It would still be possible that CXCR3 might be both directly and indirectly regulated by KLF2. However, in chimeras in which WT cells predominate, KLF2 deficient thymocytes did not express any detectable CXCR3 (Figure 3-4), suggesting that CXCR3 is only indirectly regulated by KLF2. CXCR3 was upregulated at the mRNA level in the WT bystander cells from the KLF2 deficient environment (Figure 3-3). In contrast, $S1P_1$ mRNA levels were dramatically lower in KLF2 deficient T cells than in WT bystander cells (Figure 3-3) confirming direct regulation by KLF2. Thus, it would appear that KLF2 does not directly repress chemokine receptors, but its deficiency leads to upregulation of CXCR3 on bystander cells.

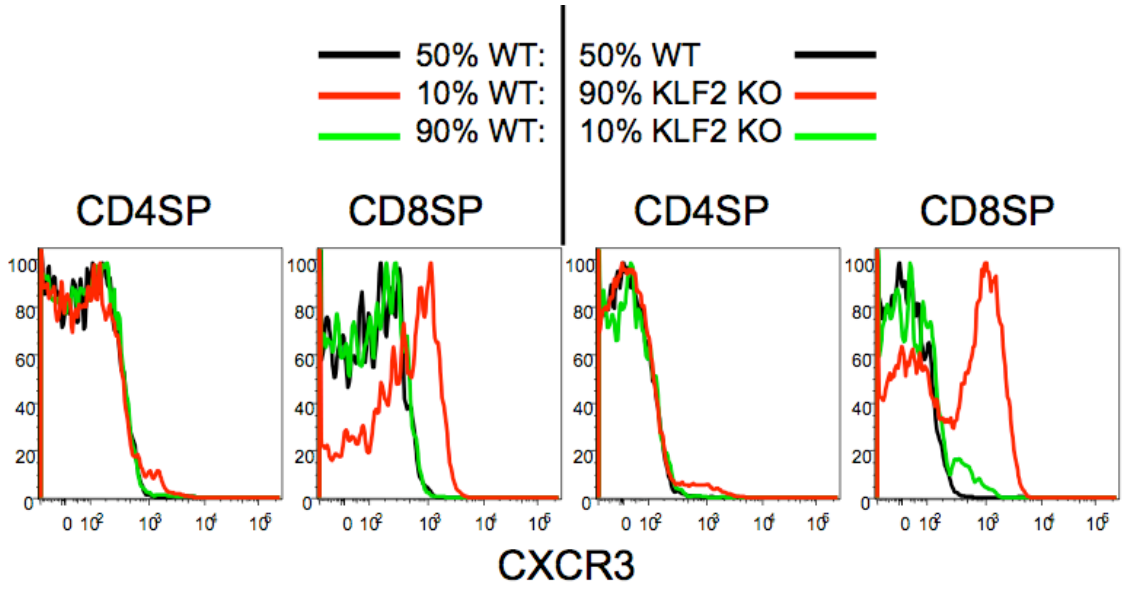
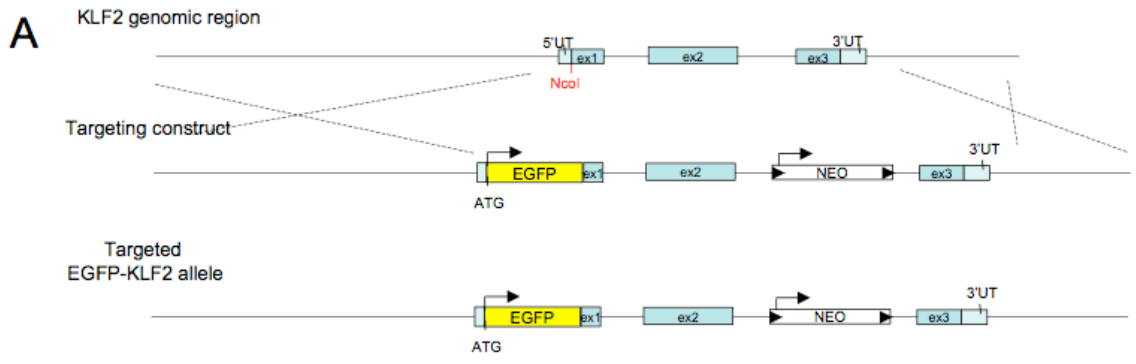


Figure 3-4. A predominance of *Klf2*$^{-/-}$ thymocytes is necessary for CXCR3 expression on *Klf2*$^{-/-}$ cells. Flow cytometry for surface expression of CXCR3 on "dump negative" SP thymocytes from the indicated mixed bone marrow chimeras. Matching colors indicate the same animal. Black= 50% WT:50% WT control, red= 10% WT: 90% *Klf2*$^{-/-}$, and green= 90% WT: 10% *Klf2*$^{-/-}$. Results are representative of greater than 5 independent chimera setups with 1-3 mice/group in each.

KLF2 and CXCR3 are co-expressed in memory phenotype cells in vivo

The hypothesis that KLF2 directly represses CXCR3 would predict that KLF2 and CXCR3 would not be mutually expressed *in vivo*. However, our findings would suggest instead that KLF2-deficiency has an indirect effect on CXCR3 expression. To explore this concept further, we wanted to assess expression of KLF2 and CXCR3 in individual T cells. The lack of detection of KLF2 by flow cytometry with commercially available antibodies has not allowed these studies. To overcome this obstacle, we generated knock-in mice where GFP was inserted in-frame at the KLF2 translational start site in exon 1, creating a GFP-KLF2 fusion protein (Figure 3-5A). The viability and similar numbers of CD4 and CD8 T cells compared to littermate nontransgenic, represented by the spleen (figure 3-5B) of homozygous KLF2^{GFP} reporter mice implies that the GFP-KLF2 fusion protein is functional, in lieu of the embryological lethality of KLF2 KO mice. Furthermore, these mice appear to be faithful reporters of KLF2 expression, as GFP was not expressed until the mature (Qa2 hi, CD69 low) stage of SP thymocyte development (Figures 3-5C-F), consistent with what was previously reported for mRNA analysis (26).



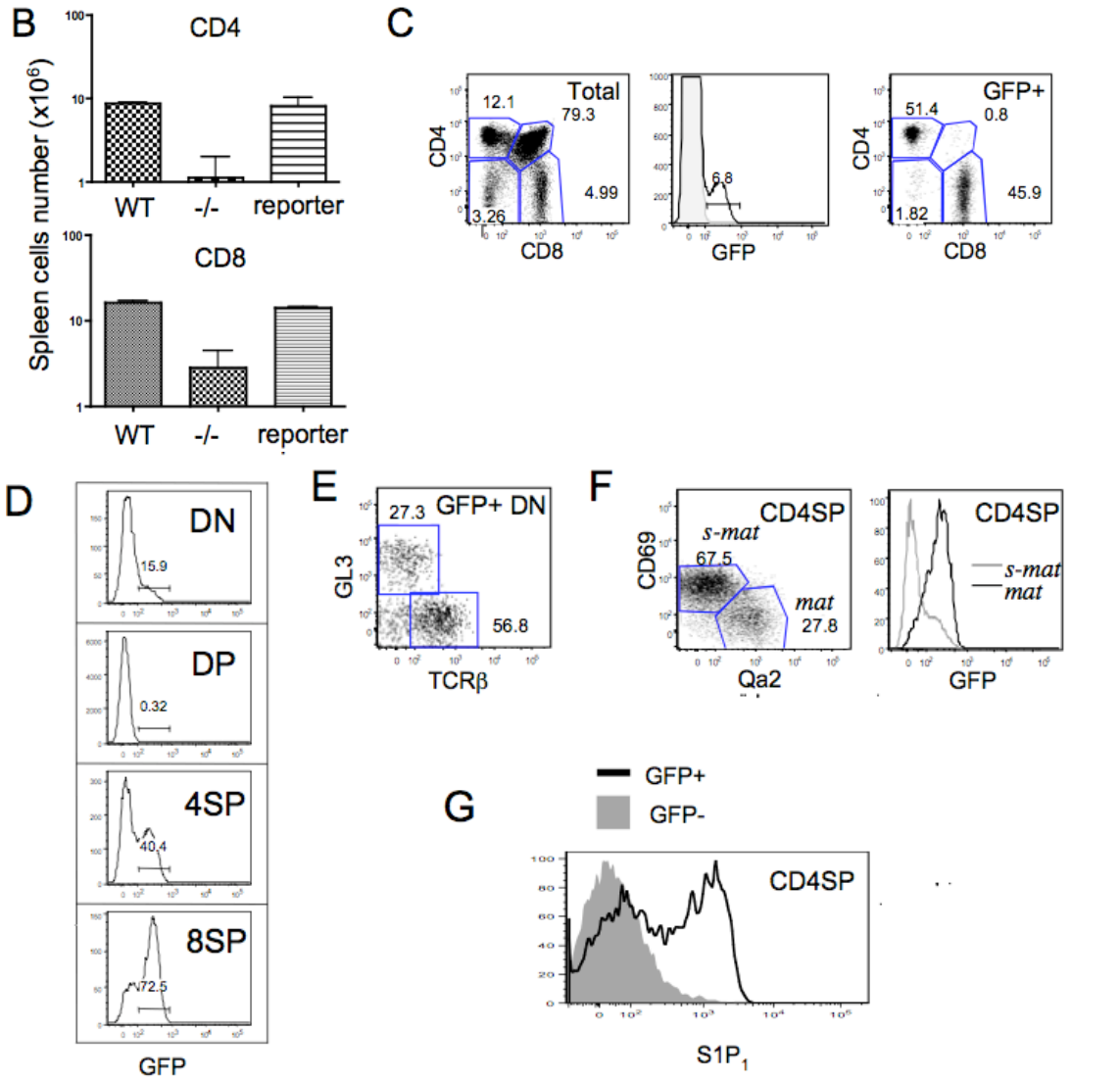


Figure 3-5. A KLF2-GFP fusion knock-in mouse faithfully reports KLF2 expression. A) Strategy used to engineer the *Klf2*GFP knock-in mouse. B) Number of TCRβ+, "dump negative" CD4 and CD8 splenocytes from age-matched wild type (WT), *Klf2*-/-, and *Klf2*GFP knock-in (reporter) mice. C-G) Flow cytometric analysis of thymus from homozygous *Klf2*GFP mice. C) Left panel shows the CD4 by CD8 profile of all thymocytes. The middle panel shows GFP expression on total thymocytes, black line= *Klf2*GFP reporter and shaded gray= WT control. Right panel shows the CD4 by CD8 profile of GFP+ thymocytes from *Klf2*GFP mice. D) GFP expression in the indicated thymocyte subset. Numbers indicate percent GFP+ cells in that subset. E) TCRβ and TCRγ expression in the GFP+ DN gate. F) Left panel shows gating for semi-mature and mature CD4SP. Histogram overlays (right) show GFP expression of semi-mature (gray) and mature (black) CD4 SP thymocytes from *Klf2*GFP mice. G) Histogram overlays show $S1P_1$ cell surface expression of GFP- (filled gray) and GFP+ (black line) in CD4 SP thymocytes. Results are representative of at least 2 mice.

Since thymocytes do not normally express CXCR3, we examined KLF2 and CXCR3 expression in peripheral T cells. Interestingly, memory T cells (CD44 high) show heterogeneity in KLF2 expression (Figure 3-6), consistent with the fact that KLF2 is downregulated during T cell activation, but can be upregulated again (42). In these memory phenotype cells, CD69 and KLF2 (GFP) are inversely correlated (Figure 3-6 middle panels). This is consistent with the upregulation of CD69 observed on KLF2 deficient T cells (34) as CD69 and $S1P_1$ have been show to antagonize each other on the cell surface (53). Thus the inverse correlation between CD69 and KLF2 may represent a positive correlation between $S1P_1$ and KLF2, as expected from mRNA analysis. We find that $S1P_1$ surface expression was limited to KLF2 expressing (GFP+) thymocytes (Figure 3-5G). In addition, we found that $S1P_1$ mRNA was expressed at greater than 40 fold higher level in GFP+ compared GFP- thymocytes (data not shown). An inverse correlation would also be expected if KLF2 directly repressed chemokine receptors, such as CXCR3. However, there was no correlation between KLF2 (GFP) expression and CXCR3 in either CD4 or CD8 memory phenotype T cells (Figure 3-6, bottom panel). Thus our data do not support direct repression of chemokine receptors by KLF2.

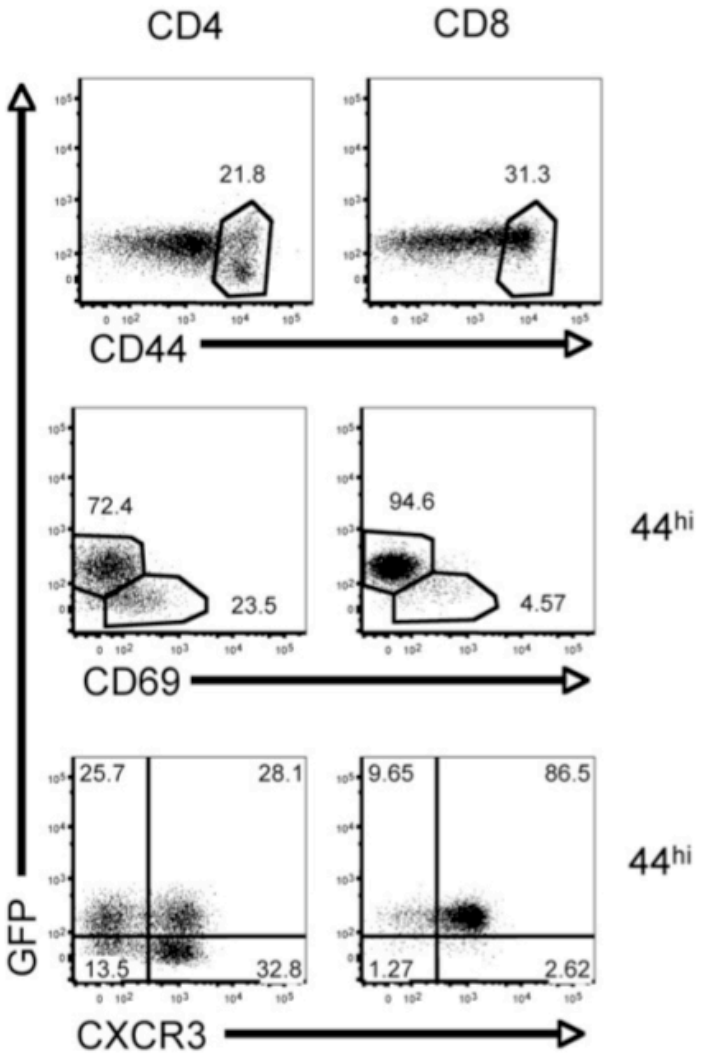


Figure 3-6. A KLF2-GFP reporter mouse shows no correlation between KLF2 and CXCR3 in CD4 or CD8 memory T cells. Top panel: CD44 expression on "dump negative" CD4 and CD8 T cells from spleen of KLF2-GFP reporter mice (see Supplementary Figure 2). Middle and bottom panels show flow cytometry dot plots from the gated CD44 high "memory phenotype" CD4 (left) and CD8 (right) populations. Similar results were observed in two other KLF2-GFP reporter mice.

Thymic retention and peripheral lymphopenia are cell intrinsic

Despite the fact that KLF2 does not directly regulate chemokine receptors, it is possible that the cell-nonautonomous upregulation of CXCR3 in KLF2 deficient mice could be responsible for lymphopenia by sequestering T cells in non-lymphoid organs.

If peripheral lymphopenia was solely due to inappropriate chemokine receptor expression, then we would expect that CXCR3-expressing WT T cells in mixed bone marrow chimeras would also be reduced in the periphery. Alternatively, if lymphopenia is due to cell-autonomous gene regulation in KLF2 deficient thymocytes (for example reduced $S1P_1$), we would expect KLF2 deficient T cells to be preferentially absent in the periphery. To answer this question we analyzed recent thymic emigrants (RTE) in mixed bone marrow chimeras. For this analysis, a covalent label is injected directly into the thymus, and peripheral tissues are evaluated two days later for cells bearing the label. The mixed bone marrow chimera approach not only allows analysis of cells with and without KLF2 in the same animal, it eliminates variability due to differential marking from the intrathymic injections. KLF2 is not expressed until the SP stage in thymocyte development so we standardized each chimeric animal to the ratio of KO:WT in the DP population. The ratio of KLF2 KO:WT increased from 1 in DP thymocytes to approximately 2 in SP thymocytes, consistent with an increased retention of mature KLF2 deficient thymocytes (Figure 3-7 and (34). In contrast, the KLF2 KO:WT ratio dramatically decreased in CD4 and CD8 RTE in peripheral lymphoid organs indicating a strong preferential emigration of WT cells. We also found that WT RTE predominated in the liver as well, arguing against the idea that KLF2 deficient T cells exit the thymus normally but are preferentially sequestered in non-lymphoid tissue (61). Thus our data support a model of cell-intrinsic lymphopenia in KLF2 deficient mice, due in large part to impaired emigration from the thymus.

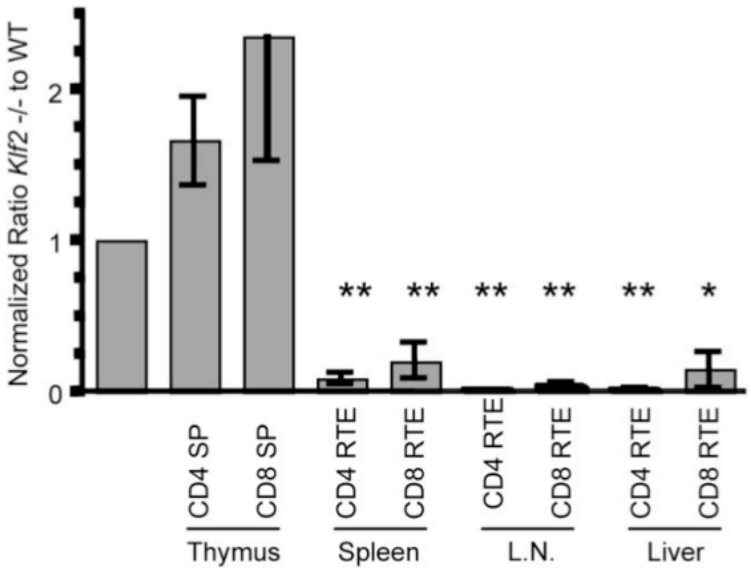


Figure 3-7. KLF2 deficient cells are defective in thymic emigration. Recent thymic emigrants (RTE) were measured in mixed bone marrow chimeras by analysis of peripheral tissues 48 hours after intrathymic injection of a biotinylating agent. The graph displays the ratio of KLF2 deficient to WT cells in various populations. To allow comparison between different experiments, the ratio in DP was normalized to one. Thus a ratio greater than one signifies an increase in KLF2 deficient cells relative to WT, and less than one means relatively more WT cells. Error bars indicate the standard deviation. N=8 chimeras for LN and spleen, and N=3 for liver. * $p < 0.05$, ** $p < 0.001$.

KLF2 deficient T cells overproduce IL-4

We next wanted to define the molecular mechanism for the cell-nonautonomous upregulation of CXCR3 in the presence of KLF2 KO T cells. Since KLF2 deficient thymocytes are retained in the thymic medulla, one possibility is that "thymic crowding" could create a situation where CXCR3 is upregulated. Therefore, we examined $S1P_1$ deficient thymocytes, which have a similar thymic emigration defect and retention of mature SP thymocytes (33). Both KLF2 and $S1P_1$ deficient thymi had an increased proportion of mature (HSA low) SP thymocytes. However, only KLF2 deficient SP expressed increased CXCR3 (data not shown). Thus, it is unlikely that cell-nonautonomous effects are due to "thymic crowding".

Another possible mechanism is that KLF2 deficient cells might secrete a factor into the thymic environment, which causes the cell-nonautonomous effect. Using microarray analysis, we found that KLF2 deficient CD4 SP thymocytes express more IL-4 mRNA compared to WT. We confirmed the IL-4 mRNA upregulation by real time PCR (Figure 3-8A). To determine if KLF2 deficient thymocytes had the ability to produce IL-4 protein, KLF2 deficient and WT thymocytes were stimulated directly *ex vivo* with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Significantly more KLF2 deficient CD4 and CD8 SPs produced IL-4 under these conditions (Figure 3-8B). Again, nonconventional T cells, such as NKT and Treg, were excluded from this analysis. Since NKTs are known to rapidly produce IL-4, we created CD4-cre/KLF2$^{fl/fl}$/CD1$^{-/-}$ mice to test if invariant NKT cells were the source (65). We observed the upregulation of CXCR3 on KLF2 deficient SP thymocytes in the presence and absence of invariant NKT cells (Figure 3-9). This suggests that KLF2 deficiency increases the potential for rapid IL-4 production in conventional T cells.

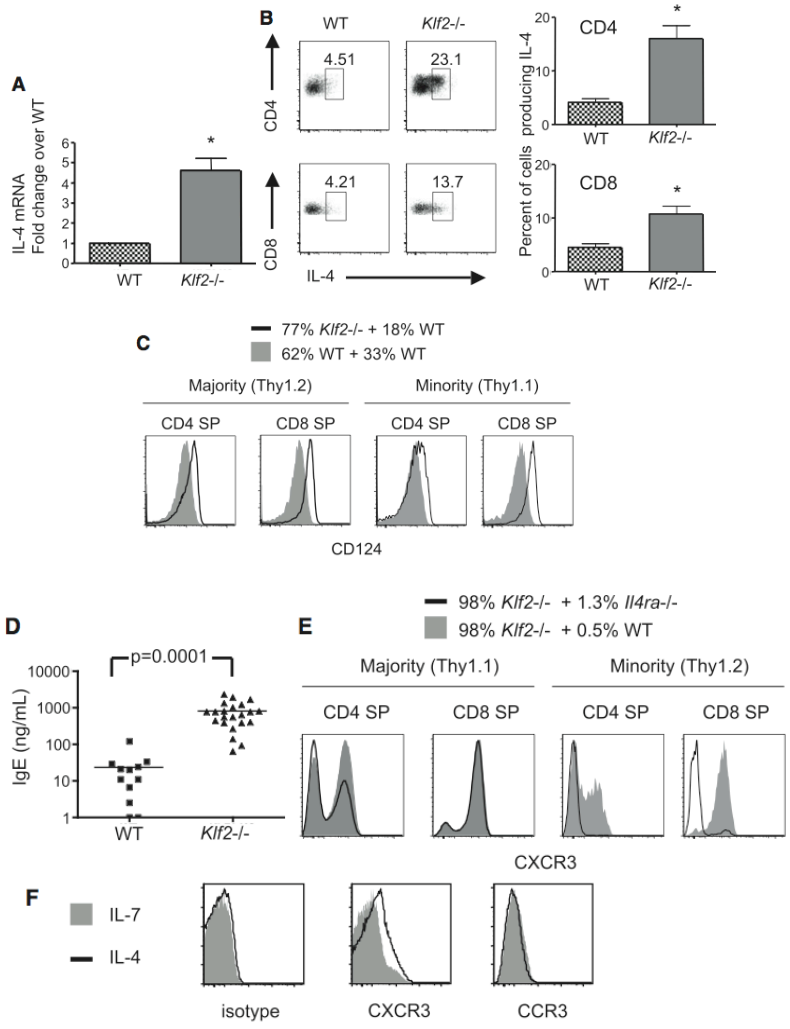


Figure 3-8. Bystander upregulation of CXCR3 is dependent on the IL-4 receptor. A) Quantitative RT PCR analysis of IL-4 mRNA from sorted "dump negative" CD4SP thymocytes from WT and KLF2 KO mice. Graph is of the mean +/- SD of 4 PCR experiments and 2 separate sorts. B) Thymocytes were stimulated *ex vivo* with PMA and ionomycin for 5 hours, and then intracellular staining was done for IL-4. "Dump negative" CD4SP and CD8SP are shown in the dot plots. The bar graph on the right shows the average percent of IL-4 secretion from 4 paired sets of animals in one experiment. The experiment was repeated 3 times with similar results. * $p < 0.05$. C) Flow cytometry histograms of CD124 surface expression on SP thymocytes from mixed chimeras, gated as in figure 2b. D) Serum IgE levels. Serum was collected from mice 40-100 days old. WT: N=12, KLF2 KO: N=22. E) Flow cytometry from the indicated mixed chimeras for surface expression of CXCR3 on "dump negative" SP thymocytes. Data are representative of greater than 5 experiments. Percentages indicate the proportion in "dump negative" CD4SP gate. F) CXCR3 and CCR3 expression on mature (Qa-2 high) CD8 SP thymocytes after 4 days of *in vitro* culture with IL-7 (filled gray) or IL-4 (black line). Results were representative of 5 independent experiments.

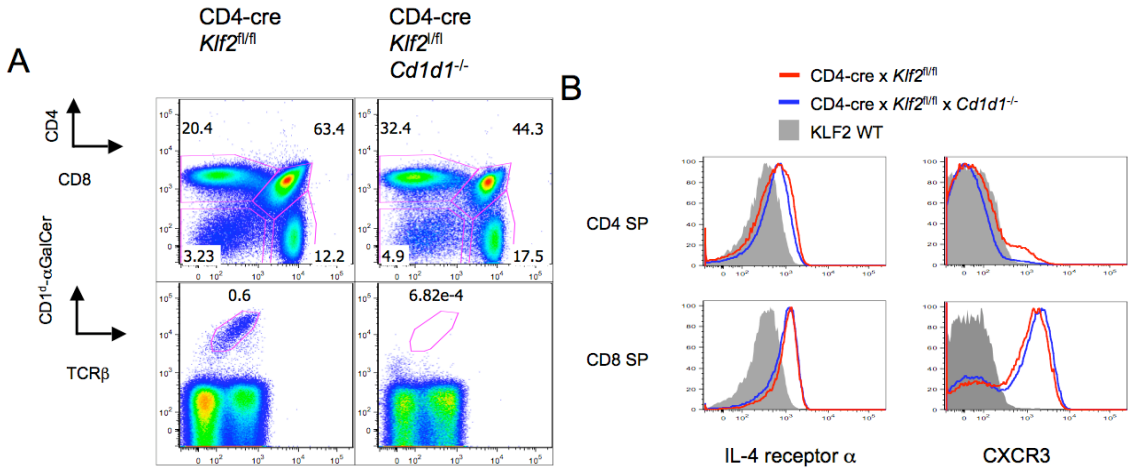


Figure 3-9. CXCR3 upregulation in the KLF2 deficient thymus occurs in the absence of CD1-selected thymocytes A) Top panel: CD4 by CD8 profile of CD4Cre-*Klf2*$^{fl/fl}$ and CD4Cre-*Klf2*$^{fl/fl}$-*Cd1d*$^{-/-}$. Bottom panel: CD1$_d$-αGalCer by TCRβ profile of all thymocytes. B) Histogram overlays of flow cytometric analysis of the IL-4 receptor α and CXCR3 on gated TCRβ+, "dump negative" CD4 SP and CD8 SP thymocyte populations, red line= CD4Cre- *Klf2*$^{fl/fl}$, blue line= CD4Cre-*Klf2*$^{fl/fl}$-*Cd1d* $^{-/-}$ and shaded gray= WT control. Results are representative of at least 2 mice per group and independent experiments.

To determine if more IL-4 is produced in vivo as well, we analyzed the level of IL-4 receptor α (CD124), since IL-4 signaling leads to increased surface expression of CD124 (66). CD124 levels were modestly, but consistently upregulated on thymocytes from KLF2 deficient mice (data not shown). This was also the case for both bystander WT and KLF2 deficient thymocytes in mixed chimeras where KLF2 deficient cells were the majority (Figure 3-8C). Since IL-4 is a major regulator of Th2 differentiation (67), we looked for systemic effects of increased IL-4 levels by analysis of serum IgE, a classical type 2 antibody (68). KLF2 deficient mice had a striking 33-fold increase in serum IgE compared to age matched controls (Figure 3-8D). To distinguish a specific IgE upregulation from broad hypergammaglobulinemia we assayed a broader range of isotypes. IgM, IgA, IgG_3, IgG_{2a} and IgG_{2b} were not significantly changed between the WT and KLF2 deficient mice. IgG_1 was significantly but more modestly upregulated

compared to IgE (Figure 3-10). This is consistent with IL-4's known role in inducing IgE and IgG_1 (69). Thus, T cell specific KLF2 deficiency leads to elevated IL-4 *in vivo* and systemically elevated IgE in the serum.

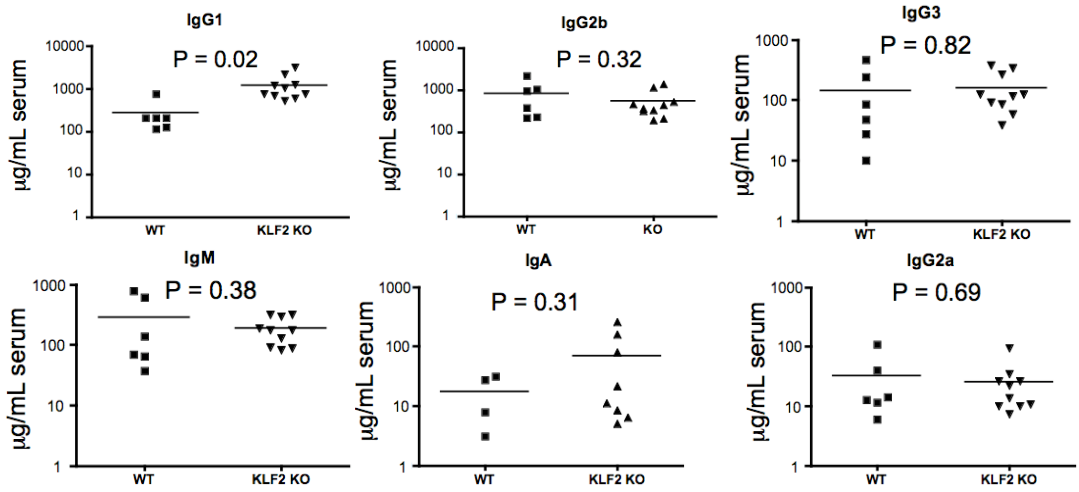


Figure 3-10. Limited overexpression of other Ig isotypes in mice with KLF2 deficient T cells. Serum IgG_1, IgM, IgA, IgG_3, IgG_{2a} and IgG_{2b} levels. Serum was collected from mice 45-160 days old. WT: N=6, *Klf2*$^{-/-}$: N=10.

To test if CXCR3 expression in the KLF2 deficient thymus was dependent on IL-4 responsiveness, we generated mixed chimeras where the majority was KLF2 deficient progenitors and the minority was either WT or IL-4Rα KO. Again, WT bystander cells in the KLF2 deficient environment expressed CXCR3. However, IL-4Rα KO bystander cells did not show CXCR3 upregulation suggesting they are resistant to this bystander effect (Figure 3-8E right panels). Importantly, the KLF2 deficient cells from the same thymus as the IL-4Rα KO cells did express CXCR3 (Figure 3-8E left panels). This indicates that CXCR3 induction is strictly dependent on IL-4.

IL-4Rα can pair with either the common γ chain to produce type I IL-4 receptor or pair with IL-13 receptor for the type II receptor (70). In mixed chimeras, IL-13R KO bystander cells in a KLF2 deficient thymus still upregulated CXCR3 (Figure 3-11), suggesting that type II IL-4 receptors are dispensable. Hence, taken together, these data suggest the type I IL-4 receptor (IL-4Rα/common γ chain) is required for bystander T cell upregulation of CXCR3.

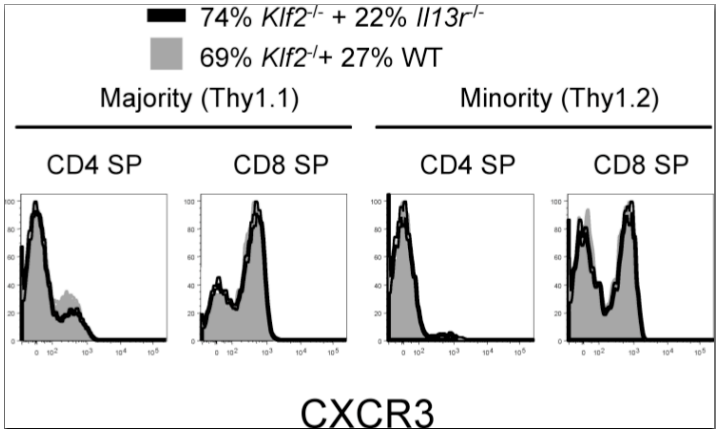


Figure 3-11. IL-13R deficient cells are susceptible to the bystander effect. Flow cytometry of CXCR3 expression on "dump negative" SP thymocytes from the indicated mixed chimeras. Percentages indicate the proportion in "dump negative" CD4SP gate. Results are representative of 3 mice in each group.

From past work, it was shown that CXCR3 is primarily induced during Th1 helper responses; yet IL-4 skews T cells toward a type 2 response (71). Thus our finding of CXCR3 upregulation due to IL-4 was unexpected. However, the paradigm of CXCR3 association with Th1 cytokines was established in activated CD4 T cells, and in our model CXCR3 is expressed most strongly on naïve CD8 thymocytes. Hence, we directly tested the impact of IL-4 influencing CXCR3 expression on naïve CD8 T cells. Interestingly, *in vitro* culture of thymocytes and splenocytes showed that IL-4 (with or without IL-7), but not IL-7 alone, induced expression of CXCR3 but not CCR3, on CD8

SP thymocytes and mature T cells (Figure 3-8F and data not shown). Thus, we propose that KLF2 deficient T cells spontaneously produce IL-4, and this cytokine efficiently upregulates CXCR3 expression on both WT and KLF2 deficient naïve CD8 T cells.

CXCR3 upregulation requires Eomesodermin

CXCR3 expression in T cells is dependent on the T box transcription factors eomesodermin (eomes) and/or T-bet (72, 73). Again, our microarray analysis identified the upregulation of eomes mRNA on KLF2 deficient SP thymocytes. We confirmed that both KLF2 deficient CD4 and CD8 SPs have higher expression of eomes mRNA compared to WT by real time PCR (Figure 3-12A). While eomes was over-expressed, the highly homologous transcription factor T-bet was not (Figure 3-12A). Previous studies showed that Tc2 skewing, using IL-4, can induce eomes in antigen stimulated CD8 T cells *in vitro* (74). Thus, we wished to determine if eomes was required for the bystander CXCR3 upregulation in KLF2 deficient mice.

A T cell specific eomes deficient mouse strain has recently been described (73), and these animals display normal thymic development. To probe the role of eomes in bystander CXCR3 induction, we again generated mixed chimeras comprising a majority of KLF2 deficient cells with a minority of WT or CD4-Cre $Eomes^{fl/fl}$ bone marrow. When KLF2 deficient thymocytes were the majority, they expressed CXCR3 (Figure 3-12B left panels). As expected WT bystander cells expressed CXCR3, however eomes deficient bystander cells did not upregulate CXCR3 (Figure 3-12B right panels). These data suggest that eomes is essential for the induction of CXCR3 in the bystander T cell population.

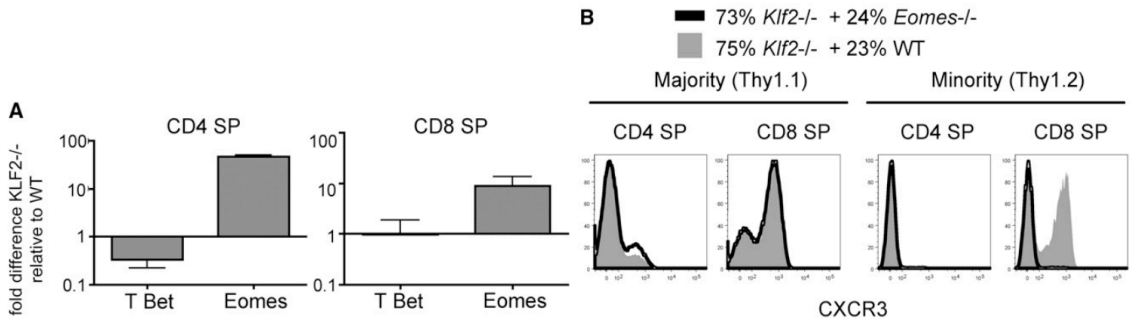


Figure 3-12. CXCR3 upregulation is dependent on Eomesodermin. A) Quantitative RT PCR on RNA from sorted "dump" negative CD4SP and CD8SP thymocytes from WT and intact KLF2 deficient mice. Displayed are KLF2 deficient mRNA levels relative to WT. Graphs represent the mean +/- SD of 4 PCR experiments and 2 separate sorts. B) Flow cytometry from indicated mixed chimeras for surface expression of CXCR3 on "dump negative" SP thymocytes. Results are representative of 3 experiments.

Together, these data suggest a model for CXCR3 upregulation in the KLF2 deficient thymus, as summarized in Figure 3-13. In the KLF2 deficient thymus, bystander CXCR3 upregulation is dependent on IL-4 derived from KLF2 deficient cells, which signals via the type I IL-4R pathway acting through the transcription factor eomesodermin.

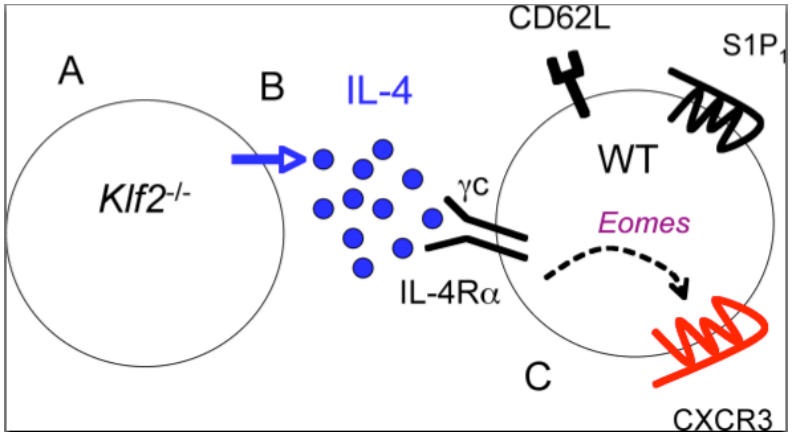


Figure 3-13. Model of KLF2 cell-autonomous and cell-nonautonomous effects. A) KLF2 deficient thymocytes lack CD62L and S1P1 and are retained in the thymus. B) KLF2 deficient thymocytes spontaneously produce IL-4. C) IL-4 signaling through the type I IL-4 receptor and dependent on the transcription factor eomesodermin leads to the upregulation of the chemokine receptor CXCR3 on both WT and *Klf2*-/- (not depicted) thymocytes.

Discussion

Our data provide compelling evidence for a cell-nonautonomous or bystander effect leading to chemokine receptor upregulation in the KLF2 deficient thymus. There is precedence for cell-nonautonomy in many gene deficient mouse models (75) and in *C. elegans* (76). Cell-nonautonomous effects have been previously observed in the thymus as well (77). When tissue specific promoters are used to direct gene deficiency to one cell type, and effects are observed on another cell type, it is easy to conclude that cell-nonautonomous effects are involved. In our case, however, T cell specific gene deletion caused cell-nonautonomous effects on other T cells, which could only be revealed by analysis of chimeric mice where both WT and gene-deleted cells were present in the same population.

The paucity of KLF2 deficient T cells in SLOs and blood has been a consistent finding in all models studied, and published studies agree that KLF2 regulates multiple factors important for T cell migration. But what molecules normally regulated by KLF2 cause the peripheral lymphopenia? Sebdza and colleagues suggested that KLF2 deficiency de-represses multiple chemokine receptors leading to tissue localization of T cells (61). We think this is unlikely to account for the lymphopenic phenotype for several reasons. First, the lack of $S1P_1$ is profound. We were unable to detect $S1P_1$ staining above background on CD4SP or CD8SP. KLF2 and $S1P_1$ T cell deficiency cause a similar accumulation of mature thymocytes (33, 34). In both cases there is a significant (two fold) increase in the number of mature SP thymocytes, which is inconsistent with normal thymocyte emigration. One distinction is the CD62L high phenotype of T cells with $S1P_1$ deficiency, whereas KLF2 deficient T cells have low

CD62L (33). The lack of CD62L on KLF2 deficient T cells is consistent with direct regulation by KLF2 (41, 63). Since CD62L is necessary for lymph node entry (64), this also explains differences in trafficking between KLF2 and $S1P_1$ deficient T cells when adoptively transferred into the blood (33, 34). Secondly, we did not observe preferential homing of KLF2 deficient T cells to the liver, in contrast to a previous report (61). Finally, we observed the upregulation of only one chemokine receptor (CXCR3) rather than an increase in 10 chemokine receptors observed by Sebzda et al. These differences may all relate to the model systems used. Sebzda et al. induced *KLF2* deletion via Cre driven by the Vav promoter (61), which is expressed in all hematopoietic cells (78), while our system (using CD4-Cre) has expression limited to the T cell lineage (79). Furthermore, our analysis was focused on "conventional" T cells, and we excluded cells expressing CD25, TCRγ, NK1.1, and capable of binding $CD1^d$/αGal-cer tetramers. We are currently exploring the role of KLF2 in the homeostasis and migration of nonconventional T cell subsets to address this point further.

While our findings do not support a model in which KLF2 directly represses chemokine receptors, they led us to discover a novel function for KLF2: the cell-intrinsic suppression of IL-4 in T cells. KLF2 deficient thymocytes had increased levels of IL-4 mRNA, and were able to rapidly produce the cytokine upon stimulation *ex vivo*. Elevated serum IgE levels suggested that IL-4 is overproduced *in vivo* as well. NKT cells are major, rapid producers of IL-4. One possibility for the increased IL-4 in the KLF2 deficient thymus is dysregulated homeostasis, trafficking and/or activation of NKT cells. To address this, we generated CD4-cre-$Klf2^{fl/fl}$/$Cd1^{-/-}$ mice, in which

deletion of CD1 drastically reduces the number of NKT cells (65). The thymocytes from these mice still had increased expression of both CXCR3 and CD124 indicating that IL-4 continued to be overproduced. This suggests that invariant NKT cells are not the sole or major provider of spontaneous IL-4 and the bystander effect in this model. We favor the hypothesis that naïve, conventional αβ T cells, which normally have high KLF2 expression and low capacity to produce IL-4, rapidly produce IL-4 in the absence of KLF2. Whether the effect of KLF2 acts directly or indirectly in regulating the IL-4 locus is currently unclear. KLF2 deficient T cells also overproduce IFNγ and TNFα (data not shown). Thus, it is interesting to note that KLF2 deficient "naïve" T cells exhibit some functions similar to "innate immune" T cells.

Overall, our findings suggest KLF2 is an important transcriptional regulator of naïve T cell identity in that it promotes the ability of T cells to recirculate through secondary lymphoid organs, while at the same time repressing rapid cytokine production in the naïve T cell pool.

MATERIALS AND METHODS:

Mice: C57BL/6 (B6) and B6.*SJL-Ptprc*^{a} *Pepc*^{b} (CD45.1 congenic B6) mice were purchased from the National Cancer Institute. B6.*PL-Thy1*^{a} (CD90.1 congenic B6) and BALB/c-*Il4ra*^{tm1Sz} (IL-4 receptor KO) mice were purchased from Jackson Labs. C57BL/6NTac-Tg(CD4-cre*)* (79) were obtained from Taconic farms. KLF2^{fl} mice were created in Jerry Lingrel's laboratory at the University of Cincinnati and are described in a manuscript in preparation. In brief, loxP sites were inserted into introns 1 and 2 of the KLF2 allele. The KLF2 floxed mice were generated in Duffy ES cells (129SvEv/Tac) and backcrossed to the B6 strain for a minimum of 5 generations before being crossed with CD4-cre mice. Ingenious Targeting Labs generated the KLF2^{GFP} reporter mice according to the strategy in figure S3A, using C57BL/6 ES cells. OT-I mice (C57BL/6 TCR transgenic strain specific for $OVA_{257-263}/K^{b}$ (80)) were maintained in our facility. All animal experimentation was conducted according to IACUC guidelines at the University of Minnesota.

Mixed Bone Marrow Chimera: Mixed bone marrow chimeras were generated by mixing T cell-depleted bone marrow preparations from Thy1 distinct, CD45.2+ strains, at various ratios and injecting 5-10x10^{6} total cells into lethally irradiated (1000 rads) CD45.1+ host animals. For chimeras with Eomes KO and IL-13R KO bone marrow, femurs and tibias were shipped overnight on wet ice in RPMI + 10% FCS from Steve Reiner's laboratory (University of Pennsylvania) and Thomas Wynn's laboratory (NIAID), respectively. For chimeras with IL-4R KO bone marrow, the recipients were

treated with NK1.1 specific antibody (clone PK136) intraperiotinally 50 μg one day prior then 25 μg days 7 and 14 after the marrow transplant to deplete NK cells and prevent graft rejection, as the IL-4R KO and control marrow were BALB/c origin and the recipients were C57BL/6 x BALB/c F1 mice. All chimeras were analyzed 8-12 weeks post transplant. Single cells suspensions of thymus, lymph node, and spleen were stained with FACS antibodies and analyzed by flow cytometry.

Flow cytometry: Single cell suspensions from spleen, lymph nodes, or thymus were prepared. Biotinylated $CD1^d$-αGalCer monomers (NIH tetramer facility) were incubated with Streptavidin-PE or Streptavidin-APC for at 4^0 C overnight to create fluorescent multimers**.** Cells were analyzed on Becton Dickinson a LSR II instrument and the data was processed using FlowJo (Tree Star) software. Antibodies to standard mouse lymphocyte surface antigens were purchased from Biolegend (San Diego, CA) or eBioscience (San Diego, CA). In addition, antibodies to CXCR3-PE (R&D Systems (Minneapolis, MN), CCR3-AF647, CCR5-biotin, and CD124-biotin (all from BD Pharmingen, San Diego, CA), were used. A polyclonal antibody to $S1P_1$ was the kind gift of Jason Cyster (University of California, San Francisco) and has been described previously (81). For detection, anti-rabbit IgG bioin (BD Pharmingen) was used followed by Streptavidin-APC (Invitrogen, Carlsbad, CA).

Cell Sorting and Real-time RT-PCR: Fluorescence-activated cell sorting (FACS) was used to purify, "dump" negative CD4SP and CD8SP. To purify the minority population from mixed chimeras, the majority population was depleted by negative selection with

anti-CD90.2 microbeads using MACS separation columns (Miltenyi Biotech, Bergisch Gladbach, Germany). Sorting was performed on a FACSAria (Becton Dickinson) and was reliably >90% of target population. RNA was isolated from sorted populations using the RNeasy kit (Qiagen, Valencia, CA) and cDNA was produced using the SuperScriptIII Platinum Two-Step qRT-PCR kit (Invitrogen), PCR products were amplified using Fast Start SYBR Green Master mix (Roche, Basel, Switzerland), and detected using a SmartCycler (Cepheid, Sunnyvale, CA). Hypoxanthine-Guanine Phosphoribosyl Transferase (HPRT) was used to normalize samples. Each group was sorted in at least two independent experiments and cDNA was prepared twice from each sort.

Primers were designed using the Ensembl database and Primer3. Primers were as follows;

HPRT: CTTCCTCCTCAGACCGCTTT & ACCTGGTTCATCATCGCTAA,

Eomes: CCCCTATGGCTCAAATTCC & GGGGTTGAGTCCGTTTATGTT,

Tbet: AGTTCAACCAGCACCAGACA & GAAGGACAGGAATGGAACA,

$S1P_1$: GTGTAGACCCAGAGTCCTGCG & AGCTTTTCCTTGGCTGGAGAG, and

KLF2: AGCCTATCTTGCCGTCCTTT & CGCCTCGGGTTCATTTC,

IL-4: GAGACTCTTTCGGGCTTTTC & TGATGCTCTTTAGGCTTTCCA,

CCR1: TCAAAGGCCCAGAAACAAAG & GCTGAGGAACTGGTCAGGAA,

CCR3: GCAGGTGACTGAGGTGATTG & GTGGAAAAAGAGCCGAAGGT,

CCR5: AGGAGGTGAGACATCCGTTC & AACTGACCCTTGAAAATCCATC,

CCR6: ATCCTGGGTCTTTCGGACTT & GGCAGTTCAACCACACTCTC,

CCR7: CAGCCTTCCTGTGTGATTTCTACA & ACCACCAGCACGTTTTTCCT,

CXCR1: CCAACAGGCAGGCTTTAGTT & CAGCATCACCAGCGAGTTT,

CXCR2: GCCACTCAGAGAACCTGGAA & ACCAAGGAGTTCCCCACAAG,

CXCR3: AGCCAAGCCATGTACCTTGA & CTCGTTTTCCCCATAATCG,

CXCR5: GGAGACCCCCATAAAGGAAA & ACACCGAGGAGGAAGATGAG,

CXCR6: GCCTGGCTTCTCTTGCATCT & CAGGCTCTTCAGCTTCTGGT,

CXCR7: AGTGTCCCACCATGCCTAAC & TATTCACCCAGACCACCACA,

XCR1: GCACTGGAGGAGATCAAAGG & ATCTGGACGCGGGATG

Quantification of RTEs: Sedated mice were intrathymically injected with up to 10 μL/lobe of 5 mg/mL sulfo-NHS-LC biotin from Pierce Chemical Co. (Rockford, IL) in PBS. 48 hours later, thymus, lymph node, spleen, and liver were harvested and stained with fluorescently conjugated streptavidin and other antibodies and analyzed by flow cytometry. Analysis of streptavidin binding on splenic B cells was routinely performed to ensure that covalent labeling was restricted to the thymus.

***In vitro* IL-4 production:** Thymocytes were isolated and plated at $1x10^6$ cells/mL. The cells were stimulated with 50 ng/mL PMA and 1.5 μM ionomycin both from (Sigma-Aldrich, St. Louis, MO) for five hours with GolgiStop Protein Transport Inhibitor (BD Pharmingen) added for the final three hours. The cells were surface stained then stained with anti-IL-4 (11B11) (eBioscience) using a fix/perm kit (BD Pharmingen) and analyzed by flow cytometry.

Serum IgE: A mouse IgE ELISA MAX kit (Biolegend) was used to quantify IgE from serum samples. Samples were run in duplicate.

Serum immunoglobulin isotype quantification

Serum immunoglobulin isotypes were measured with Beadlyte mouse immunoglobulin isotyping kit from Millipore (St. Charles, MO) according to manufacturer's recommendations. This was used to quantify IgG_1, IgM, IgA, IgG_3, IgG_{2a} and IgG_{2b} from serum samples of mice between 45 and 160 days old. Measurements and calculations of standard curves were made using the Bio-Plex 200 instrument and software from Bio- Rad (Hercules, CA).

In vitro **cytokine stimulation:** Splenocytes and thymocytes from OT-I mice were cultured (1×10^6 splenocytes/ml, 5×10^6 thymocytes/ml) with IL-7 (10 ng/ml), IL-4 (20 ng/ml) or both for 4 days. Cells were analyzed by flow cytometry using an anti-CXCR3-PE (220803) antibody. Cytokines and antibody were purchased from R&D Systems.

Statistical analysis: Statistical analysis was performed using Prism (Graphpad software) unpaired T tests were used for IgE data and paired T tests were used for RT PCR and normalized RTE analysis.

Chapter 4

CXCR3 is necessary for persistence of memory T cells in the thymus

Abstract

T cell migration is a dynamic process dependent on the inflammatory and activation state of the T cell and organism. The first migration step for mature, naïve T cells is emigration from the thymus. We describe a thymic emigration defect for wild type (WT) T cells in mixed bone marrow chimeras where Kruppel-Like Factor (KLF2) deficient T cells were the majority. We found this effect to be dependent on IL-4 signaling. KLF2 and the KLF2-regulated receptor $S1P_1$ are necessary for thymocyte emigration and both KLF2 and $S1P_1$ were reduced in WT cells in the KLF2 deficient environment. However, we found that transgenic expression of $S1P_1$ was not able to correct this defect. To investigate another possible basis for the emigration defect, we show that WT thymocytes in the KLF2 deficient environment also upregulated the chemokine receptor CXCR3. Although CXCR3 ligands are typically expressed in inflamed environments, we found that the CXCR3 ligand CXCL10 is constitutively expressed in the thymus. CXCR3 is normally upregulated after T cell activation and continues to be expressed on memory T cells. We used an infection model, to test if there was a role for CXCR3 on activated T cells in thymic localization. We found that CXCR3 deficient T cells were equivalent to WT in homing to the thymus at the peak of the response but were not retained at later time points, suggesting that S1P1 and CXCR3 can both regulate thymic emigration. Our findings point to a role for CXCR3 in maintenance of memory T cells in the thymus.

Introduction

Our understanding of the mechanisms of T cell trafficking has grown in recent years. Therapeutics to take advantage of this knowledge for immunomodulation are showing promise in the clinic(82, 83). Emigration of developing T cells from the thymus shares many mechanisms with T cell migration elsewhere in the body(32, 84). Thus, findings in the regulation of thymocyte emigration may be applicable to T cell biology in general. For example, the sphingosine-1-phosphate receptor, $S1P_1$, is required for thymocyte emigration(33). $S1P_1$ serves a similar role in regulating T cell egress from the lymph node as it does in the thymus(33, 85).

T cell activation has been shown to inhibit thymocyte emigration(86). However, the role of cytokines in the process of thymocyte emigration has not been as well studied. Observations in transgenic mice have indicated that IL-4 may play a role. Tepper et al. reported an increased proportion of single positive (SP) thymocytes in IL-4 transgenic mice indicating an accumulation of more mature cells, consistent with an emigration defect. However, in this model IL-4 was produced by both T and B cells leading to systemic inflammation which may have complicated the findings in the thymus(87). Additional support for an IL-4 dependent emigration defect came from Lewis et al. In their model IL-4 was primarily detected in the thymus. They reported a striking accumulation of mature cells in the thymus and fewer T cells, particularly CD8s, in the periphery(88).

We have shown using mixed bone marrow chimeras that deficiency in the transcription factor kruppel-like factor 2 (KLF2) leads to cell extrinsic effects in the

thymus(58). In this model WT bystander cells upregulated the chemokine receptor CXCR3 in the KLF2 deficient thymic environment dependent on IL-4 and the transcription factor eomesodermin. Here we show that thymic emigration of the bystander cells was also impaired, and herein explored the molecular mechanism by which this occurred.

Materials and Methods

MATERIALS AND METHODS:

Mice: C57BL/6 (B6) and B6.*SJL-Ptprc*^{a} *Pepc*^{b} (CD45.1 congenic B6) mice were purchased from the National Cancer Institute. B6.*PL-Thy1*^{a} (CD90.1 congenic B6) and BALB/c-*Il4ra*^{tm1Sz} (IL-4 receptor KO) mice were purchased from Jackson Labs. C57BL/6NTac-Tg(CD4-cre*)* (79) were obtained from Taconic farms. KLF2^{fl} mice were created in Jerry Lingrel's laboratory at the University of Cincinnati. The KLF2^{GFP} reporter mice have been described previously(58). Human S1P1 transgenic mice were obtained from and described by Edward Goetzl's laboratory (89). All animal experimentation was conducted according to IACUC guidelines at the University of Minnesota.

Mixed Bone Marrow Chimera: Mixed bone marrow chimeras were generated by mixing T cell-depleted bone marrow preparations from Thy1 distinct, CD45.2+ strains, at various ratios and injecting 5-10x10^{6} total cells into lethally irradiated (1000 rads) CD45.1+ host animals. For chimeras with IL-4R KO bone marrow, the recipients were treated with NK1.1 specific antibody (clone PK136) intraperiotinally 50 μg one day prior then 25 μg days 7 and 14 after the marrow transplant to deplete NK cells and prevent graft rejection, as the IL-4R KO and control marrow were BALB/c origin and the recipients were C57BL/6 x BALB/c F1 mice. All chimeras were analyzed 8-12 weeks post transplant. Single cells suspensions of thymus, lymph node, and spleen were stained with FACS antibodies and analyzed by flow cytometry.

Flow cytometry: Single cell suspensions from spleen, lymph nodes, or thymus were prepared. Cells were analyzed on Becton Dickinson a LSR II instrument and the data was processed using FlowJo (Tree Star) software. Antibodies to standard mouse lymphocyte surface antigens were purchased from Biolegend (San Diego, CA) or eBioscience (San Diego, CA). In addition, antibodies to CXCR3-PE (R&D Systems (Minneapolis, MN) were used. A polyclonal antibody to $S1P_1$ was the kind gift of Jason Cyster (University of California, San Francisco) and has been described previously (81). For detection, anti-rabbit IgG bioin (BD Pharmingen) was used followed by Streptavidin-APC (Invitrogen, Carlsbad, CA).

Cell Sorting and Real-time RT-PCR: Fluorescence-activated cell sorting (FACS) was used to purify, "dump" negative CD4SP and CD8SP. To purify the minority population from mixed chimeras, the majority population was depleted by negative selection with anti-CD90.2 microbeads using MACS separation columns (Miltenyi Biotech, Bergisch Gladbach, Germany). Sorting was performed on a FACSAria (Becton Dickinson) and was reliably >90% of target population. RNA was isolated from sorted populations using the RNeasy kit (Qiagen, Valencia, CA) and cDNA was produced using the SuperScriptIII Platinum Two-Step qRT-PCR kit (Invitrogen), PCR products were amplified using Fast Start SYBR Green Master mix (Roche, Basel, Switzerland), and detected using a SmartCycler (Cepheid, Sunnyvale, CA). Hypoxanthine-Guanine Phosphoribosyl Transferase (HPRT) was used to normalize samples. Each group was sorted in at least two independent experiments and cDNA was prepared twice from each sort.

Primers were designed using the Ensembl database and Primer3. Primers were as follows;

HPRT: CTTCCTCCTCAGACCGCTTT & ACCTGGTTCATCATCGCTAA,

$S1P_1$: GTGTAGACCCAGAGTCCTGCG & AGCTTTTCCTTGGCTGGAGAG, and

KLF2: AGCCTATCTTGCCGTCCTTT & CGCCTCGGGTTCATTTC

Immunohistochemistry:

The immunohistochemistry protocol used was previously described in detail (90). Briefly, thymi were embedded in O.C.T. Compound (Tissue-Tek), snap frozen in a dry ice bath, and stored at −80°C. TSA Fluorescence Systems kit (PerkinElmer) was used for staining. Images were obtained using a microscope (AX70; Olympus) with a camera (MRC 1024; Bio-Rad Laboratories) operating with the LaserSharp software (Bio-Rad Laboratories) and analyzed using ImageJ (National Institutes of Health) and Photoshop (Adobe) software.

***Listeria monocytogenes* infection:** Naïve, CD44 low OT-I T cells were harvested and purified from WT and *Cxcr3*-/- OT-I TCR transgenic using negative selection with the Macs magnetic bead system (Miltenyi). $1x10^5$ OT-I cells of both WT and *Cxcr3*-/- which were CD90 distinct but both CD45.2+ were was mixed with an equal number of adoptively transferred to a CD45.1 host. The next day cells the mice were infected with $3x10^6$ of an attenuated, $ActA^{-/-}$ strain of Listeria monocytogene expressing the ovalbumin protein. Spleen and thymus were harvested and single cell suspensions were

enriched by panning over anti-CD45.1 antibody (A20) coated plates. After enrichment the cells the ratio of OT-I cells was assessed by flow cytometry in the thymus and spleen.

Quantification of RTEs: Sedated mice were intrathymically injected with up to 10 μL/lobe of 5 mg/mL sulfo-NHS-LC biotin from Pierce Chemical Co. (Rockford, IL) in PBS. 48 hours later, thymus, lymph node, spleen, and liver were harvested and stained with fluorescently conjugated streptavidin and other antibodies and analyzed by flow cytometry. Analysis of streptavidin binding on splenic B cells was routinely performed to ensure that covalent labeling was restricted to the thymus.

Statistical analysis: Statistical analysis was performed using Prism (Graphpad software) unpaired T tests were used for IgE data and paired T tests were used for RT PCR and normalized RTE analysis.

Results

KLF2 deficiency causes a cell extrinsic, IL-4 dependent thymic emigration defect

We previously showed using mixed bone marrow chimeras that CD4cre/KLF2$^{fl/fl}$ thymocytes elicit cell extrinsic effects on WT thymocytes(58). We also showed that these cell extrinsic or bystander effects are dependent on IL-4 receptor signaling(58). Previous work with IL-4 transgenic mice has shown that IL-4 in the thymus leads to an increased proportion of mature single positive (SP) thymocytes, consistent with an emigration defect(87, 88). Indeed, we found a similar increased proportion of WT SPs in the IL-4 rich, KLF2 deficient thymus (Fig. 4-1a). Like other IL-4 dependent effects, CD8 SPs were found to have a more severe emigration defect compared to CD4s.

Next we examined the maturation state within the CD8 SP population. We found the CD8s from the KLF2 deficient environment had lower expression of CD24, which decreases as thymocytes mature (Fig. 4-1b). While it is possible that CD24 could be directly regulated by IL-4, we independently assessed the "age" of the SP population using RAG2p-GFP mice. In these mice GFP is expressed under the control of the RAG2 promoter so after thymocytes have been positively selected, GFP production is stopped and GFP expression reflects the kinetics of GFP decay. These mice have been used as a molecular timer to study the age of SP thymocytes and recent thymic emigrants with lower GFP indicating an older cell(26, 50). We found that RAG2p-GFP thymocytes had lower GFP in the KLF2 deficient environment, matching the CD24 data (Fig. 4-1c). We directly analyzed the emigration of thymocytes by intrathymic labeling

and quantifying labeled cells found in the spleen and lymph node 42 hours later. While not statistically significant, there is a trend of less WT recent thymic emigrants from the KLF2 deficient thymus (Fig. 4-1d).

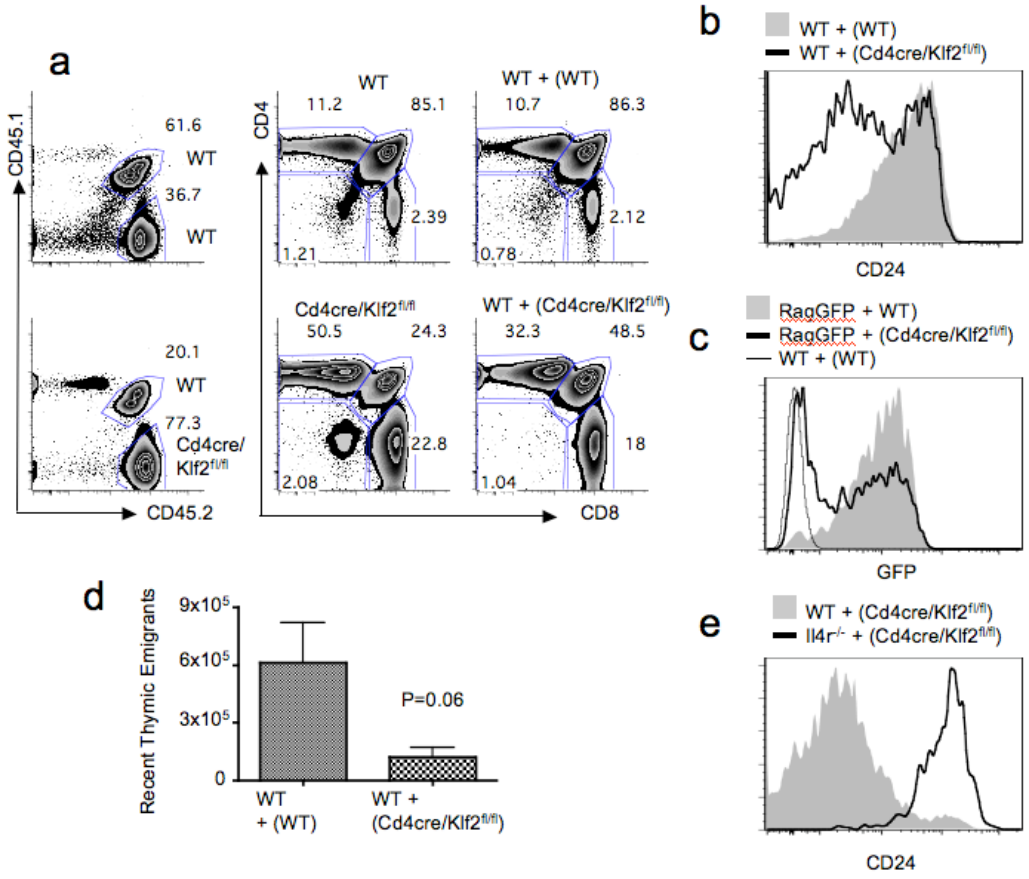


Figure 4-1. IL-4-dependent bystander emigration defect in the KLF2 deficient thymus. a, Thymus of CD4cre/KLF2$^{fl/fl}$ + WT and WT + WT mixed bone marrow chimeras. Left column depicts thymic gating strategy and middle and right columns show CD4 by CD8 profile of the relevant gated populations. b, CD24 expression on WT CD8 SP thymocytes from indicated chimeras. Gray solid = WT + WT and black line = CD4cre/KLF2$^{fl/fl}$ + WT. c, GFP expression on RAG2p-GFP CD8 SP thymocytes from indicated chimeras. Gray solid = WT + RAG2p-GFP, thick, black line = CD4cre/KLF2$^{fl/fl}$ + RAG2p-GFP, and thin black line = GFP negative WT control. d, Quantification of WT bystander CD8 recent thymic emigrants in spleen and lymph nodes. Numbers are normalized for chimerism and intrathymic labeling and numbers indicate calculated RTE number per mouse. n=4, error bars show standard deviation (SD). e, CD24 expression on WT or Il4r$^{-/-}$ CD8 SP thymocytes from CD4cre/KLF2$^{fl/fl}$ majority chimeras. Gray solid = WT and black line = Il4r$^{-/-}$.

Finally to determine if the emigration defect was dependent on IL-4 we generated mixed bone marrow chimeras with a CD4cre/KLF2$^{fl/fl}$ majority and either WT or *Il4rα*$^{-/-}$ as the minority. While WT bystander cells had decreased CD24, *Il4rα*$^{-/-}$ CD8 SPs were found to be CD24 high indicating unaltered thymic emigration (Fig. 4-1e). These results indicate that KLF2 deficiency leads to a bystander, IL-4 dependent retention of mature thymocytes.

IL-4 overexpression leads to a reduction in KLF2 and S1P$_1$ in WT thymocytes

We sought to better understand the mechanisms by which IL-4 regulates thymocyte emigration using the KLF2 deficient mixed bone marrow chimera model. KLF2 has been shown to regulate the sphingosine-1-phosphate receptor S1P$_1$ in T cells(34). Since S1P$_1$ is necessary for emigration from the thymus both KLF2 and S1P$_1$ control thymocyte emigration(33). To determine if KLF2 and S1P$_1$ were affected in WT cells in the KLF2 deficient environment, we sorted WT CD8 SPs from bone marrow chimeras that were either majority CD4cre/KLF2$^{fl/fl}$ or completely WT and assessed RNA expression. We found the bystander CD8 SPs had a modest (two-fold) but significant reduction in both KLF2 and S1P$_1$ mRNA expression (Fig. 4-2a). This finding is particularly surprising since WT cells in the KLF2 deficient environment are more mature and KLF2 and S1P1 usually increase with SP maturation(26). Using the KLF2^{GFP} reporter mouse(58) as bystander cells, we found that protein expression of KLF2 was also decreased on mature CD8 SPs (Fig. 4-2b). S1P$_1$ staining also showed decreased S1P$_1$ surface expression on bystander CD8 SPs (Fig. 4-2c). Together this

data indicates that excess IL-4 signaling leads to a decreased transcription of important T cell migration regulators KLF2 and $S1P_1$.

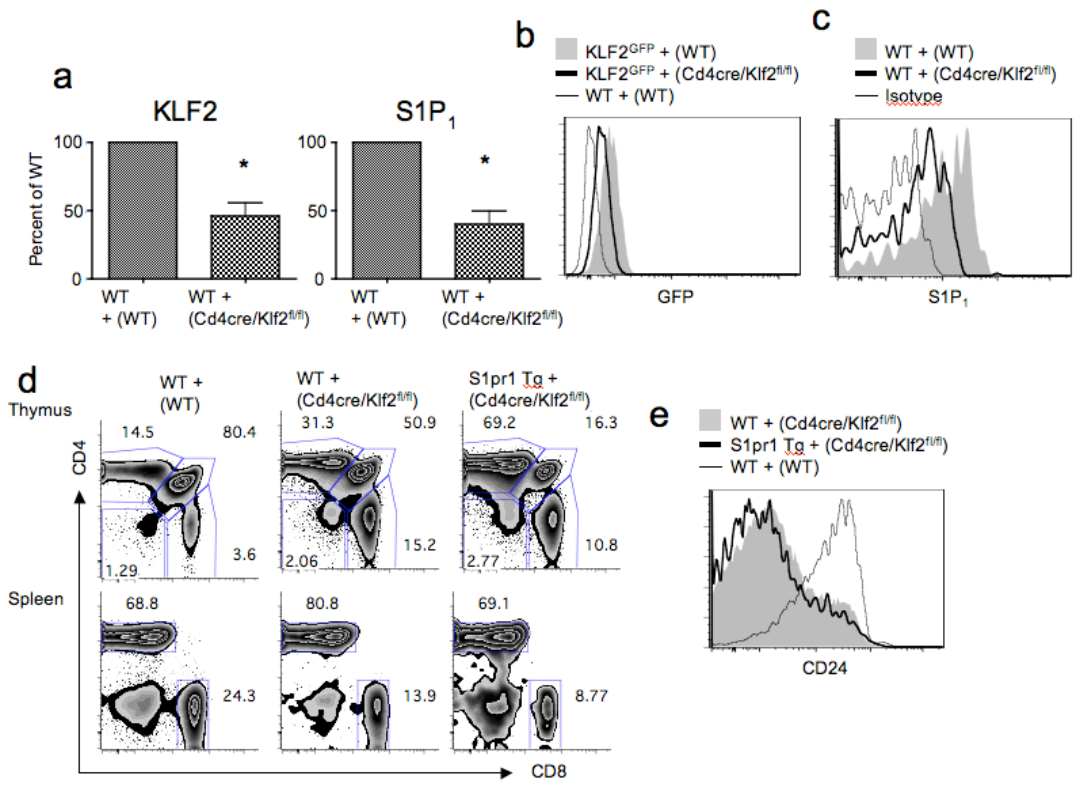


Figure 4-2. KLF2 and $S1P_1$ are reduced on WT CD8s in KLF2 deficient thymus. a, WT CD8 SPs were sorted from CD4cre/$KLF2^{fl/fl}$ + WT and WT + WT mixed bone marrow chimeras. RNA expression for KLF2 and $S1P_1$ relative to HPRT and graphed as percentage of WT + WT value. N=3, error bars show SD. b, GFP expression on $Klf2^{gfp}$ CD8 SP thymocytes from indicated chimeras. Gray solid = WT + $Klf2^{gfp}$, thick, black line = CD4cre/$KLF2^{fl/fl}$ + $Klf2^{gfp}$, and thin, black line = GFP negative WT control. c, $S1P_1$ expression on WT CD8 SP thymocytes from indicated chimeras. Gray solid = WT + WT, thick, black line = CD4cre/$KLF2^{fl/fl}$ + WT, and thin, black line = isotype control. d, CD4 by CD8 profile of the relevant gated populations from mixed bone marrow chimeras. Total thymus show on the upper row and total spleen on the lower. e, CD24 expression on WT or S1pr1 Tg CD8 SP thymocytes from CD4cre/$KLF2^{fl/fl}$ majority chimeras. Gray solid = WT + CD4cre/$KLF2^{fl/fl}$, black line = S1pr1 Tg + CD4cre/$KLF2^{fl/fl}$, and thin line = WT + WT chimera.

To determine if the reduction in KLF2 and $S1P_1$ expression is the cause of the thymic emigration defect, we wanted to overexpress $S1P_1$ on the bystander cells. We obtained mice that have T cell specific expression of human $S1P_1$ generated in the

Goetzl laboratory(89). It has previously been shown that T cells from these mice have increased chemotaxis to the $S1P_1$ ligand S1P. In addition, multiple changes to the T cell response have been described(89, 91-93). We found that $S1P_1$ transgenic and WT T cells had similar accumulation of mature, CD24 low SP thymocytes, when present as bystander cells in KLF2 deficient mixed bone marrow chimeras (Figure 4-2D). Thus, transgenic $S1P_1$ expression was unable to correct the thymic emigration defect.

It is possible that the human $S1P_1$ transgene is not functional to allow migration *in vivo*. While increased trafficking to the blood has been reported with these $S1P_1$ Tg mice, early emigration of thymocytes has not been reported(89). To test the function of this transgene in the thymus, we crossed the S1pr1 Tg to CD4cre/$Klf2^{fl/fl}$ mice. Since $S1P_1$ is downstream of KLF2, if the human $S1P_1$ transgene is functional to cause emigration we would expect the transgene to correct the CD4cre/$KLF2^{fl/fl}$ thymic emigration defect. While CD4cre/$Klf2^{fl/fl}$/S1pr1 Tg continued to have an increased accumulation of mature SPs in the thymus, there was a clear increase in CD8 T cells found in the spleen (Fig. 4-3), thus this S1pr1 transgene showed a partial ability to restore the KLF2 defect. Recently, the Cyster laboratory has published another transgenic expressing mouse S1pr1 that does cause early emigration of thymocytes(31). This transgene caused emigration of thymocytes prior to KLF2 being expressed and it restored the thymic emigration defect of KLF2 deficient T cells more dramatically. The differences seen between these two S1pr1 Tg models may have to do with expression levels, as only the highest expressing mouse S1pr1 transgene restored the KLF2 deficiency effectively. Nonetheless our findings indicate that the S1pr1 Tg used here has the capacity to partially correct a $S1P_1$ dependent emigration defect in a KLF2

deficient model where $S1P_1$ is undetectable(58). Since the S1pr1 Tg did not correct the bystander thymic emigration defect where S1P1 was more modestly decreased, it seems likely other mechanisms might also be at play in this defect.

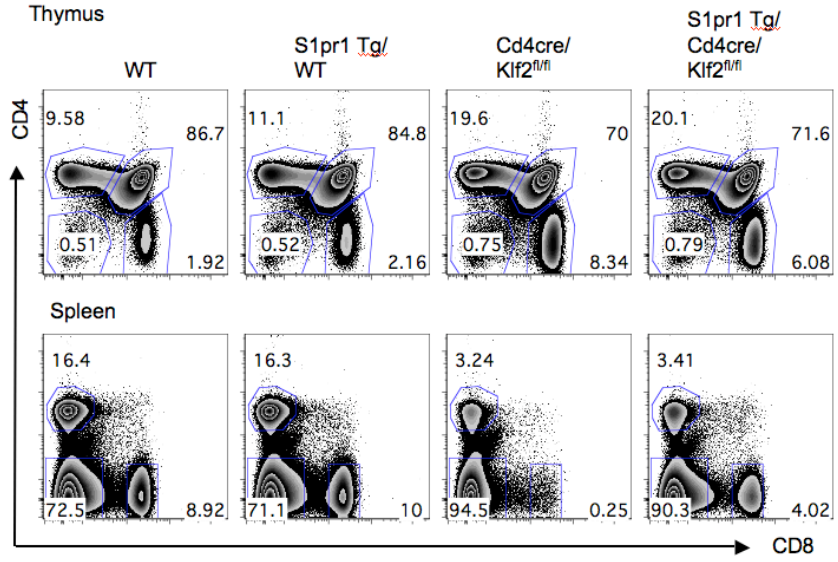


Figure 4-3. Human $S1P_1$ transgene leads to increased emigration of KLF2 deficient CD8 T cells. CD4 by CD8 profiles of indicated mice. Upper row = thymus, lower row = spleen.

The role of the CXCR3/CXCL10 axis in the IL-4 overexpressing thymus

Both NKT cells and conventional T cells derive from the thymus but they differ in a number of ways, including rapid cytokine production and migration behavior(94). While virtually all conventional T cells exit the thymus within days of being functionally mature(26), many mature NKT cells become long-term residents of the thymus(95). Recently, it was shown that the expression of the chemokine receptor CXCR3 is necessary for the retention of NKT cells in the thymus(96). We previously showed that CXCR3 upregulation was a characteristic of the IL-4 dependent bystander effects in the KLF2 deficient thymus(58). In agreement with previous findings(96), we

found that the CXCR3 ligand, CXCL10, is expressed in the thymic medulla by immunohistochemistry (Fig. 4-4a).

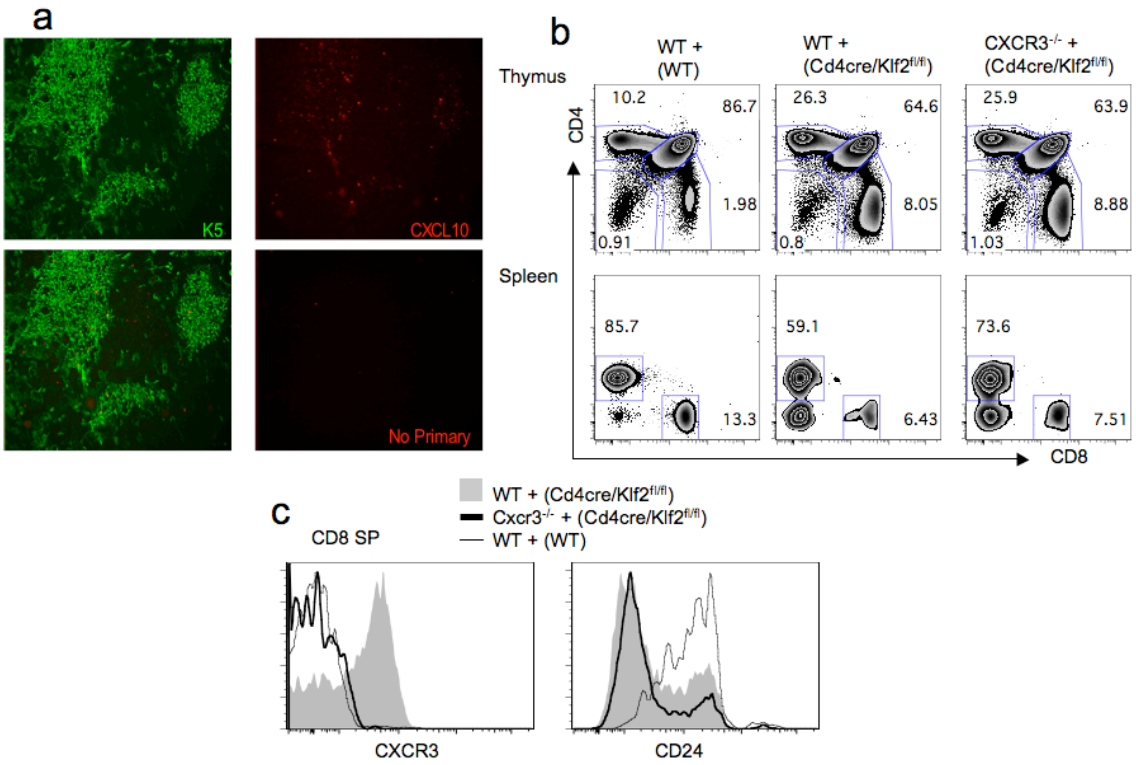


Figure 4-4. Emigration defect in KLF2 deficient thymus is not due to CXCR3 expression on T cells. a, Immunoflorescence of a WT thymus for medullary marker keratin 5 (K5) and chemokine, CXCL10. b, Thymus (upper) and spleen (lower) CD4 by CD8 profiles of WT + WT, CD4cre/KLF2$^{fl/fl}$ + WT and CD4cre/KLF2$^{fl/fl}$ + Cxcr3$^{-/-}$ mixed bone marrow chimeras. c, CXCR3 and CD24 expression on WT or Cxcr3$^{-/-}$ CD8 SP thymocytes from CD4cre/KLF2$^{fl/fl}$ majority chimeras. Gray solid = WT + CD4cre/KLF2$^{fl/fl}$, black line = Cxcr3$^{-/-}$ + CD4cre/KLF2$^{fl/fl}$, and thin line = WT + WT chimera.

To test if the emigration defect was due to CXCR3 expression on bystander cells, we generated majority CD4cre/KLF2$^{fl/fl}$ bone marrow chimeras with WT or Cxcr3$^{-/-}$ as the minority. Mature CXCR3 deficient bystander SPs accumulated in the thymus comparable to WT SPs (Fig. 4-4b,c). It therefore appears that CXCR3 is not solely responsible for the IL-4 dependent thymic emigration defect either.

CXCR3 is necessary for the maintenance of memory CD8 T cells in the thymus

Given the lack of effect on CXCR3 deficient bystander cells, we wanted to test the if CXCR3 could mediate retention of conventional T cells in the thymus during other physiological situations. CXCR3 is upregulated on activated and memory T cells in the context of infections(97). To directly determine the role of CXCR3, we generated Cxcr3$^{-/-}$/OT-I transgenic T cells, specific for ovalbumin (OVA). We cotransferred 10^5 WT and Cxcr3$^{-/-}$ OT-I into congenically marked WT hosts. The next day mice were infected with $3x10^6$ ActA- Listeria monocytogenes expressing OVA. At day 7, the ratio of WT to CXCR3 deficient OT-I cells was similar in the spleen and the thymus (Fig. 4-5 left graph). However, by day 28 the WT OT-I CD8s were found at a significantly higher proportion compared to CXCR3 deficient OT-I T cells (Fig. 4-5 right graph). These findings suggest that CXCR3 is dispensable for entry of activated T cells into the thymus but required for the retention of memory CD8 T cells in the thymus.

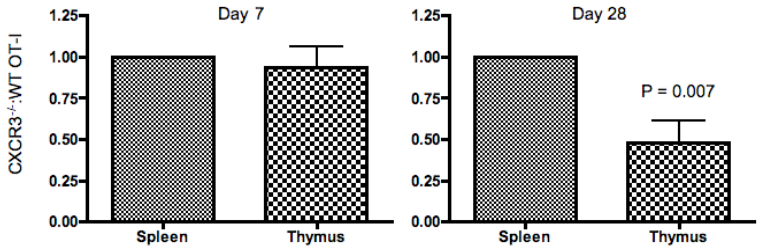


Figure 4-5. CXCR3 is required for maintenance of memory CD8 T cells in the thymus. Cxcr3$^{-/-}$:WT OT-I CD8 T cells ratio in the thymus relative to the ratio in the spleen. Animals were harvested day 7 and 28. Graphs represent 7 animals from 2 separate experiments.

Discussion

We have previously described that cell extrinsic effects caused by KLF2 deficient T cells in the thymus are mediated by IL-4(58). In this manuscript, we show that the KLF2 deficient thymic environment causes WT CD8 T cells to be retained in the thymus. This effect is consistent with previous findings in IL-4 transgenic mice(87, 88).

In attempting to determine the root of this retention effect, we came to the surprising finding that KLF2 deficient thymocytes lead to the downregulation of KLF2 at both the transcriptional and protein level in WT thymocytes. Our data suggest a model in which IL-4 signaling in T cells dramatically increases CXCR3 expression and modestly decreases KLF2, the later of which subsequently lowers the expression of S1P1.

Our findings show that IL-4 can affect important regulators of T cell migration in an antigen-independent fashion. It has recently been shown that IL-4, in the context of Th2-generating, helminth infection, leads to effects on non-antigen specific bystander T cells. IL-4 altered the response of these bystander T cells to a subsequent infection(98). However considering the evidence presented here the regulation of CXCR3 and S1P1 should also be considered in this situation. Cytokines altering the migration of bystander T cells away from sites of antigen presentation may increase the presentation and resources available to T cells responding to antigen.

The significance of activated and memory T cells returning to the thymus is a matter of debate(99). It has been shown in mouse models that recirculating T cells do

have the ability to induce central tolerance(100, 101). It has also been shown that DC migrating to the thymus can mediate negative selection(102). In addition, memory T cells have been shown in one model to mediate positive selection in the thymus(103). Of particular interest, considering maintenance of T cells in the thymus is a role in protection from viral infections of the thymus for tissue-resident memory CD8 T cells. Viruses, including human immunodeficiency virus (HIV) and lymphocytic choriomeningitis virus (LCMV), have been shown to infect the thymus(104, 105). Adoptive transfer of T cells from LCMV-immune mice was able to clear virus from the thymus of chronically infected mice(104). While our findings do not help answer the question of the significance of memory cells in the thymus, better understanding the molecular basis of this retention may provide insight into their function in the future.

The migration and maintenance of tissue specific memory T cells is regulated by multiple cell surface receptors(106). These receptors are often redundant and their importance vary in different tissues. We found that during an infection activated T cells migrate to the thymus independently of CXCR3. However, CXCR3 appears to play a non-redundant role in memory T cell maintenance in the thymus. CXCR3 has been shown to be required for protection from cutaneous leishmaniasis(107). It will be interesting in the future to compare the regulation of T cell migration to the thymus to that of other tissues. In summary, we show a novel function for CXCR3 for the regulation of conventional, memory T cell thymic migration.

Chapter 5

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

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Weinreich, M. A., Odumade, O.A., Jameson, S. C., Hogquist, K. A. "T cells expressing the transcription factor PLZF regulate the development of memory-like CD8$^+$ T cells",

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 KLF2, but rather to interleukin 4 (IL-4) produced by an expanded population of T cells expressing the PLZF transcription factor. The development of $CD8^+$ T cells previously described in ITK and CBP transcription factor deficient mice is also attributable to this IL-4-dependent mechanism. Finally, the same mechanism drives innate $CD8^+$ T cells differentiation in BALB/c mice. These findings reveal a novel mechanism for the regulation of $CD8^+$ T cells via $PLZF^+$ T cell production of IL-4.

Introduction

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)(108).

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(94, 109) and have multiple and sometimes conflicting roles in protection from infection versus pathogenesis resulting from autoimmunity(16). Promyelocytic leukemia zinc finger protein (PLZF) was recently identified as a key transcription factor involved in the development of NKT cells(19, 20). Yet it remains unclear if and how such $PLZF^+$ populations affect the homeostasis of conventional T cells.

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(38). This can be explained by the requirement of KLF2 for the expression of $S1P_1$ in T cells(34), as $S1P_1$ is a receptor necessary for T cell egress from the thymus and lymph nodes(33). In addition KLF2 regulates naïve T cell migration by binding to the L-selectin promoter and inducing its expression(34, 41). KLF2 deficient T cells express many inflammatory-type chemokine receptors, such as CXCR3(61). 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(58). 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. We showed that 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(58).

The current study examines further the bystander effects caused by KLF2 deficiency and determines 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(58). Thus, WT cells develop in a KLF2 deficient environment. In such a setting, bystander WT CD8$^+$ single positive (SP) thymocytes upregulated CXCR3. This effect was dependent on the IL-4Rα and the transcription factor Eomes(58), suggesting that KLF2 deficiency causes IL-4 overproduction 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. 5-1a**). Increased expression of CXCR3 is found in activated and memory T cells(110). 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. 5-1b**) 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(73, 111). 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(58) and protein (**Fig. 5-1c**). 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. 5-1b**). 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(88).

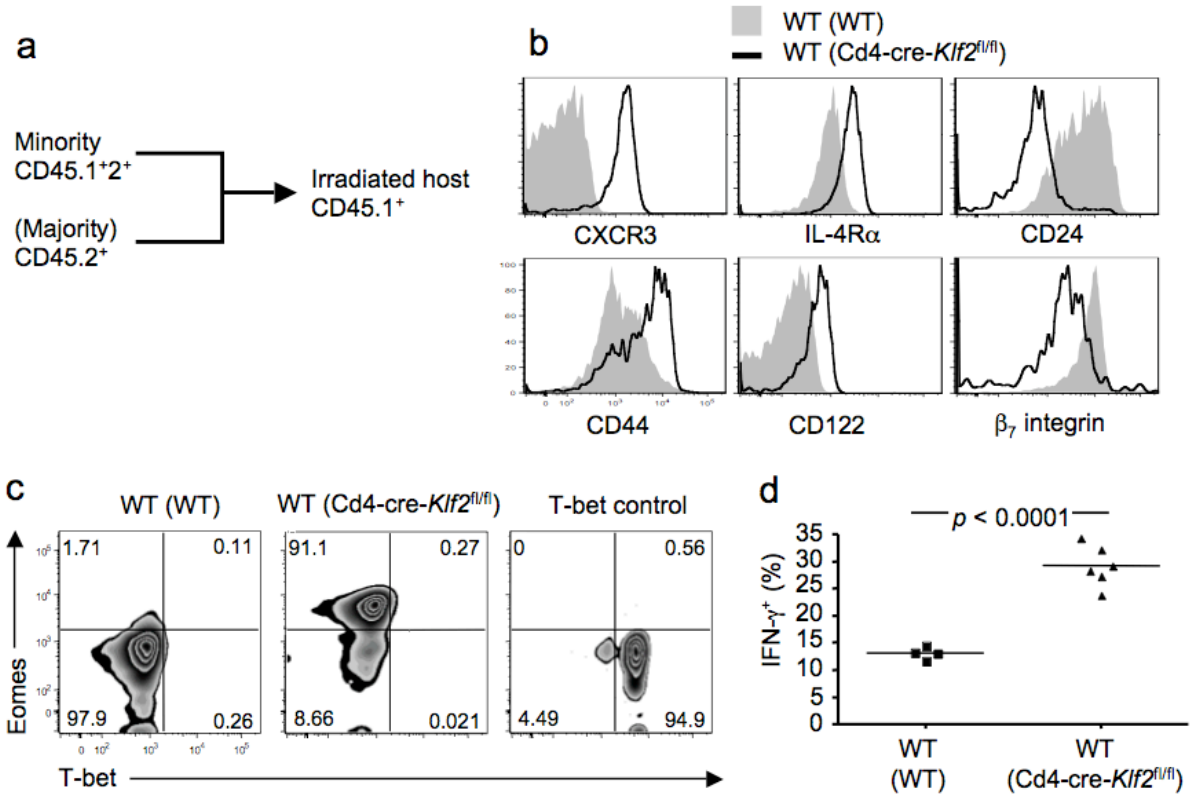

Figure 5-1 KLF2-deficient T cells induce a memory-like phenotype on bystander $CD8^+$ thymocytes. (**a**) Schematic of the bone marrow chimera set-up used in the following experiments. The majority bone marrow is indicated with parentheses. (**b**) Analysis of the cell surface phenotype of wildtype (WT) 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.

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. 5-1d**). 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 **Fig. 5-2**). **Fig. 5-3a** diagrams the bone marrow chimera set-up, where OT-I and WT cells were mixed with the indicated majority population.

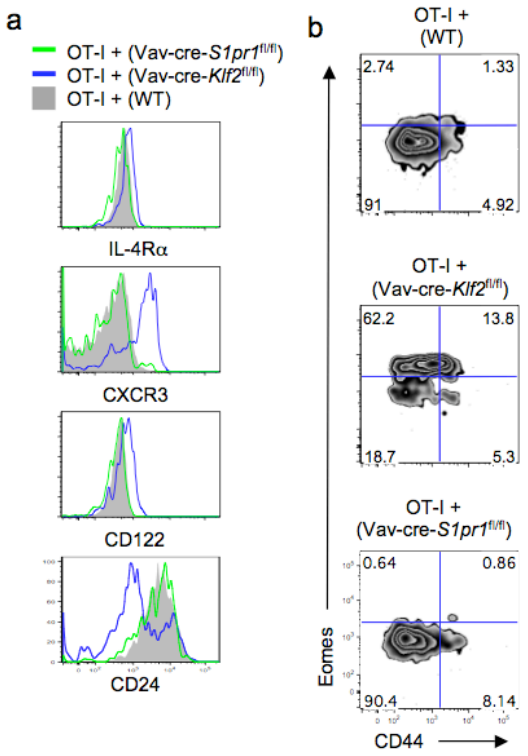

Figure 5-2. OT-I CD8 T cells exhibit bystander phenotype in KLF2 deficient thymus. (**a**) Analysis of cell surface phenotype of bystander CD8 SP OT-I thymocytes from bone marrow chimeras with majority populations indicated in parentheses. (**b**) Eomesodermin and CD44 expression on bystander OT-I thymocytes. n=7

Bystander OT-I thymocytes had a similar memory-like phenotype to WT bystander cells when Cd4-cre-*Klf2*$^{fl/fl}$ cells were the majority (**Fig. 5-2**) 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(112). While not as dramatically as in the thymus (**Fig. 5-2**), 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. 5-3b**).

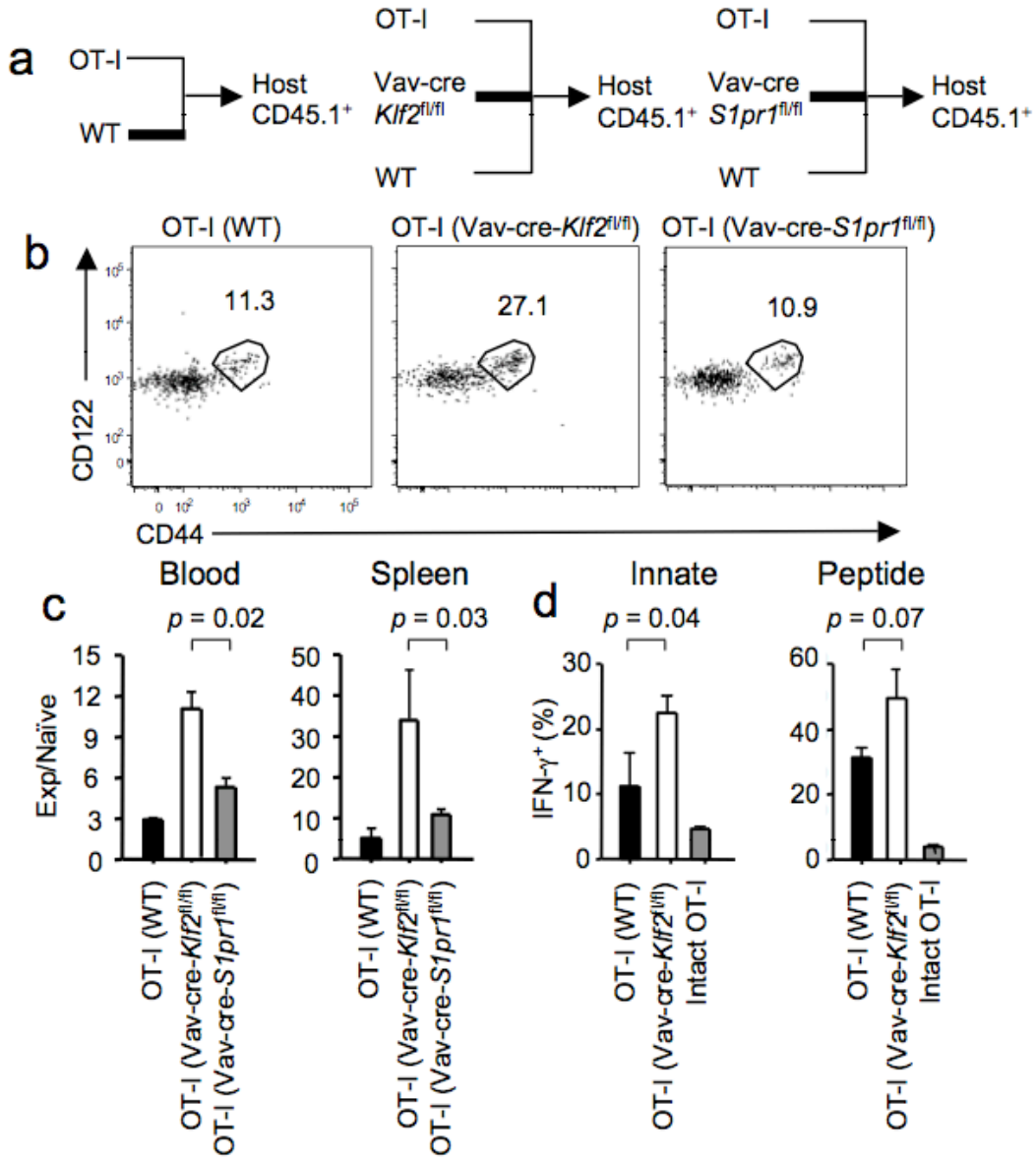

Figure 5-3 Bystander CD8$^+$ cells have greater *in vivo* function. (**a**) Mixed–bone marrow chimeras with OT-I *Rag1*$^{-/-}$ CD8$^+$ T cells as minority bystander population; thick lines indicate the majority population. (**b**) Expression of CD122 and CD44 by bystander OT-I CD8$^+$ cells from combined spleen and lymph node. Numbers adjacent to outlined areas indicate percent CD122$^+$CD44$^+$ cells. (**c**) OT-I T cells in blood (left) analyzed at day 4 and spleen (right) at day 7 of recipients (n = 3 mice per group) of 3 × 10^5 OT-I T cells from various chimeras (horizontal axis) plus an equal number of naive OT-I T cells, then infected with *L. monocytogenes* expressing OVA (*ActA*$^{-/-}$ strain), presented as the ratio of OT-I cells from experimental (exp) chimera to naive OT-I cells, analyzed at day 4 (blood) or day 7 (spleen). (**d**) Frequency of IFN-g-producing OT-I T cells from various chimeras (horizontal axis) or intact OT-I *Rag1*$^{-/-}$ splenocytes after stimulation with IL-2, IL-12 and IL-18 (left) or OVA peptide (right). Data are from two separate experiments with two mice each (error bars, s.d.).

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. 5-3c**).

Memory $CD8^+$ T cells have been shown to produce IFN-γ antigen-independently as a result of inflammatory cytokines(113). 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. 5-3d**). TCR-dependent cytokine production also appeared increased. Similar to our findings with thymocytes (**Fig. 5-1d**), 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. 5-3d**). Together these results show that the KLF2-deficient environment leads to the expansion of a subset of $CD8^+$ T cells with increased function.

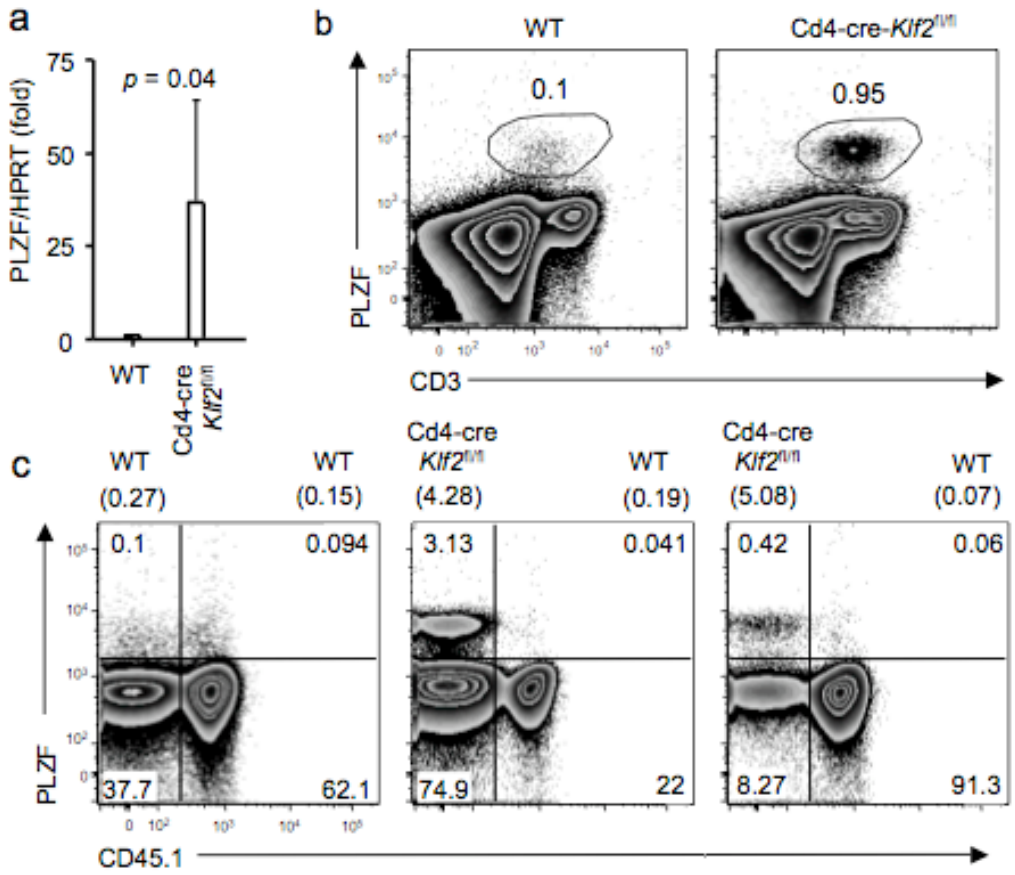

Figure 5-4 KLF2 deficiency leads to an expansion of PLZF expressing T cells. (**a**) PLZF mRNA expression on sorted CD4 SP thymocytes, quantified by real time PCR. n=5 (**b**) PLZF and CD3 expression on thymocytes from intact WT and Cd4-cre-*Klf2*$^{fl/fl}$ mice. Results are representative of more than 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.

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(58). 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(19, 20). We found a dramatic increase in *Plzf* mRNA expression in the Cd4-cre-*Klf2*$^{fl/fl}$ thymus (**Fig. 5-4a**). PLZF was not increased on all thymocytes but rather an increased percentage of thymocytes expressed PLZF (**Fig. 5-4b**). 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. 5-4c**). 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(22, 114). Within the PLZF^{+} populations, the proportion of these subsets is similar comparing WT and Cd4-cre-*Klf2*$^{fl/fl}$ (**Table 1 and Fig. 5-5a**). 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 (**Fig. 5-5b**). These findings together with KLF2 expression being limited to the mature SP stage of thymocyte development(58) 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.

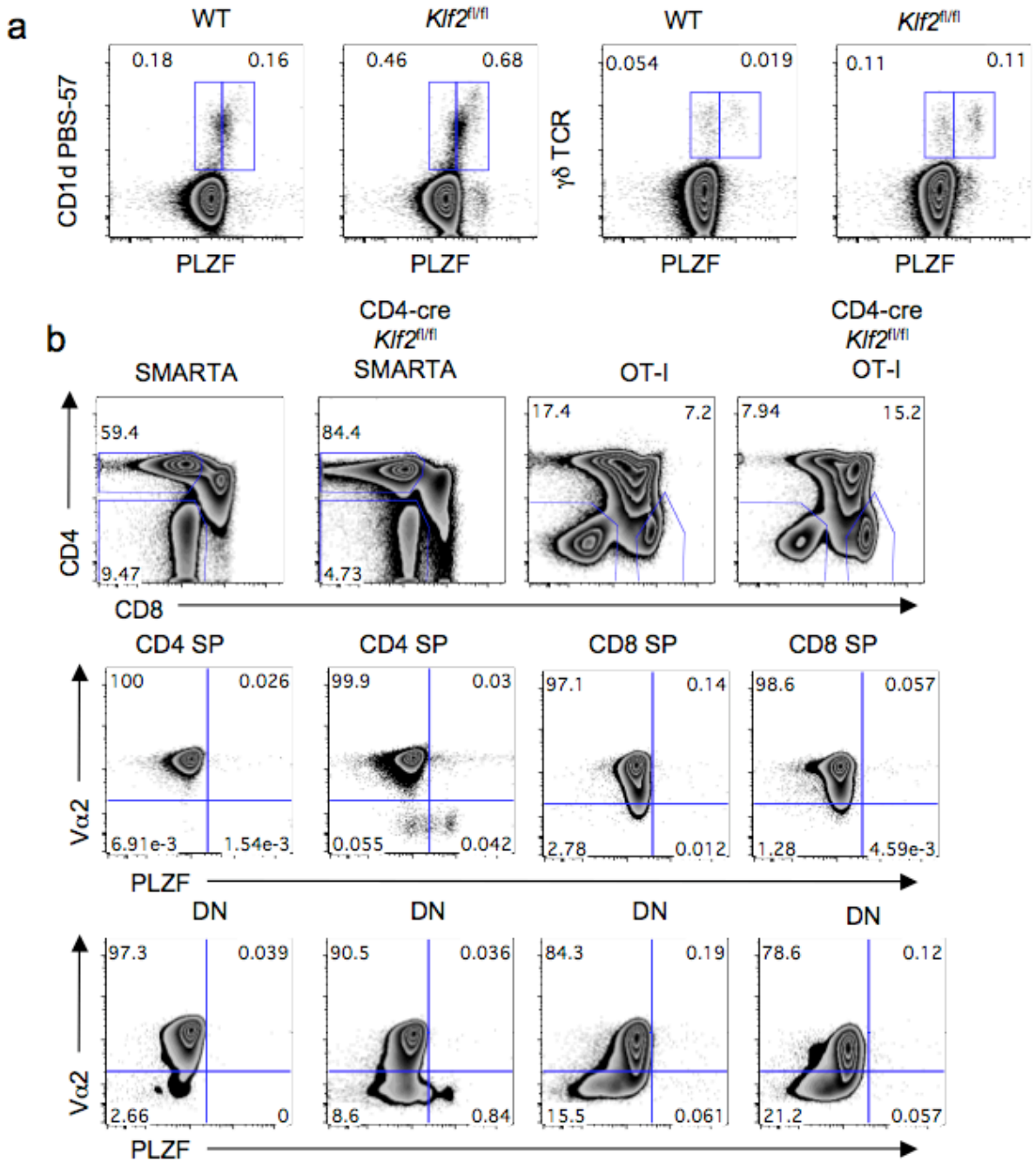

Figure 5-5. KLF2 deficiency expands but does not alter the repertoire of the PLZF+ population. (**a**) Expression of PLZF versus CD1d-tetramer binding, top, and PLZF versus TCR, bottom in WT and CD4cre*Klf2*$^{fl/fl}$ thymus. (**b**) KLF2 deficiency does not cause PLZF expression on T cells with a fixed T cell receptor. SMARTA, a CD4 TCR transgenic, and SMARTA/CD4cre*Klf2*$^{fl/fl}$ (left) and OT-I, a CD8 TCR transgenic, and OT-I/CD4cre*Klf2*$^{fl/fl}$ (right) were evaluated for expression of their transgenic V TCR chain (V2) and PLZF in indicated thymic subsets. n=3

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. 5-6a**). Within the PLZF^{+} subset, both γδ^{+} and CD1^{d} tetramer binding cells produced IL-4 (data not shown).

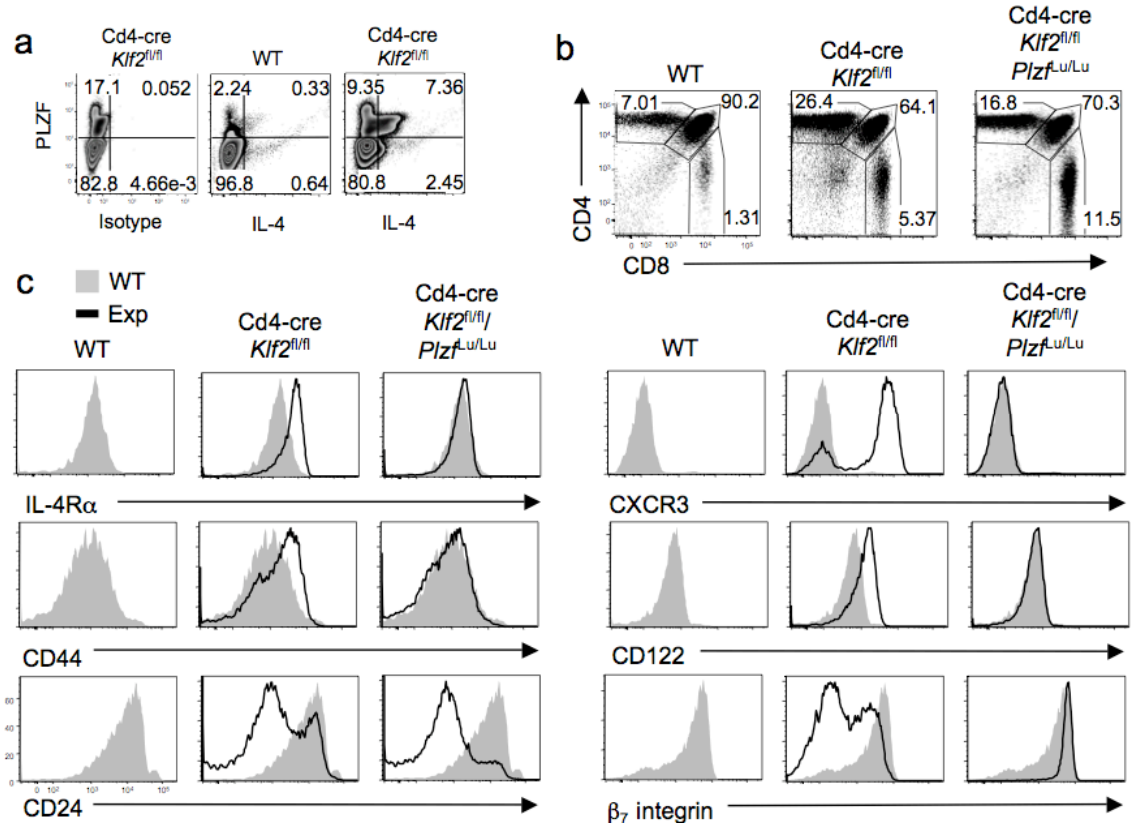

Figure 5-6 PLZF^{+} cells are responsible for excess IL-4 production and CD8^{+} bystander effects in KLF2 deficient mice. (**a**) IL-4 and PLZF expression in thymocytes from intact WT and Cd4-cre-*Klf2*$^{fl/fl}$ mice. Thymocytes were isolated and stimulated with PMA and ionomycin for 5 hours then intracellular 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.

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(115). *Plzf*$^{lu/lu}$ and *Plzf*$^{-/-}$ mice have a similar phenotype and NKT defect(19, 20). 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. 5-6c**). 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 (**Fig. 5-7a**). In addition, WT bystander cells were not affected in a chimera where the majority of transferred BM cells were Cd4-cre-*Klf2*$^{fl/fl}$-*Plzf*$^{lu/lu}$ (**Fig. 5-7b**). This indicates that PLZF is important for the induction but not the response to the bystander effects.

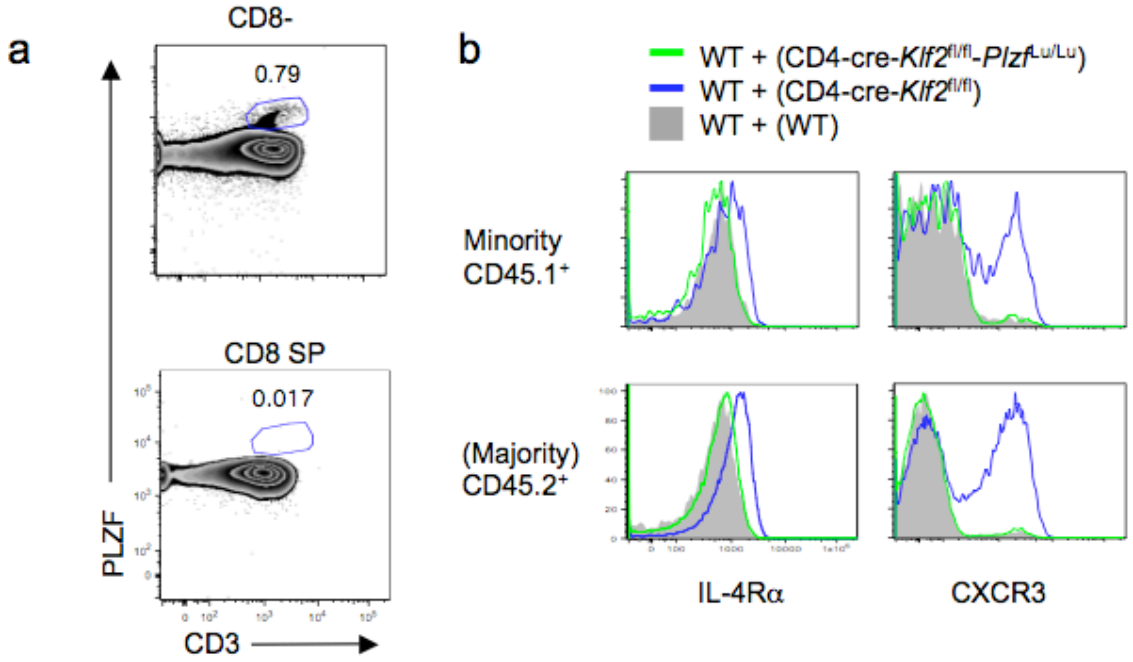

Figure 5-7. PLZF is not expressed in CD8 T cells, but is necessary for alteration of the CD8 phenotype on bystander cells. (**a**) Intracellular stain for PLZF on CD8 negative (CD4SP and DN) or CD8SP thymocytes in CD4cre/KLF2$^{fl/fl}$ mice. n=5 (**b**) The expression of IL-4R and CXCR3 on CD8 SP thymocytes in mixed bone marrow chimeras where WT, CD4cre*Klf2*$^{fl/fl}$, or CD4cre/*Klf2*$^{fl/fl}$/*Plzf*$^{Lu/Lu}$ are the majority. n=3

Evidence to date supports KLF2 controlling T cell migration by directly regulating the cell surface receptors $S1P_1$ and CD62L in T cells(34, 41). 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. 5-6c**). This phenotype and high CD69 expression are consistent with KLF2 deficiency leading to a lack of $S1P_1$ and a thymic emigration defect(33). 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.

Similar mechanism occurs with other deficiencies

We were interested if these effects occur in other gene deficiency models. ITK is a Tec family kinase with a role in TCR signaling(116). 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(117, 118). 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 (22, 23). 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. 5-1a**. 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. 5-8a,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.

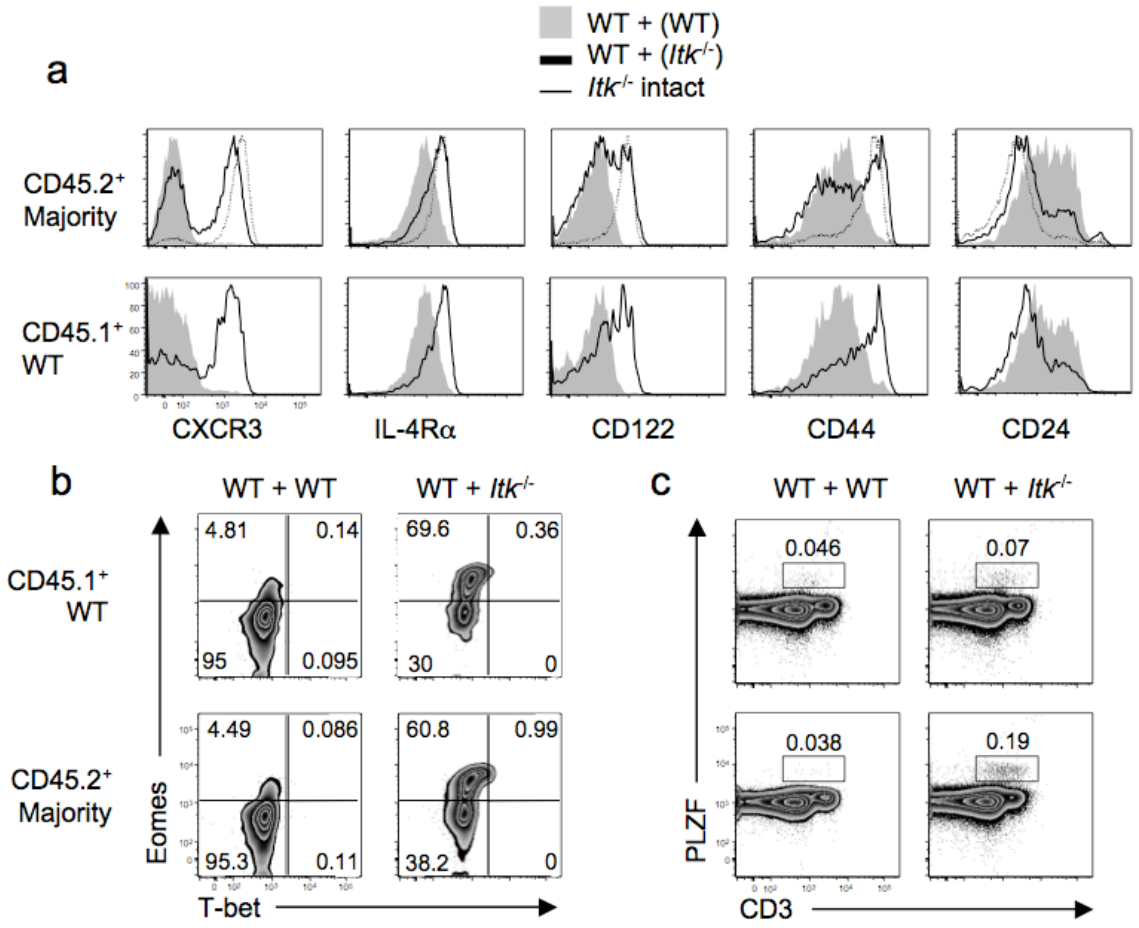

Figure 5-8. *Itk*$^{-/-}$ mice have a cell-extrinsic CD8^{+} phenotype and expanded PLZF^{+} population. (**a**) CD8^{+} SP phenotype from intact *Itk*$^{-/-}$ mice (thin line), mixed chimera with *Itk*$^{-/-}$ majority (thick line) and an all WT chimera (shaded). Top row shows the majority population 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.

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. 5-8c**). 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(24, 119) and enhanced γδ NKT cell development (22, 23).

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. 5-9**). 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.

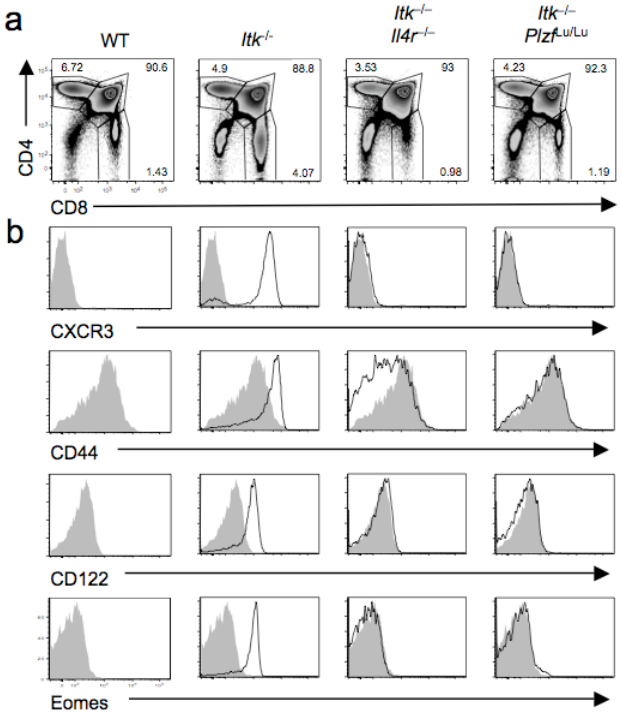

Figure 5-9. The memory-like CD8^{+} phenotype in *Itk*$^{-/-}$ mice is dependent on IL-4 and PLZF. Thymocyte analysis from WT, *Itk*$^{-/-}$, *Itk*$^{-/-}$/*Il4ra*$^{-/-}$, and *Itk*$^{-/-}$/*PLZF*$^{Lu/Lu}$ mice. (**a**) CD4 by CD8^{+} expression. (**b**) Histogram overlays of CD8^{+} SP phenotype. 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.

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(120). 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 (**Fig. 5-10**). 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.

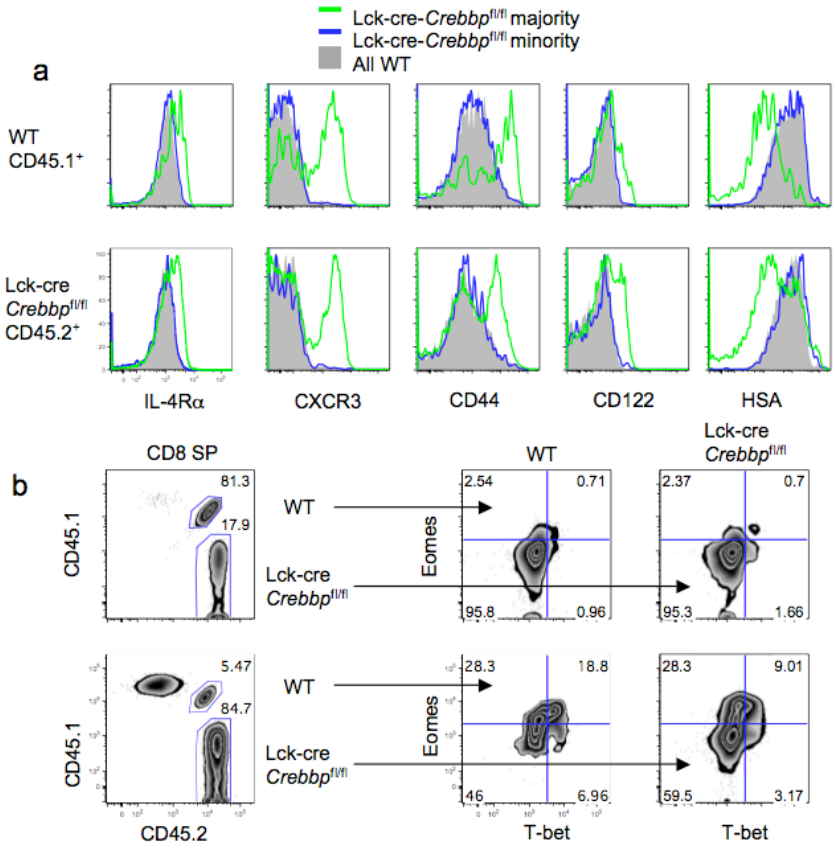

Figure 5-10. The memory-like CD8 phenotype in Lck-cre-*Crebbp* is a cell-nonautonomous effect. (**a**) CD8 SP thymocyte phenotype from bone marrow chimeras with Lck-cre-*Crebbp*fl/fl majority, Lck-cre-*Crebbp*fl/fl minority or all WT. (**b**) The ratio of WT to Lck-cre-*Crebbp*fl/fl cells in the CD8 SP population detected by flow cytometry for CD45 congenic markers, left column. Eomesodermin and T-bet expression on WT and Lck-cre-*Crebbp*fl/fl (middle and right columns, respectively) CD8 SPs from mixed bone marrow chimeras. n=3

Similar mechanism occurs with 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(121-123). 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. 5-11a**). Interestingly, a higher percentage of BALB/c $CD8^+$ SP were memory phenotype ($CD44^{hi}$, $CD122^{hi}$) compared to age-matched B6 (**Fig. 5-11b**). We also observed, in BALB/c mice, that the memory phenotype $CD8^+$ SP expressed high levels of Eomes but not T-bet (**Fig. 5-11c**). This is consistent with PLZF-IL-4 dependent memory-like $CD8^+$ T cell generation in BALB/c mice.

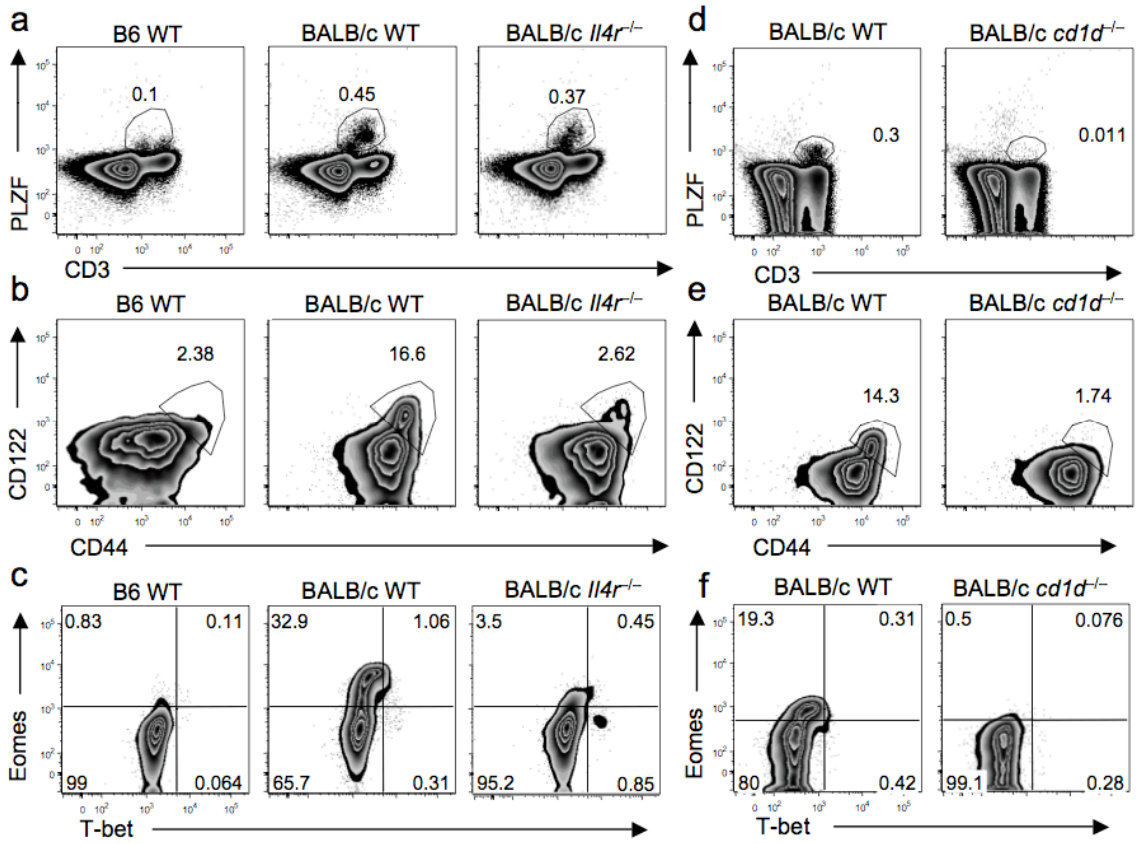

Figure 5-11. An expanded PLZF^{+} population and an IL-4 and NKT dependent memory-like population in BALB/c mice. (**a**) PLZF and CD3 expression of thymocytes from B6, BALB/c and *Il4ra*$^{-/-}$ on BALB/c background and (**d**) BALB/c and *CD1d*$^{-/-}$ on BALB/c background. (**b, e**) CD44 by CD122 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.

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. 5-11b**). Strikingly, Eomes expression in *Il4rα*$^{-/-}$ BALB/c mice was reduced to levels comparable to WT B6 mice (**Fig. 5-11c**). 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. 5-11d**). In the *Cd1d*$^{-/-}$ BALB/c thymus, we also found a decreased percentage of memory-like CD8$^+$ T cells (**Fig. 5-11e,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 (**Fig. 5-12**). 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.

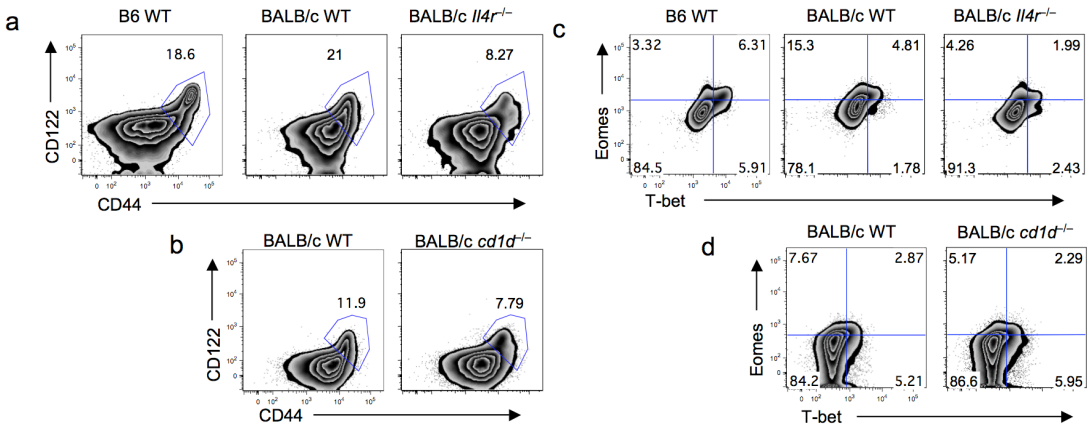

Figure 5-12. Effects of IL-4 and CD1d deficiency on memory-like CD8 T cells in the BALB/c spleen. (**a**) CD44 by CD122 expression on CD8 T cells from the spleen of B6, BALB/c and *Il4ra*$^{-/-}$ on BALB/c background and (**b**) BALB/c and *CD1d*$^{-/-}$ on BALB/c background. (**c, d**) Eomesodermin by T-bet expression on CD8 T cells from the spleen of indicated mice. n=3

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 (116, 124). 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(21, 125-127) 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(22, 23). 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(120). 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(58) 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(128), 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(19, 20). 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(58) 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(19, 129). 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(16). The potential of NKT cells in therapeutics is also actively being investigated(130). 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(121-123). 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(131, 132), including IL-4 from activated NKT(133), 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(134). 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(135), 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(136). 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(137-140). 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(58). *S1pr1*$^{fl/fl}$ mice were obtained from Richard Proia (National Institutes of Health NIDDK, Maryland). *Itk*$^{-/-}$ mice(118) 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 Lck-cre-*Crebbp*$^{fl/fl}$ 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(19). 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 $3x10^5$ 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 $3x10^6$ 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: $1.5x10^6$ cells/mL were incubated with

$5x10^2$ 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.).

Table 1

	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***

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

Chapter 6

Discussion

Conclusions

The work presented in this thesis makes three main, novel contributions to the field of T cell development. First, it provides new understanding of the role of KLF2 in both conventional and innate-like T cell development. Second we find a mechanism of indirect regulation that we discovered in KLF2 deficient mice also occurred in other gene deficiency models and dramatically alter the interpretation of those models. Lastly, these mechanisms were also responsible for the development of memory-like T cells in WT strains of mice, indicating that it may play a role in immunity of normal mice and possibly humans.

When we began this project it was thought that KLF2 was a master regulator of naïve T cell migration. KLF2 deficient T cells have dysregulated expression of CD62L, S1P1, CD44, β7 integrin, and CXCR3 all of which play a role in migration. We showed for the first time that KLF2 deficiency leads to both direct and indirect effects. While CD62L and S1P1 are directly regulated by KLF2, we show the other receptors are changed in a cell extrinsic fashion. Thus, KLF2 has a critical but limited role in regulateing naïve T cell migration. KLF2 is vital for the positive regulation certain naïve T cell homing molecules but does not regulate the repression of inflammatory chemokine receptors.

The regulation of CCR7 by KLF2 was somewhat of an enigma in this study. It was not changed at the mRNA level in the intact KLF2 KO but its surface expression was decreased. WT bystander cells affected by the KLF2 deficiency also have low levels of CCR7. However, CCR7 was down in KLF2, PLZF double deficient mice, which lack the bystander effect. CCR7 ligands CCL19 and 21 were expressed at a high

level in thymus so it is possible that increased thymic retention time leads to the surface downregulation of CCR7, which occurs in all three scenarios described above. However CCR7 was not downregulated in the S1P1 KO thymus, which also has an extended thymic retention time. WT bystander affected thymocytes did have a partial deficiency in KLF2 so direct regulation of CCR7 by KLF2 could be occurring in all of these situations. KLF2 is a transcription factor so it would be expected to regulate genes at the transcriptional level but only CCR7 protein expression and not mRNA were decreased. Thus, it is possible that KLF2 directly regulates the transcription of another molecule that subsequently controls CCR7.

The mechanism that we have revealed for these cell-extrinsic effects starts with the expansion of PLZF+ T cells in the KLF2 deficient thymus. These PLZF+ T cells produce IL-4 and signaling through the type I IL-4 receptor and the transcription factor eomesodermin leads to the constellation of bystander effects.

The timing of KLF2 expression and the expansion of all lineages of PLZF+ T cells argue against KLF2 working at the point of selection. Another simple way that the absence of KLF2 could increase PLZF+ T cells is if KLF2 directly repressed PLZF expression. Genetic deficiency in KLF2 would relieve this repression and expand the PLZF+ population. The high expression of PLZF in immature NKTs and subsequent decrease of PLZF with NKT maturation and KLF2 increase fits this model. To explore this possibility we have done preliminary chromatin immunoprecipitation assays on conventional T cells to see if KLF2 binds to the PLZF promoter. There are multiple consensus KLF2 binding sites upstream of PLZF but we have been unable to detect KLF2 binding to sites we have examined thus far. This model could be tested in future

studies examining the effect of forced KLF2 expression on the generation of PLZF+ T cells.

An alternative to direct repression is if KLF2 deficiency leads to the increased proliferation or survival of PLZF+ cells. KLF2 has been associated with T cell quiescence so this could also be explored in PLZF+ T cells in the future.

If KLF2 was found to directly repress PLZF, an interesting model would mutual repression by these transcription factors. In support of this model PLZF transgenic CD8 T cells decreased CD62L consistent with the possibility of decreased KLF2. Interestingly we found that CD1d-binding NKTs that survive in PLZF deficient mice have increased levels of KLF2. Although further work needs to be done to test if this model of corepression is true, KLF2 and PLZF may play a role in distinguishing conventional from innate-like T cells.

The second part of this cell extrinsic process is the production of IL-4 by the PLZF+ cells. We and others have been unable to detect the production of IL-4 from unstimulated PLZF+ cells. However, our data clearly shows that PLZF+ cells are the source of IL-4. Some NKT cells have been shown to be reactive to self-lipids so it is possible that stimulation with these peptides cause the production of IL-4. We may not be able to detect this production ex vivo because the number of PLZF+ cells at any one time producing IL-4 or amount of IL-4 produced per cell is so low. Identifying the ligands that cause IL-4 production in vivo will be important to understanding the role of PLZF+ T cells in homeostasis of other T cell subsets.

We have found that these IL-4-dependent bystander effects are more pronounced on CD8 compared to CD4 thymocytes. One possible explanation is the

differential regulation of the transcription factor eomesodermin in CD8 and CD4 thymocytes. Eomes is upregulated on the bystander cells and is necessary for the other cell-extrinsic effects. Memory CD8 T cells express eomes and a related transcription factor T-bet while memory CD4 T cells express T-bet exclusively. It is possible that differences in susceptibility to bystander effects are due to the threshold for eomesodermin expression in CD4s being higher compared to CD8s.

The second main point is how the cell extrinsic effects are seen in multiple gene deficiency models and how this has led to misinterpretation of the effects of these genes. KLF2 was thought to play a wider role in the regulation of cell surface emigration markers prior to this work. For example it had be incorrectly been assumed that KLF2 directly regulated a large number of inflammatory chemokine receptors(61).

More dramatically is how this mechanism alters the interpretation for the role of ITK in T cell development. ITK is a tyrosine kinase that signals downstream of the TCR. The increase in CD8 T cells that rapidly produce cytokine was interpreted as ITK deficiency leading to the generation of "innate-like" CD8 T cells. Thus, it was thought that ITK worked to repress the "innate-like" lineage and allow the development of conventional T cells(116). Our work supports an explanation that the expansion of PLZF+ T cells and IL-4 production drives the production of "innate-like" CD8 T cells secondarily. It puts into question the idea of separate differentiation pathways for conventional and "innate-like" CD8 T cells. CBP KO and ID3 KO also show this phenotype so any new KO that generates an "innate-like" CD8 phenotype warrants investigation with mixed bone marrow chimeras(120). Since the nexus for this

mechanism is an expansion of a PLZF+ T cell population, it will be interesting to find out how PLZF+ T cells are dysregulated in each model.

The potentially most significant finding from this thesis is that in "normal" BALB/c mice this same mechanism generates CD8 T cells with the phenotype and increased function of memory cells. This memory-like T cell expansion appears to be independent of TCR specificity and we found that these cells have both increased antigen specific and innate-like responses. If this model is correct and IL-4 drives the development of IFNγ producing CD8 T cells, we would expect mice deficient in IL-4 to be more susceptible to intracellular pathogen for which CD8s and IFNγ are necessary for protection. This seems counterintuitive because IL-4 is known to inhibit IFNγ production thus loss of IL-4 would be expected to cause a more robust IFNγ response. Infection experiments comparing IL-4 receptor deficient and WT BALB/c should be able to test if these memory-like CD8 T cells are functionally significant.

We think that the fact that this mechanism occurs in at least some strains of mice brings up the possibility that it also takes place in humans. There is at least 100 fold variation in the percentage of NKTs in the blood within the human population(141). Additonally, there is evidence that humans have a higher proportion of total PLZF+ T cells(135). This may be due to a pathway for PLZF+ T cell expression dependent on Class II expression on DP thymocytes, which occurs in humans but not mice(142, 143). If the mouse teaches us anything about PLZF+ T cell frequency then it is expected that there is diversity in this frequency within the human population. Once the function for these PLZF/IL-4 dependent memory-like T cells is known, it will be interesting to see

how the frequency of PLZF+ cells correlates to human T cell function and susceptibility to infection.

In conclusion, this thesis expands our basic understanding of both conventional and nonconventional T cell development. It also causes reinterpretation of previous findings and will alter the course of future studies in our lab and others. Finally, we discovered a novel mechanism for the development of memory-like T cells with improved function in normal mice. Further understanding of development and interactions between conventional and nonconventional T cells could lead to new approaches for improving and regulating the human immune system.

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