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Transcription factor KLF2 regulates the migration of naive T cells by restricting chemokine receptor expression patterns

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The migration patterns of naive and activated T cells are associated with the expression of distinct sets of chemokine receptors, but the molecular basis for this regulation is unknown. Here we identify Krupple-like factor 2 (KLF2) as a key transcriptional factor needed to prevent naive T cells from expressing inflammatory chemokine receptors and acquiring the migration patterns of activated T cells. Lineage-specific deletion of KLF2 resulted in fewer naive T cells in the blood and secondary lymphoid organs, whereas it expanded naive T cell numbers in nonlymphoid tissues; these effects were associated with altered expression of inflammatory chemokine receptors on naive T cells. KLF2 repressed the expression of several chemokine receptors, including CCR3 and CCR5. We thus conclude that KLF2 maintains proper T cell migration patterns by linking T cell movement and transcriptional regulation of chemokine receptor expression patterns.

Kruppel-like factors (KLFs) constitute a family of zinc-finger transcriptional regulators, many of which have critical functions in cell lineage development[1,2]. KLF2 is expressed in endothelial cells and a limited subset of leukocytes, including monocytes and B and T lymphocytes. KLF2 is first expressed in the T cell lineage at the single-positive thymocyte stage, and high expression is maintained in naive T cells[3,4]. KLF2 expression is downregulated after T cell activation and is subsequently reactivated in memory T cells, which suggests involvement of this transcription factor in quiescent T cells.

KLF2-deficient mice die *in utero* because of cardiovascular abnormalities[5–7], and recombination-activating gene 2–deficient ($Rag2^{-/-}$) blastocyst complementation experiments incorporating $Klf2^{-/-}$ embryonic stem cells were originally used to examine lymphocyte lineages[4]. Thymocyte development was described as normal, although a small increase in single-positive thymocytes was evident. In contrast, the spleen and blood of chimeric mice had many fewer mature KLF2-deficient T cells. Examination of the few remaining T cells suggested that these cells were activated and undergoing spontaneous apoptosis in a FasL-mediated way, and it was concluded that KLF2 is necessary to maintain naive T cells in a quiescent state. A subsequent study reported that overexpression of KLF2 in a Jurkat T cell line suppressed proliferation in a c-Myc-dependent way, a finding consistent with regulation of T cell quiescence by KLF2 (ref. 8). Therefore, KLF2 was thought to act as an inhibitory transcription factor that suppresses spontaneous naive T cell cycling and activation.

Further studies have led to a reinterpretation of KLF2-regulated T cell homeostasis[9]. The initial finding that KLF2 is needed to maintain normal peripheral T cell numbers was confirmed with lethally irradiated $Rag2^{-/-}$ mice reconstituted with KLF2-deficient fetal liver[9]. However, it was noted that adoptively transferred KLF2-deficient single-positive thymocytes were relatively long lived, which suggested that these cells were not undergoing spontaneous apoptosis. Instead, it was proposed that $Klf2^{-/-}$ single-positive thymocytes fail to leave the thymus, a phenotype similar to that of mice deficient in sphingosine 1-phosphate receptor 1 ($S1P_1$)[10,11]. $S1P_1$ is a G protein–coupled, seven-transmembrane receptor that responds to the chemotactic factor S1P[12]. Mature thymocytes and naive T cells express $S1P_1$, which is necessary for the exit of T cells from the thymus and from peripheral lymph nodes[13]. In the absence of $S1P_1$, thymocytes are trapped in the thymus, and adoptive transfer of these cells to wild-type recipient mice results in their accumulation in lymph nodes. Consistent with the hypothesis that KLF2 is required for thymic egress, KLF2-deficient $CD4^+$ thymocytes are reported to have less expression of $S1P_1$ mRNA, and KLF2, when heterologously expressed in a Jurkat cell line, has been shown to bind the promoter of *Edg1* (which encodes $S1P_1$)[9]. These data suggest that KLF2 is required for proper expression of $S1P_1$ and that KLF2-deficient single-positive thymocytes are unable to respond to peripheral S1P gradients and thus remain trapped in the thymus.

However, outstanding issues remain regarding the hypothesis of $S1P_1$-mediated peripheral T cell hypocellularity in KLF2-deficient mice. Most notably, *in vivo* migration patterns of KLF2-deficient

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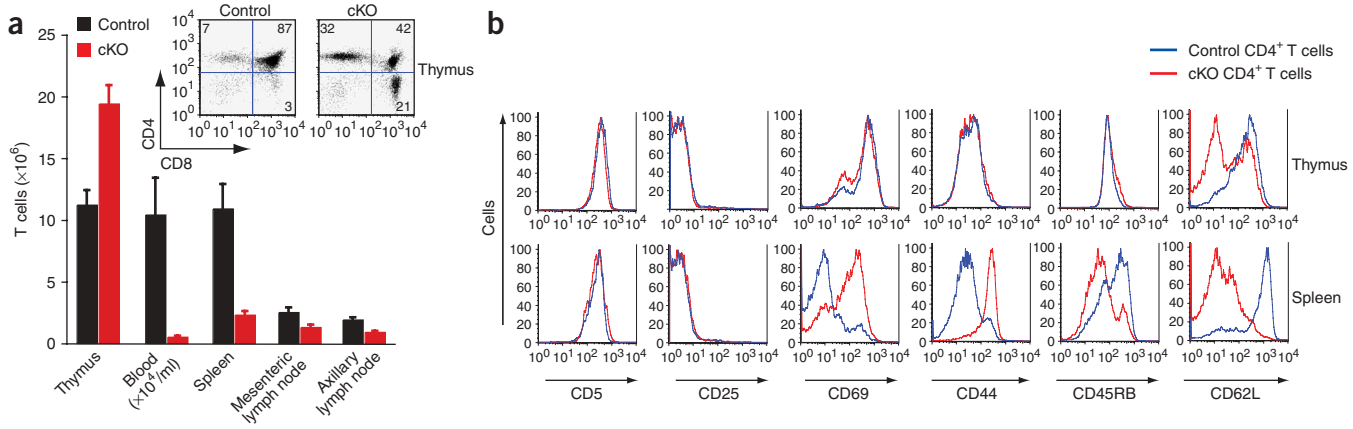

Figure 1 *Klf2*$^{-/-}$Vav-Cre^{+} mice have more mature thymocytes but many fewer T cells in the peripheral blood and lymphoid organs. (**a**) Single-positive Thy-1.2^{+} T cells in various tissues of *Klf2*$^{fl/fl}$Vav-Cre^{-} mice (Control) and *Klf2*$^{-/-}$Vav-Cre^{+} mice (cKO; $n = 4$ mice per group; age-matched littermates). $P < 0.001$ for each tissue. Inset, typical CD4 and CD8 flow cytometry of Thy-1.2^{+} thymocytes from *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} littermates. Numbers in quadrants indicate percent cells in each. (**b**) Flow cytometry of Thy-1.2^{+}CD4^{+} thymocytes and splenocytes from *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} littermates, stained for a panel of activation markers. Data are representative of four experiments.

and S1P$_1$-deficient thymocytes are distinctly different: adoptively transferred S1P$_1$-deficient thymocytes become trapped in lymph nodes because S1P$_1$ is required for the exit of T cells from lymph nodes into the lymph[10], whereas adoptively transferred KLF2-deficient thymocytes fail to appear in the lymph nodes[9]. That same study indicated that a defect in KLF2-deficient thymic egress was responsible for the failure of labeled thymocytes to appear in peripheral secondary lymphoid organs. However, consistent with those KFL2-dependent phenotypes is the possibility that KLF2-deficient thymocytes do leave the thymus but primary peripheral T cell migration is defective. Thus, although there seems to be a correlation between expression of KLF2 and S1P$_1$, the function of S1P$_1$ in the KLF2-deficient T cell phenotypes (fewer peripheral lymphocytes in secondary lymphoid organs in KLF2-deficient mice and after adoptive transfer of KLF2-deficient cells) remains unclear.

To better understand how KLF2 affects peripheral T lymphocytes, we generated stable genetic mouse models in which *Klf2* is conditionally excised in the hematopoietic compartment by Cre recombinase expressed by the promoter of the gene encoding the guanine nucleotide–exchange factor Vav (Vav-Cre). We found that KLF2-deficient single-positive thymocytes and T cells had less S1P$_1$, but functional studies demonstrated that these amounts of receptor were sufficient for *Klf2*$^{-/-}$ thymocytes to respond to S1P *in vitro* and to exit the thymus *in vivo*. Unexpectedly, we found that lower numbers of naive KLF2-deficient T cells in secondary lymphoid organs were associated with much higher numbers of naive T cells in nonlymphoid peripheral tissues. Thus, the absence of KLF2-deficient T cells in peripheral lymphoid organs resulted from a defect in peripheral migration and not a block in thymic egress. Homeostatic T cell migration is directed by chemokine gradients, and we found that naive KLF2-deficient CD4^{+} thymocytes and T cells aberrantly expressed many functional inflammatory chemokine receptors. These receptors conferred new migration responses to KLF2-deficient T cells *ex vivo*, and blockade of chemokine receptor signaling with the nonspecific inhibitor pertussis toxin or the antagonist Met-RANTES, specific for chemokine receptors CCR1 and CCR5, inhibited migration to nonlymphoid sites. Thus, the chief function of KLF2 in regulating T cell homeostasis is to restrict chemokine receptor expression and T cell movement before T cell activation.

RESULTS

Naive KLF2-deficient T cells are absent from lymphoid organs

To better understand how KLF2 affects peripheral T lymphocytes, we bred mice with *lox*P-flanked *Klf2* (*Klf2*$^{fl/fl}$ mice) with Vav-Cre–transgenic mice to generate an animal model with excision of *Klf2* at the hematopoietic stem cell stage[7,14]. The results we describe here are T cell intrinsic, as identical phenotypes were produced with a Lck-Cre transgene that produced deletion of *Klf2* exclusively in T cells (data not shown). We used the Vav-Cre system for our studies here because this transgene provides earlier and more efficient excision. Hematopoietic excision of *Klf2* (called '*Klf2*$^{-/-}$Vav-Cre^{+}' or 'KLF2-deficient' here) resulted in significant accumulation of single-positive thymocytes and many fewer peripheral T cells in the blood and secondary lymphoid organs (**Fig. 1a**). Further examination of the few KLF2-deficient T cells collected from the spleen showed a 'pseudo-activated' expression pattern of surface markers (**Fig. 1b**). These cells had high expression of CD69, a surface marker typically associated with early cell activation, but did not have other phenotypic characteristics of T cell activation, such as less CD5 or upregulation of the interleukin 2 receptor α-chain (CD25). Peripheral KLF2-deficient T cells also expressed memory T cell markers; this included high expression of CD44 and less surface expression of CD45RB and CD62L. Noticeably absent were naive T lymphocytes, normally the dominant T cell population in secondary lymphoid organs.

A published study of the reconstitution of *Rag2*$^{-/-}$ mice with *Klf2*$^{-/-}$ embryonic stem cells reported a similar activated phenotype in peripheral T cells and concluded that KLF2 is needed to maintain naive T cells in a quiescent state[4,15]. That study also suggested that KLF2-deficient peripheral T cells become activated, upregulate the cytokine FasL (CD95L) and undergo spontaneous apoptosis. We examined peripheral T cells in our KLF2-deficient mice and failed to find either upregulation of FasL or substantially more spontaneous apoptosis, as measured by intracellular staining with 7-amino-actinomycin D and annexin V (**Fig. 2a**). Terminal deoxynucleotide transferase–mediated dUTP nick end labeling (TUNEL) analysis also failed to detect more apoptotic T cells in the thymi, spleens or livers of *Klf2*$^{-/-}$ Vav-Cre^{+} mice (**Fig. 2b**). As a positive control, we sublethally irradiated a control (*Klf2*$^{fl/fl}$Vav-Cre^{-}) littermate mouse before TUNEL staining, as thymocytes are particularly sensitive to radioactivity and

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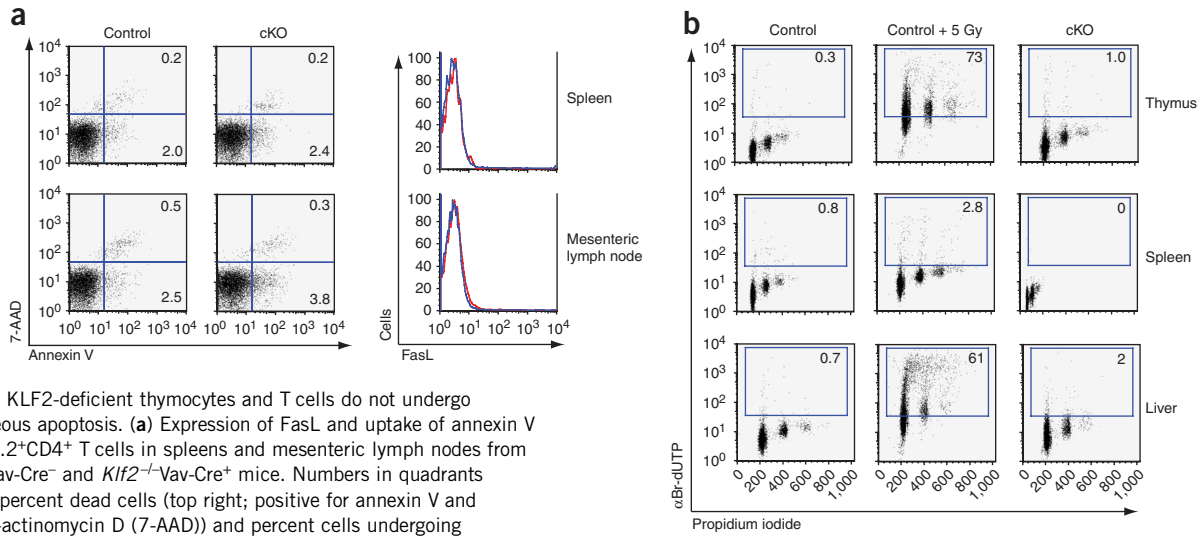

Figure 2 KLF2-deficient thymocytes and T cells do not undergo spontaneous apoptosis. (**a**) Expression of FasL and uptake of annexin V by Thy-1.2$^+$CD4$^+$ T cells in spleens and mesenteric lymph nodes from *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$Vav-Cre$^+$ mice. Numbers in quadrants indicate percent dead cells (top right; positive for annexin V and 7-amino-actinomycin D (7-AAD)) and percent cells undergoing apoptosis (bottom right; positive for annexin V and negative for 7-amino-actinomycin D). Right, surface expression of FasL by Thy-1.2$^+$CD4$^+$ T cells from *Klf2*$^{-/-}$Vav-Cre$^+$ control mice (blue) and *Klf2*$^{-/-}$Vav-Cre$^+$ mice (red). Data are representative of two experiments. (**b**) Flow cytometry of Thy-1.2$^+$CD4$^+$ T cells from *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$Vav-Cre$^+$ mice, as well as a sublethally irradiated *Klf2*$^{fl/fl}$Vav-Cre$^-$ mouse (control + 5 Gy; positive control). Numbers in outlined areas indicate percent apoptotic Thy-1.2$^+$CD4$^+$ T cells. αBr-dUTP, 5-bromo-2′-deoxyuridine 5′-triphosphate. This experiment was repeated twice with similar results.

apoptotic T cells are known to accumulate in the liver. The lack of peripheral T cells in our KLF2-deficient mice was therefore not due to apoptosis resulting from spontaneous T cell activation.

KLF2-deficient T cells respond functionally to S1P

The lack of KLF2-deficient T cells in secondary lymphoid organs and blood was similar to that in mice lacking $S1P_1$ (refs. 10,11). Moreover, a published study has attributed this phenotype to an inability of KLF2-deficient thymocytes to upregulate $S1P_1$, thus preventing mature thymocytes from exiting the thymus[9]. Consistent with that report, we found that KLF2-deficient single-positive thymocytes expressed approximately fivefold less $S1p_1$ mRNA than did cells from littermate control mice (**Fig. 3a**). As the amount of $S1P_1$ is regulated at the protein level by ligand-induced receptor clearance[16–18], we directly examined $S1P_1$ surface expression on KLF2-deficient thymocytes with an antibody to $S1P_1$ that recognizes the receptor ectodomain[19]. In agreement with the amount of mRNA, there was much less surface $S1P_1$ on KLF2-deficient CD4$^+$ thymocytes, although we reproducibly detected low expression of $S1P_1$ protein (**Fig. 3b**). These small amounts of $S1P_1$ were even lower on thymocytes from mice treated with FTY720, a powerful $S1P_1$ agonist that rapidly downregulates surface receptor expression[10,20,21] (**Fig. 3c**), which provided additional evidence that KLF2-deficient thymocytes express amounts of $S1P_1$ that are responsive.

The limited amounts of $S1P_1$ mRNA and surface receptor detected on thymocytes from KLF2-deficient mice suggested that functional studies were needed to determine if S1P-responses were intact in KLF2-deficient T cells. To directly test $S1P_1$ function, we first measured the migration of KLF2-deficient thymocytes toward S1P *ex vivo*. In a Transwell assay, S1P induced the migration of CD4$^+$ KLF2-deficient thymocytes, and this migration was eliminated by CD4$^+$ cells from FTY720-treated mice (**Fig. 3d**). To test whether KLF2-deficient T cells could respond to S1P *in vivo*, we treated *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$Vav-Cre$^+$ mice with FTY720 to block $S1P_1$-induced migration and then measured CD4$^+$ T cell numbers in the thymus, blood, spleen, and mesenteric and axillary lymph nodes. Consistent with the *ex vivo* experiments, treatment with FTY720 resulted in the accumulation of KLF2-deficient CD4$^+$ T cells in the thymus and peripheral lymph nodes (**Fig. 3e,f**). These findings show that KLF2-deficient thymocytes and peripheral T cells respond to S1P gradients, which suggests that the substantially fewer naive T cells in the blood and secondary lymphoid organs of KLF2-deficient mice might not reflect a failure of the cells to exit the thymus.

Naive KLF2-deficient T cells in nonlymphoid peripheral tissues

We obtained an initial indication of how KLF2 regulates peripheral T lymphocytes by examining T cells trapped in the lymph nodes of FTY720-treated KLF2-deficient mice. In contrast to the small number of 'pseudo-activated' (CD44^{hi}CD62L^{lo}) T cells in untreated KLF2-deficient mice, cells from FTY720-treated mice had a normal, naive phenotype (CD44^{lo}CD62L^{hi}; **Fig. 4**). Drug treatment did not directly modify these activation markers, as KLF2-deficient splenocytes cultured in the presence of FTY720 maintained their activated, memory phenotype (**Supplementary Fig. 1** online). Instead, the rapid accumulation of KLF2-deficient lymphocytes in lymph nodes suggested the presence of a large population of previously unidentified naive KLF2-deficient peripheral T cells.

To assess the distribution of peripheral T cells in *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$Vav-Cre$^+$ mice, we measured CD4 mRNA expression in a panel of tissues, including both T cell–rich secondary lymphoid organs (such as thymus, spleen and lymph nodes) and tissues typically devoid of naive T cells (such as bone marrow, kidney and skeletal muscle). We used this technique because of the difficulty in analyzing some peripheral tissues by more conventional means, such as flow cytometry; it also allowed us to screen a broad array of peripheral organs. Although expression of CD4 in the secondary lymphoid organs of *Klf2*$^{-/-}$Vav-Cre$^+$ mice was lower than that in *Klf2*$^{fl/fl}$Vav-Cre$^-$ (control) mice, expression of CD4 was much higher in nonlymphoid tissues of *Klf2*$^{-/-}$Vav-Cre$^+$ mice (**Fig. 5a**). That finding was supported by quantitative flow cytometry of CD4$^+$ T cells in livers from these mice: *Klf2*$^{fl/fl}$Vav-Cre$^-$ mice had

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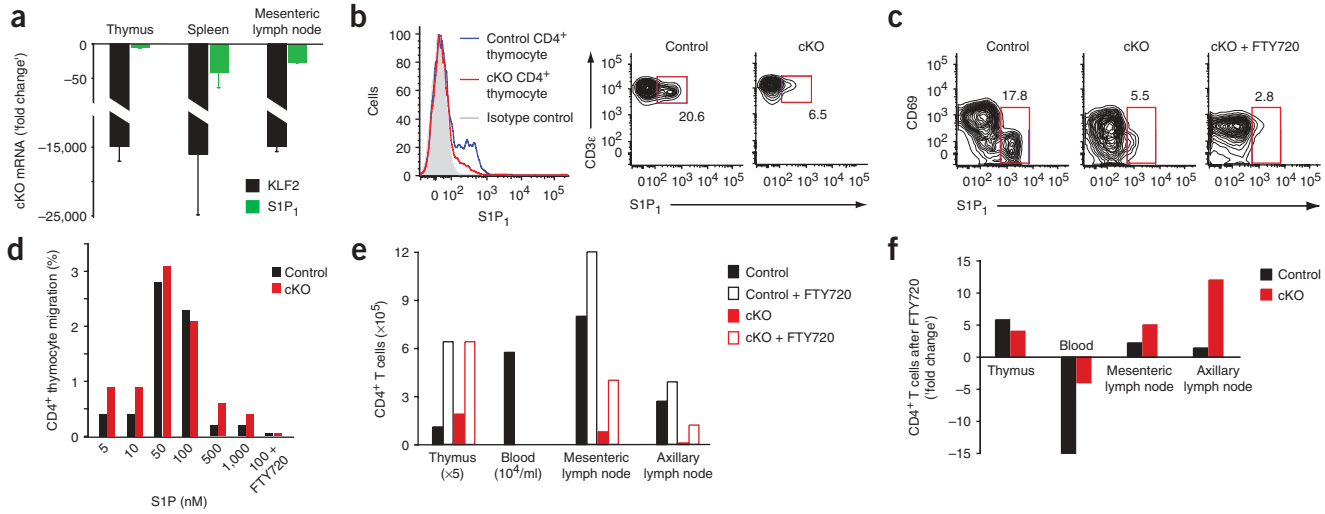

Figure 3 CD4+ T cell migration in response to S1P and inhibition of migration by FTY720 indicates the presence of peripheral KLF2-deficient T cells in *Klf2*$^{fl/fl}$Vav-Cre^{-} mice. (**a**) RT-PCR analysis of the expression of KLF2 and S1P$_1$ mRNA by Thy-1.2^{+}CD4^{+} thymocytes and T cells from *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} mice. Results are presented as the 'fold change' in mRNA in KLF2-deficient cells relative to that in *Klf2*$^{fl/fl}$Vav-Cre^{-} cells. (**b**,**c**) Flow cytometry of CD4^{+} thymocytes from *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} littermates, stained with antibody to the ectodomain of S1P$_1$. (**b**) Right, S1P$_1$ surface expression on mature CD4^{+} thymocytes; numbers adjacent to outlined areas indicate percent S1P$_1^{+}$ cells among mature (CD3ε^{hi}) thymocytes. (**c**) Cells stained with anti-CD69; cells collected from *Klf2*$^{-/-}$Vav-Cre^{+} mice after treatment with FTY720 (far right) serve as a negative control. (**d**) Transwell assay of the migration of *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} thymocytes toward various concentrations of S1P after 4 h. Thymocytes previously treated with FTY720 (far right) serve as a negative control. Percent migration = (migration of experimental CD4^{+} T cells – background migration of CD4^{+} T cells) / total CD4^{+} thymocyte input. (**e**,**f**) CD4^{+} T cell numbers in various tissues of *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} mice left untreated or treated for 24 h with FTY720 (+ FTY720). $P < 0.005$ (for all results except $P < 0.05$, control axillary lymph node), before versus after FTY720. (**f**) Change in CD4^{+} T cell numbers after FTY720 treatment. Data are representative of three (**a**), two (**b**–**d**) or four (**e**,**f**) independent experiments.

$7.0 \times 10^4 \pm 2.0 \times 10^4$ cells, and *Klf2*$^{-/-}$Vav-Cre^{+} littermate mice had $18.2 \times 10^4 \pm 4.9 \times 10^4$ cells ($P < 0.03$; **Fig. 5b**). These findings show that despite the many fewer KLF2-deficient T cells in the blood and secondary lymphoid tissues, KLF2-deficient naive T cells were present in nonlymphoid organs, which suggested that a trafficking or migration defect might be responsible for the peripheral T cell phenotypes of the KLF2-deficient mice.

To directly track naive T cell migration, we injected fluorescein isothiocyanate (FITC) into the thymuses of *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} mice and characterized the *in vivo* homing of naive T cells that exited the thymus. As anticipated, 24 h after intrathymic injection of FITC, we detected labeled wild-type T cells in secondary lymphoid organs such as the spleen, whereas we detected few wild-type T cells in the liver (**Fig. 5c**). In contrast, most FITC-labeled KLF2-deficient T cells were present in nonlymphoid organs such as the liver. These findings demonstrate that KLF2 is not required for thymic exit but is necessary to maintain naive T cells in a homeostatic migratory pattern restricted to secondary lymphoid organs.

Klf2$^{-/-}$ T cells express inflammatory chemokine receptors

The migration of naive T cells is restricted to the blood and secondary lymphoid organs to maximize the opportunity for these cells to interact with appropriate antigen-presenting cells. This migration pattern is governed by the expression of a limited set of chemokine receptors such as CCR7 and CXCR4, which direct the entry of naive T cells into lymph nodes across high endothelial venules[22–24]. To determine if the abnormalities in the homing of KLF2-deficient T cells reflected a change in chemokine-directed movement, we examined chemokine receptor expression in *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} CD4^{+} thymocytes. Although there was minimal change in CCR7

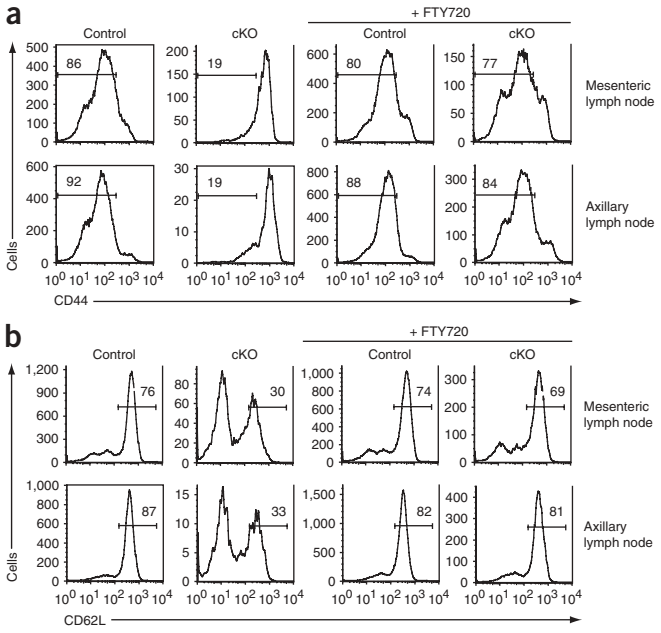

Figure 4 KLF2-deficient T cells trapped in lymph nodes by FTY720 treatment have a naive phenotype. Flow cytometry of the activation-memory markers CD44 (**a**) and CD62L (**b**) on Thy-1.2^{+}CD4^{+} T cells from lymph nodes of *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} littermates before and after FTY720 treatment. Numbers above bracketed lines indicate percent CD44^{lo} T cells (**a**) or CD62L^{hi} T cells (**b**). Data are representative of four experiments.

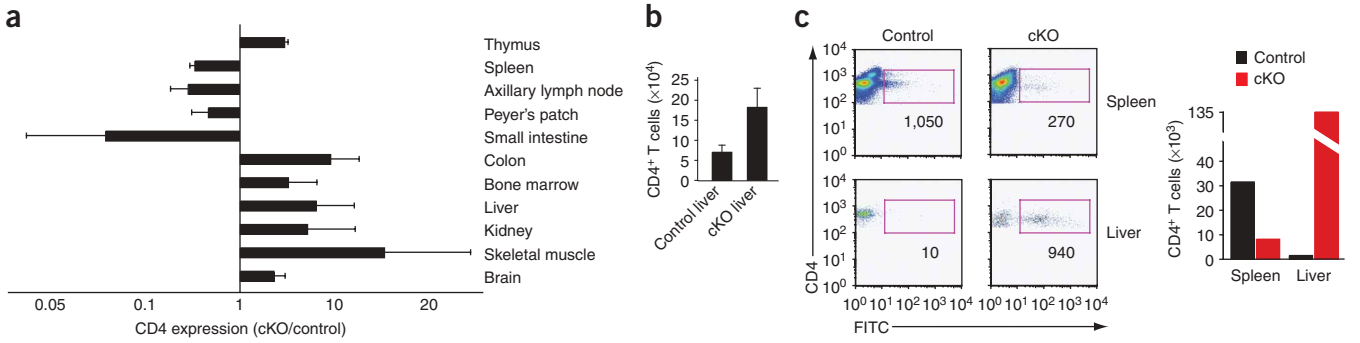

Figure 5 KLF2-deficient T cells home to nonlymphoid peripheral tissues. (**a**) Real-time PCR analysis of CD4 mRNA in a panel of tissues from *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} mice. Data are presented as 'fold change' in expression in *Klf2*$^{-/-}$Vav-Cre^{+} tissues relative to that in *Klf2*$^{fl/fl}$Vav-Cre^{-} tissues. Data are representative of two experiments (error bars indicate s.d. in each individual experiment). (**b**) Flow cytometry of CD4^{+} cells in liver tissues from *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} mice ($n = 3$ per group). $P < 0.03$. Data are representative of four experiments. (**c**) Flow cytometry of FITC-labeled CD4^{+} cells in the spleens and livers of *Klf2*$^{fl/fl}$Vav-Cre^{-} and *Klf2*$^{-/-}$Vav-Cre^{+} mice at 24 h after intrathymic injection of FITC. Numbers below outlined areas indicate numbers of Thy-1.2^{+}CD4^{+} T cells. Right, absolute numbers of Thy-1.2^{+}CD4^{+}FITC^{+} T cells. $P < 0.01$ (spleen) or $P < 0.005$ (liver). Data are representative of two experiments.

or CXCR4, KLF2-deficient CD4^{+} thymocytes had higher expression of many chemokine receptors not associated with naive T cells, including CCR1, CCR3, CCR5, CCR6, CxCR1 (interleukin 8 receptor α-chain), CXCR2 (interleukin 8 receptor β-chain), CXCR3, CXCR5, CXCR7 and XCR1 (**Fig. 6a**). To further characterize the expression patterns of chemokine receptors in naive KLF2-deficient T cells, we isolated CD4^{+}CD62L^{hi} T cells from the lymph nodes of KLF2-deficient and control mice that had been treated with FTY720 previously. The aberrant expression pattern of chemokine receptors noted in KLF2-deficient thymocytes was also present in peripheral KLF2-deficient T lymphocytes (**Fig. 6b**).

To determine if KLF2 repressed the expression of inflammatory chemokine receptors, we compared KLF2-mediated regulation of luciferase reporter constructs generated with the promoter regions of genes encoding the chemokine receptors CCR3 (*Ccr3*) and CCR5 (*Ccr5*), as well as S1P$_1$ (*Edg1*). KLF2 has been shown to upregulate expression of *Edg1* reporter constructs[9,25], and we also noted positive regulation of *Edg1* by KLF2 (approximately 2.5-fold; $P = 0.001$; **Fig. 6c**). In contrast, KLF2 repressed expression of a *Ccr3* reporter by 75% ($P = 0.0001$) and of a *Ccr5* reporter by 40% ($P = 0.0003$; **Fig. 6c**). These findings are consistent with published studies demonstrating repressor regions in genes encoding these chemokine receptors that contain consensus KLF-binding sequences[26–28]. We also detected upregulated chemokine receptor expression at the protein level in the absence of KLF2, as both untreated thymocytes and T cells from FTY720-treated KLF2-deficient mice had higher surface expression of CCR3 than did those from littermate control mice (**Fig. 6d**). Finally, to determine if expression of these atypical chemokine receptors was able to confer changes in T cell migration, we did *in vitro* migration assays with the eosinophil-recruiting chemokine CCL11 (eotaxin), a CCR3 ligand[29,30]. We chose this chemokine receptor because CCL11 is a highly specific ligand for CCR3 and CCR3 is rarely expressed on T cells, even in an activated state. Thus, testing cellular responses to CCL11 provides a stringent functional test of whether the upregulated chemokine receptor mRNA detected in KLF2-deficient thymocytes and T cells resulted in newly acquired migration potential. KLF2-deficient CD4^{+} thymocytes migrated in response to this chemokine, but control CD4^{+} thymocytes did not (**Fig. 6e**), which demonstrated that upregulation of chemokine receptors on naive T cells lacking KLF2 was sufficient to drive new migratory responses. These studies demonstrate that KLF2 is able to repress chemokine receptor expression and that loss of KLF2 in T cells results in the upregulated expression of many chemokine receptors capable of altering T cell migration responses.

Chemokine receptor signaling mediates the migration of *Klf2*$^{-/-}$ T cells

KLF2-deficient T cells are located in nonlymphoid peripheral tissues, a phenotype consistent with the expression of inflammatory chemokine receptors, but we also detected changes in the expression of adhesive receptors such as CD44, CD62L and β$_7$ integrin that may contribute to this migration pattern, as reported before[4,9,25] (**Fig. 1a** and **Supplementary Fig. 2** online). To functionally assess the involvement of abnormal chemokine receptor expression in the homing of KLF2-deficient naive T cells *in vivo*, we labeled CD4^{+} thymocytes from *Klf2*$^{fl/fl}$Vav-Cre^{-} CD4^{+} and *Klf2*$^{-/-}$Vav-Cre^{+} mice and injected them together into wild-type C57BL/6 recipient mice with or without prior treatment with pertussis toxin to block the G$_i$ protein–coupled signals mediated by chemokine receptors. Consistent with the results obtained with thymic injection of FITC (**Fig. 5b**), *Klf2*$^{fl/fl}$Vav-Cre^{-} CD4^{+} (control) thymocytes homed to secondary lymphoid organs such as the spleen and lymph nodes, whereas KLF2-deficient CD4^{+} thymocytes homed mainly to nonlymphoid tissues such as the liver (**Fig. 7a**). Notably, we found few KLF2-deficient CD4^{+} thymocytes in peripheral lymph nodes (**Fig. 7a**), a result identical to that reported for KLF2-deficient thymocytes collected from fetal liver chimeras[9] but in contrast to S1P$_1$-deficient thymocytes that become trapped in lymph nodes[10]. Control (*Klf2*$^{fl/fl}$Vav-Cre^{-} CD4^{+}) and KLF2-deficient thymocytes treated with pertussis toxin showed identical migration patterns and were retained in the blood and spleen, failing to enter the lymph nodes or liver (**Fig. 7b**). These findings demonstrate that the migration of KLF2-deficient naive T cells is not conferred by changes in the expression of adhesive receptors or S1P$_1$ deficiency but instead requires G$_i$ protein–coupled receptor signaling, such as that 'downstream' of chemokine receptors.

Although loss of abnormal T cell migration in pertussis toxin–treated cells is consistent with chemokine receptor–directed responses, that finding did not specifically address the contribution of those receptors to the phenotype of KLF2-deficient peripheral T cells. To directly test the function of inflammatory chemokine receptors, we

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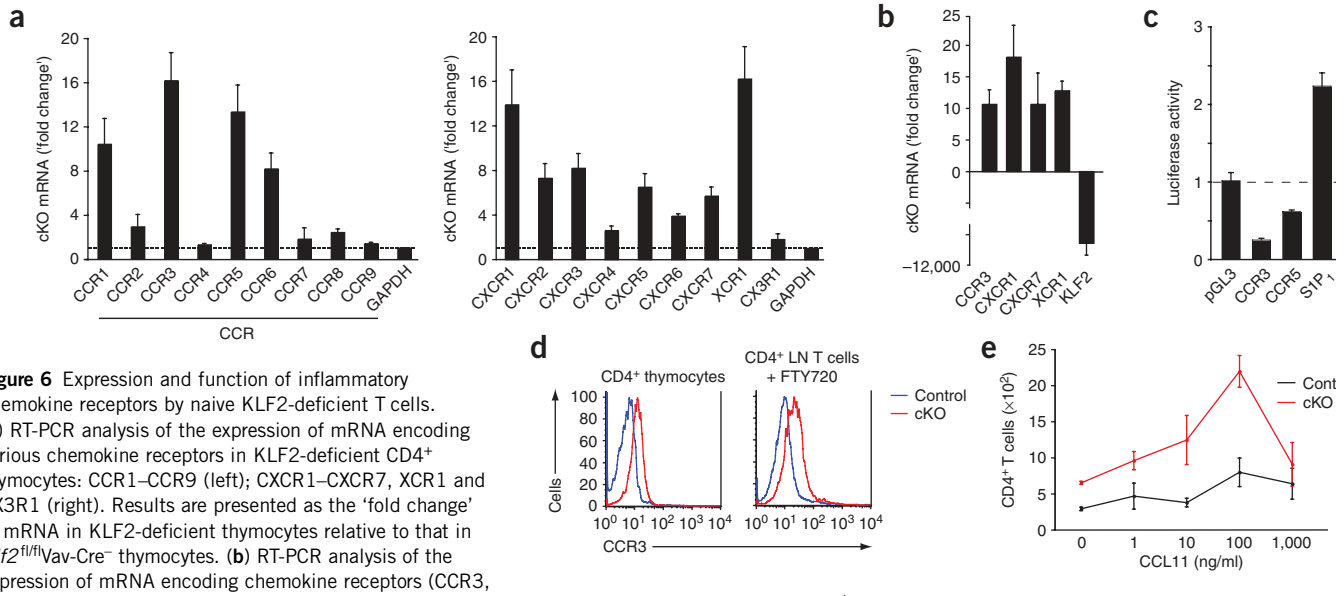

Figure 6 Expression and function of inflammatory chemokine receptors by naive KLF2-deficient T cells. (**a**) RT-PCR analysis of the expression of mRNA encoding various chemokine receptors in KLF2-deficient CD4$^+$ thymocytes: CCR1–CCR9 (left); CXCR1–CXCR7, XCR1 and CX3R1 (right). Results are presented as the 'fold change' in mRNA in KLF2-deficient thymocytes relative to that in *Klf2*$^{fl/fl}$Vav-Cre$^-$ thymocytes. (**b**) RT-PCR analysis of the expression of mRNA encoding chemokine receptors (CCR3, CXCR1, CXCR7 and XCR1) and KLF2 in peripheral CD4$^+$CD62L$^+$ T cells from FTY720-treated *Klf2*$^{-/-}$Vav-Cre$^+$ mice relative to that in FTY720-treated *Klf2*$^{fl/fl}$Vav-Cre$^-$ mice. (**c**) Luciferase assay of *Ccr3*, *Ccr5* and *Edg1* (S1P$_1$) promoter activity after cotransfection of reporter plasmids along with pcDNA3 vector or pcDNA3-*Klf2* in HeLa cells ($n = 3$ samples for each group). Luciferase activity is presented relative to that of the pcDNA3 control. pGL3, empty luciferase vector. $P = 0.5$ (pGL3); $P = 0.0001$ (*Ccr3*); $P = 0.0003$ (*Ccr5*); and $P = 0.001$ (*Edg1*). (**d**) Flow cytometry of surface CCR3 expression on CD4$^+$ thymocytes and CD4$^+$CD62L$^+$ peripheral T cells from *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$Vav-Cre$^+$ mice. Mean fluorescent intensity (mean ± s.d.): thymocytes, 21 ± 8 (*Klf2*$^{fl/fl}$Vav-Cre$^-$) and 35 ± 8 (*Klf2*$^{-/-}$Vav-Cre$^+$; $P < 0.1$); peripheral T cells, 74 ± 4 (*Klf2*$^{fl/fl}$Vav-Cre$^-$) and 137 ± 15 (*Klf2*$^{-/-}$Vav-Cre$^+$; $P < 0.05$). (**e**) Transwell assay of the migration of CD4$^+$ thymocytes toward various concentrations of the CCR3-specific chemokine CCL11, measured in triplicate *ex vivo*. $P < 0.005$ at maximum migration (100 ng/ml). Data are representative of two independent experiments (**a**, error bars indicate s.d. in each individual experiment), or two (**b**,**c**,**e**) or three (**d**) experiments.

treated thymocytes from KLF2-deficient mice with Met-RANTES, an amino-terminal-modified derivative of the chemokine RANTES (CCL5) that antagonizes signaling by the inflammatory chemokine receptors CCR1 and CCR5, both of which have been shown to mediate cellular homing to the liver[31,32]; Met-RANTES has been shown to attenuate T cell–induced tissue damage in animal models of hepatitis by reducing the homing of T cells to the liver[33]. Treatment of KLF2-deficient thymocytes with Met-RANTES did not alter cellular migration to the spleen or lymph node but it significantly inhibited migration to the liver (**Fig. 7c**). As anticipated, given the large repertoire of chemokine receptors expressed by KLF2-deficient thymocytes, Met-RANTES inhibition was less complete than that noted with pertussis toxin. The blockade of abnormal migration by both a broad inhibitor of G$_i$ protein–coupled signaling and a specific inhibitor of chemokine receptors suggests that the homing of naive KLF2-deficient T cells is mediated by chemokine responses.

DISCUSSION

Studies have established that KLF2 is required for mature T cell homeostasis, but the mechanism involved has remained elusive. Here we have used *Klf2*$^{-/-}$Vav-Cre$^+$ mice, which have conditional excision of *Klf2* in the hematopoietic compartment, to demonstrate a function for KLF2 in the repression of inflammatory chemokine receptor expression and migration. As KLF2 expression is tightly coupled to T cell activation state, our findings suggest that KLF2 may serve as a molecular link between T cell movement and activation state, an essential feature of the adaptive immune response.

The lack of KLF2-deficient T cells in peripheral lymphoid organs has been the subject of several studies. KLF2 was first proposed to maintain naive T cells in a quiescent state, and KLF2 deficiency was proposed to result in upregulation of FasL and induction of apoptosis[4,15], thereby explaining the lack of naive *Klf2*$^{-/-}$ T cells as a consequence of cell death. Using a stable genetic mouse model to examine KLF2-deficient T cells, we found that neither single-positive

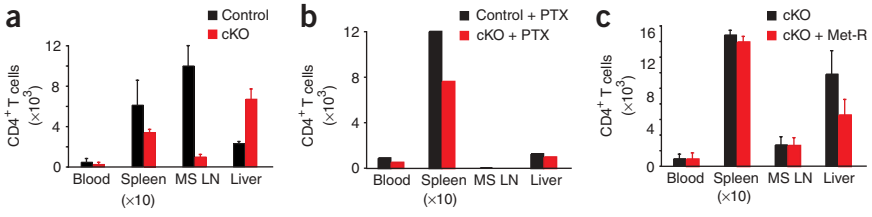

Figure 7 Altered homing by KLF2-deficient T cells *in vivo* requires G$_i$ protein–coupled chemokine receptor signaling. (**a**) Flow cytometry of CD4$^+$ T cells in peripheral tissues 16 h after thymocytes from *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$Vav-Cre$^+$ mice were mixed at a 1:1 CD4$^+$ cell ratio and injected together intravenously into wild-type recipient mice ($n = 3$ mice). $P > 0.2$ (blood), or $P < 0.1$ (other tissues). (**b**) Repeat of the experiment in **a** after treatment with pertussis toxin (PTX) to block G$_i$ protein–coupled signaling 'downstream' of chemokine receptors (**b**). MS LN, mesenteric lymph node. Data (mean and s.d.) are representative of two experiments in **a**,**b**. (**c**) CD4$^+$ T cells in wild-type recipient mice 16 h after adoptive transfer of Met-RANTES treated KLF2-deficient thymocytes mixed at a ratio of 1:1 with untreated KLF2-deficient thymocytes ($n = 2$ mice). $P < 0.005$, liver of treated versus untreated thymocytes. Data (mean and s.d.) are representative of two experiments.

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thymocytes nor $CD4^+$ T cells from KLF2-deficient mice had more FasL surface expression or any evidence of more apoptosis. Similar negative findings have also been reported with radiation chimeras[9]. Thus, it is unlikely that the lack of KLF2-deficient T cells in peripheral lymphoid organs is due to unregulated apoptosis.

Another study has suggested that KLF2-deficient T cells are absent from secondary lymphoid organs because they fail to leave the thymus[9]. According to this model, the main function of KLF2 is to upregulate $S1P_1$ expression in mature thymocytes, a necessary step in thymic exodus. Inconsistent with this model, however, is the fact that adoptively transferred $S1P_1$-deficient thymocytes accumulate in lymph nodes, whereas almost no KLF2-deficient thymocytes are detected in lymph nodes with the same transfer technique. As suggested by those observations, we demonstrated here that the smaller amount of $S1P_1$ on KLF2-deficient thymocytes was sufficient to confer migratory responses to S1P *ex vivo* and thymic egress *in vivo*: after FTY720 treatment, similar numbers of single-positive thymocytes accumulated in the thymuses of *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$Vav-Cre$^+$ mice and an equivalent number of tagged single-positive thymocytes were detected in the periphery of *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$Vav-Cre$^+$ mice after intrathymic injection of FITC. Most notably, we detected substantial numbers of peripheral naive T cells in *Klf2*$^{-/-}$Vav-Cre$^+$ peripheral organs, albeit not in secondary lymphoid organs. These results suggest that the lack of naive KLF2-deficient T cells in peripheral lymphoid organs results from a defect in peripheral T cell homing rather than thymic exit.

If KLF2-deficient T cells exit the thymus, why are none found in secondary lymphoid organs? Serendipitously, the first clue to this unusual phenotype arose through studies of $S1P_1$ function. In addition to demonstrating thymic exit, treatment of *Klf2*$^{-/-}$Vav-Cre$^+$ mice with the pharmacological $S1P_1$ inhibitor FTY720 unexpectedly trapped a large number of naive T cells in lymph nodes, which suggested that a population of KLF2-deficient naive T cells exists in peripheral tissues outside its expected location in secondary lymphoid organs. This unusual distribution of naive KLF2-deficient T cells is directly attributable to aberrant migration patterns, as demonstrated by assays of intrathymic injection of FITC and the homing pattern of KLF2-deficient thymocytes injected into wild-type recipient mice. Thus, the chief means by which KLF2 regulates peripheral naive T cells is control of cellular migration and movement.

The migration of naive T cells is directed by chemokine receptors responding to homeostatic chemokine gradients[34–36]. We found that KLF2-deficient thymocytes and naive T cells expressed inflammatory chemokine receptors not normally associated with such cells, which provided a molecular explanation for aberrant homing to nonlymphoid peripheral tissues. The movement of naive T cells between the blood and lymph nodes is regulated mainly by CCR7 and CXCR4, homeostatic chemokine receptors whose mRNA expression was unchanged in KLF2-deficient thymocytes. In contrast, KLF2-deficient thymocytes and naive $CD4^+$ T cells upregulated many chemokine receptors normally expressed only on activated T cells or non–T cells. Studies addressing the expression and function of one such receptor, CCR3, showed that expression of these chemokine receptors was able to confer new migration responses. More notably, we found that specific inhibition of CCR1 and CCR5 with Met-RANTES blocked the migration of KLF2-deficient thymocytes to the liver, as did nonspecific inhibition of chemokine receptor signaling by pertussis toxin. Together these studies support a mechanism in which atypical chemokine receptor expression in KLF2-deficient T cells drives aberrant migration to nonlymphoid tissues.

Redistribution of peripheral T cells to nonlymphoid organs requires extravasation, an active process that is consistent with chemokine receptor gain of function but could also be caused by changes in adhesive receptor function. Some subsets of KLF2-deficient T cells have altered expression of adhesive receptors, but these changes are not consistent with the observed migration phenotype. For example, a population of $CD4^+$ KLF2-deficient thymocytes has low expression of CD62L, a receptor that helps T cells enter lymph nodes[37], but this phenotype does not explain how KLF2-deficient T cells home to and extravasate into nonlymphoid tissues. In addition, we noted that a subset of peripheral T cells found in the secondary lymphoid organs of KLF2-deficient mice seemed 'pseudo-activated' and had higher surface expression of CD44 and β_7 integrin. However, KLF2-deficient $CD4^+$ thymocytes had normal surface expression of those receptors, which demonstrated that they were not required for migration to nonlymphoid peripheral tissues such as the liver. Changes in the expression of adhesive receptors may therefore be another aspect of the KLF2-deficient T cell phenotype but are not sufficient to explain the aberrant migration patterns of KLF2-deficient T cells.

A notable issue raised by our studies is the nature of the inflammatory chemokines to which naive KLF2-deficient T cells respond in an uninfected mouse. Chemokine receptors defined as 'inflammatory' in the context of T lymphocytes are constitutively expressed by a variety of quiescent leukocyte populations, presumably to regulate their own unique migration patterns. For example, although only a subset of activated wild-type T cells express CCR3, this chemokine receptor is constitutively expressed by eosinophils, basophils and mast cells[38–40]. Likewise, CCR1 and CCR5 are both constitutively expressed on tissue-invading macrophages, whereas these receptors are restricted to activated T cell subsets in wild-type animals. As the migratory patterns of eosinophils, basophils, mast cells and macrophages are distinct from those of naive T cells, we speculate that KLF2-deficient T lymphocytes respond to some of these non–T cell homeostatic chemokine gradients and thereby acquire their abnormal migration patterns.

The adaptive immune system requires that naive T cells survey for potential pathogens in a restricted circulatory loop composed of blood and secondary lymphoid organs. Once activated, T cells proliferate, acquire effector functions and migrate to sites of inflammation. Memory T cells generated during this immune response either 'revert' to a naive pattern of circulation (central memory cells) or continue to migrate to peripheral tissues (effector memory cells)[36,41,42]. Thus, the range of T cell movement is tightly linked to T cell functional state. Here we have identified KLF2 as a molecular component of that link, which is needed to prevent naive T cells from expressing inflammatory chemokine receptors and acquiring broader migration patterns in the body. KLF2 is first expressed in mature, single-positive thymocytes before they enter the naive T cell pool. T cell activation rapidly downregulates KLF2, and this transcription factor is re-expressed only if the lymphocyte becomes a memory T cell[3,43]. KLF2 downregulation after T cell activation is therefore likely to release cells from the constraint of naive T cell trafficking and facilitate migration to peripheral tissues. Further studies addressing the function of KLF2 in the migration and function of effector memory T cells will determine the extent to which this transcription factor links T cell movement and activation state.

Finally, the identification of KLF2 as a regulator of T cell chemokine receptor expression and migration raises issues regarding the function of T cell movement in controlling immune responses *in vivo*. The biological effects of unrestrained T cell migration by KLF2-deficient

T cells are unknown. Impaired circulation of naive T cells may delay antigen recognition, resulting in an immune compromised state. Aberrant stimulation of naive T cells in a nonlymphoid environment may result in T cell anergy and also impair immune responses. Alternatively, exposure of naive T cells to 'cloistered' self antigens may result in autoimmunity. The results of future studies addressing these issues may identify new approaches for modulating immune responses in human disease states.

METHODS

Mouse models. *Klf2*$^{fl/fl}$ mice were generated as outlined[7]. Vav-Cre–transgenic mice were provided by T. Graf (Albert Einstein College of Medicine)[14]. Lck-Cre–transgenic mice were purchased from Taconic[44]. All mice were housed in pathogen-free conditions at the University of Pennsylvania in accordance with National Institute of Health guidelines and approved animal protocols.

Antibodies. Surface antibodies included antibody to mouse CD4 (anti–mouse CD4; GK1.5), anti-CD8α (53-6.7), anti-CD24 (M1/69), anti-CD25 (7D4), anti-CD44 (1M7), anti-CD45 (30-F11), anti-CD45RB (16A), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD90.2 (50-2.1), anti-CD178 (MFL3) and anti-CD193 (polyclonal CCR3; all from BD PharMingen). Anti–mouse CD5 (53-7.3) was from eBioscience. $S1P_1$-specific antiserum was generated as described[19].

Flow cytometry. Cells were stained according to standard protocols and were examined on a FACSCalibur (Becton Dickinson). For counting, 2.5×10^5 CaliBRITE Beads (BD Biosciences) were placed in each individual tube before flow cytometry. FlowJo software (Tree Star) was used to analyze the data.

Analysis of T cell apoptosis. Cells from spleen and mesenteric lymph node were collected and spontaneous T cell apoptosis was measured with the Annexin V:PE Apoptosis Detection Kit I (BD Biosciences). Alternatively, cells were collected from thymus, spleen and liver and an APO-BRDU kit from Phoenix Flow Systems was used for TUNEL assays. As a positive control, a wild-type littermate mouse was sublethally irradiated with a Gamma Cell 40 Exactor (MDS Nordion) 24 h before examination.

***In vitro* migration assay.** Thymocytes from control (*Klf2*$^{fl/fl}$Vav-Cre$^-$) mice or KLF2-deficient (*Klf2*$^{-/-}$Vav-Cre$^+$) mice were stained with CellTracker Green BODIPY (8-chloromethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) or CellTracker Orange CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl) amino)tetramethylrhodamine), respectively (Molecular Probes). Thymocytes were washed and were resuspended in serum-free media (Iscove's modified Dulbecco's media, 1× Nutridoma-SP (Roche) and 2-mercaptoethanol), and 5×10^6 thymocytes from each group were placed in 12-well Transwell tissue culture plates with 3.0-μm polycarbonate membranes (Corning). The bottom chambers were filled with various concentrations of S1P (Sigma) or CCL11 (PeproTech) suspended in serum-free media. Plates were cultured for 4 h at 37 °C, at which point transmigrated thymocytes were collected from the lower chamber, stained for CD4 and CD8 and analyzed by flow cytometry.

FTY720-treated mice. *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$Vav-Cre$^+$ littermates were given drinking water containing FTY720 (2 μg/ml; Cayman Chemical) for 4 d before tissue collection.

Real-time PCR of mouse tissues. Tissues from control and KLF2-deficient littermates were mechanically homogenized and passed through 70-μm nylon cell strainers. Tissues were placed in Eppendorf tubes, were 'spun down' and were resuspended in 750 μl Trizol (GibcoBRL). RNA was extracted according to the manufacturer's instructions and was resuspended in 50–100 μl RNAse-free water. A SuperScript II First-Strand Synthesis kit (Invitrogen) was used to generate cDNA. Sybr Green master mix (Applied Biosystems) plus forward and reverse primers for mouse CD4 were used for real-time PCR of cDNA, done in triplicate. An ABI Prism 7900HT Sequence Detection System was used for the PCR, and data were analyzed with SDS 2.0 software (Applied Biosystems).

Real-time analysis of chemokine receptor expression. CD4$^+$ thymocytes or CD4$^+$CD62L$^+$ T cells were isolated from *Klf2*$^{fl/fl}$Vav-Cre$^-$ (control) and *Klf2*$^{-/-}$ Vav-Cre$^+$ (KLF2-deficient) littermates with a FACSAria (BD Biosciences). For maximization of cell numbers, CD4$^+$CD62L$^+$ T lymphocytes were collected from mice that had previously been treated with FTY720. Real-time PCR was used to generate cDNA from these cells (chemokine receptor primer pairs, **Supplementary Table 1** online). Data are expressed as follows: $2^{[(\text{CT for KLF2-deficient chemokine receptor gene} - \text{CT for KLF2-deficient } \textit{Gapdh}) - (\text{CT for control chemokine receptor gene} - \text{CT for control } \textit{Gapdh})]}$, where CT is the cycling threshold and *Gapdh* encodes glyceraldehyde phosphate dehydrogenase.

Luciferase reporter assays. Luciferase reporter plasmids were generated in the luciferase expression vector pGL3-basic (Promega) with PCR to amplify the following promoter and/or regulatory regions from mouse genomic DNA: *Ccr3*, 1.6 kilobases extending 5′ from the start codon; *Ccr5*, 1.27 kilobases extending 5′ from the start codon and 2.7 kilobases of the first intron extending 3′ of exon 1; and *Edg1* (encoding $S1P_1$), 450 base pairs extending 5′ from the start codon. HeLa cells (4×10^4) were cotransfected with 2.5 ng phRL-TK (renilla luciferase plasmid), 250 ng reporter plasmids and 200 ng empty pcDNA vector or pcDNA-HA-KLF2 (a gift from the laboratory of L.H Glimcher) with Lipofectamine LTX transfection reagent (Invitrogen Life Technologies). At 24 h after transfection, luciferase activity was assessed with the Dual-Luciferase Reporter Assay system (Promega E1910).

Intrathymic FITC injection. Three age-matched *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$ Vav-Cre$^+$ mice were anesthetized and 50 μl FITC isomer I (350 μg/ml; Sigma) resuspended in saline was injected into each thymic lobe. The Mouse Cardiovascular Physiology and Microsurgery Core of the University of Pennsylvania did this surgery. Mice were killed 24 h later and FITC-stained CD4$^+$ T cells were detected by flow cytometry.

***In vivo* T cell migration assay.** Thymocytes from *Klf2*$^{fl/fl}$Vav-Cre$^-$ and *Klf2*$^{-/-}$ Vav-Cre$^+$ mice were stained with CellTracker Green BODIPY or CellTracker Orange CMTMR (Molecular Probes), then thymocytes were mixed at a CD4$^+$ ratio of 1:1 and resuspended at a final concentration of 5×10^7 cells per ml, and 200 μl of this cell suspension was injected into three wild-type recipient mice. At 16 h after injection, tissues were collected and examined by flow cytometry. For this, 400 μl blood was collected from the inferior vena cava of each anesthetized recipient mouse, then was washed in media containing 20 mM EDTA and lysed with Red Blood Cell Lysing Buffer (Sigma) after antibody staining. After blood was collected, the hepatic vein was nicked and mice were perfused by passage of 10 ml media through the left ventricle; in this way, blood was drained from the liver, thus allowing more accurate analysis of T cell homing to this tissue. A similar technique was used for analysis of thymocytes treated with pertussis toxin, except that thymocytes were cultured for 3 h at 37 °C with pertussis toxin (20 ng/ml; Sigma) before being stained with CellTracker dye. Because of lower thymocyte numbers, a single recipient was used instead of the three used before. Alternatively, thymocytes were pooled from three *Klf2*$^{-/-}$Vav-Cre$^+$ mice and stained with CellTracker Green BODIPY or CellTracker Orange CMTMR, and half the cells were cultured for 40 min at 37 °C with 160 nM recombinant human Met-RANTES (R&D Systems). Cells were thoroughly washed, combined with the remaining cells and injected together intravenously into three wild-type recipient mice.

Statistics. Where applicable, means and s.d. are presented. *P* values were calculated with an unpaired Student's *t*-test.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

E.S. designed and did experiments and wrote the manuscript; Z.Z., J.S.L. and T.W. designed and did experiments; and M.L.K. designed experiments and wrote the manuscript.

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