Kruppel-Like Factor 2 Is Required for Trafficking but Not Quiescence in Postactivated T Cells


*J Immunol* 2011; 186:775-783; Prepublished online 15 December 2010; doi: 10.4049/jimmunol.1000094
http://www.jimmunol.org/content/186/2/775

**Supplementary Material** http://www.jimmunol.org/content/suppl/2010/12/15/jimmunol.1000094.DC1

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Kruppel-Like Factor 2 Is Required for Trafficking but Not Quiescence in Postactivated T Cells


The transcription factor Kruppel-like factor 2 (KLF2) was proposed to regulate genes involved in cell cycle entry and T cell trafficking; however, the physiological role of its expression in postactivated T cells is not well defined. Previous studies suggested that the cytokines IL-2 and IL-15 differentially regulate KLF2 re-expression in postactivation T cells and that these cytokines also influence effector versus memory T cell differentiation. Using conditional and inducible KLF2-knockout model systems, we tested the specific role of KLF2 expression in activated CD8+ T cells cultured with these cytokines. KLF2 was required for effective transcription of sphingosine-1-phosphate receptor-1 (SIP1) and CD62L in postactivation T cells. However, although different cytokines dramatically altered the expression of cell-cycle–related genes, endogenous KLF2 had a minimal impact. Correspondingly, KLF2-deficient T cells showed dysregulated trafficking but not altered proliferative characteristics following in vivo responses to Ag. Thus, our data help to define KLF2-dependent and -independent aspects of activatedCD8+ T cell differentiation and argue against a physiological role in cell cycle regulation. The Journal of Immunology, 2011, 186: 775–783.
KLF2 IS DISPENSABLE FOR ACTIVATED T CELL QUIESCENCE

KLF2 has a direct and profound effect on expression of key cytokines. Although this role relates primarily to its impact on T cell trafficking rather than its control of cell cycle.

Materials and Methods

Mice

B6, B6. PL (Thyl.1 congenic B6), and B6. SJL (CD45.1 congenic B6) mice were purchased from National Cancer Institute. Klf2−/− mice have been described (13). CD4Cre transgenic (Tg) mice were purchased from Taconic Farms (Germantown, MD). ROSA26-floxSTOP-YFP mice were a kind gift of Dr. Frank Costantini (Columbia University). OT-I Tg mice with or without Rag−/− background were bred in our colony at the University of Minnesota. Klf2-null mice were described previously (10, 21).

Cell culture

Spleen cells from Rag−/− OT-I mice were incubated with 10 nM OVA peptide (OVAp, SIINFEKL) for 1 h at 37°C. After extensive washing, cells were cultured in RPMI 1640 media (Cellgro, Herndon, VA), supplemented with 10% FCS for stimulation. Polyclonal spleen cells and thymocytes were stimulated with plate-bound anti-CD3 Ab (145-2C11; BD Pharmingen, San Diego, CA) at 1 μg/ml. To stimulate the thymocytes, irradiated (3000 rad) spleen cells from normal mice were also added to the culture. Two days later, viable cells were enriched by centrifugation with Cellgro lymphocyte separation medium (Mediatech, Herndon, VA) and cultured in the presence of 20 ng/ml IL-2, IL-7, or IL-15 (R&D Systems, Minneapolis, MN) for another 6 d. Cytokine-containing media were replaced every other day. Before isolating total RNA from cultured thymocytes, CD8+ cells were purified by positive selection with anti-CD8 magnetic beads, following the manufacturer’s directions (Miltenyi Biotec, Auburn, CA), which yielded >90% CD8+CD4− cells. In some experiments, cells were subjected to sorting on FACSARia (Becton Dickenson, Mountain View, CA) at the end of the culture, yielding >90% purity of the desired target population.

Real-time PCR analysis

Total RNA was extracted from the indicated cell populations using the RNeasy kit (Qiagen, Valencia, CA), and cDNA was synthesized with a SuperScript III RT-PCR kit (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. The cDNA was amplified on a SmartCycler (Cepheid, Sunnyvale, CA) by PCR with FastMaster SYBR Green Master (Roche, Basel, Switzerland) and the following primers: Self (CD62L), 5′-CTAATTCCCCCTCGGTATCAT-3′; 5′-GACATTACGTCCTGAC-3′; 5′-TGAAGGCTGGATTTCCTTTG-3′; 5′-AGCCTATCTGGCGTCCAA-3′; 5′-TCCATGAGCCCCTCTGTTAT-3′; Gapdh, 5′-TGTCCTACATGGCCCTTC-3′; 5′-AGCCTATCTGGCGTCCAA-3′; and 5′-AGCCTATCTGGCGTCCAA-3′; and Edjl (SIP1), 5′-TGTTAGACCCAGAGTAGCTCGTG-3′; and 5′-ACCTTTTCTTGGCCAGAG-3′. RNA levels of all samples were normalized to the detected amount of GAPDH mRNA.

Apoptosis and proliferation analysis

To examine the apoptosis and proliferation induced by T cell activation, thymocytes were placed in plates that had been coated with anti-CD3 (145-2C11) at 1.0 μg/ml and anti-CD28 (37.51; BD Pharmingen) at 20 μg/ml. To detect apoptotic cells, cells were analyzed by flow cytometry with Annexin V/PI (BD Pharmingen). To detect proliferation, cells were analyzed by flow cytometry after culture for 24–72 h.

Transactivator of transcription Cre treatment

Transactivator of transcription (TAT)Cre was a kind gift from Dr. Donna Farber, University of Maryland. Viable cells, separated from spleen cells stimulated for 48 h with plate-bound anti-CD3, were intensively washed in Hyclone ADCF-Mab serum-free media (Logan, UT) and reseeded in the media, at 5 × 106 cells/ml, containing Tat-Cre (50 μg/ml) and incubated for 45 min at 37°C. After washing with RPMI 1640 media supplemented with 10% FCS, cells were subjected to culture with the cytokines for an additional 6 d, as described above.

Vaccinia virus infection

Fetal liver chimeras were generated from the neonates of time-mated KLF2−/− breeders at day 12.5 of gestation, as previously described (10). Thyl.1 CD8+CD4− thymocytes from KLF2−/− or KLF2−/− OT-I Tg fetal liver chimeras were transferred to B6 mice (0.75 × 106 cells/mouse). One day later, the mice were injected i.p. with 5 × 106 PFU vaccinia virus-expressing OVA. Five days postinfection, donor cells in various tissues were analyzed by flow cytometry with Thy1 congenic markers.

Listeria monocytogenes infection

Thymocytes were obtained from KLF2−/−CD4Cre (Thyl.1+Thyl.2+) and wild-type (WT) (Thyl.1+Thyl.2+) F1 hybrid mice. To detect apoptotic cells, cells were analyzed by flow cytometry with Annexin V/PI (BD Pharmingen). Specific apoptosis induced by TCR stimulation was calculated according to a previous report (22). To evaluate apoptosis and proliferation analysis, cells were labeled with CFSE and analyzed by flow cytometry after culture for 24–72 h.

Flow cytometry

Cells were resuspended in PBS plus 1% FCS and incubated with fluorochrome-labeled Abs: anti-CD4 (L3T4), CD8 (53-6.7), CD24 (M1/69), and Qa-2 (1, 1-2) were purchased from BD Pharmingen; anti-CD25 (PC6.15) CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), Thy1.1 (HS51), and Thy1.2 (53-2.1) were purchased from e Bioscience (San Diego, CA). OVApeptide tetramer was prepared as previously described (24). Cells were analyzed using FACSCalibur or LSR II flow cytometer (Becton Dickenson), and the data were processed using FlowJo software (Tree Star, San Carlos, CA).

In vivo migration assay

To evaluate the T cell migration to secondary lymphoid organs and the blood, we applied a previously reported protocol, with slight modifications (25, 26). Equal numbers (5 × 106) of IL-15–treated cells (CD45.2+Thyl.2+) and naive splenocytes from B6.PL mice (CD45.2+Thyl.1+) were mixed so that 2 × 10^6 mature CD8+ T cells from each donor was transferred (by i.v. injection) into normal B6 mouse recipients. One day later, the host mice were injected i.p. with an attenuated (ActA−) strain of Listeria monocytogenes-expressing OVA (3 × 10^6 CFU/mouse) (23). At different time points postinfection, donor cells in various tissues were analyzed by flow cytometry with Thy1 congenic markers and the binding of OVApeptide tetramer.

Results

Expression of KLF2-related molecules in different CD8+ T cell stages

Previous studies reported that activated CD8+ T cells cultured with high-dose IL-2 differentiate into a state that phenotypically and
functionally mimics effector cells, whereas treatment with IL-7 or IL-15 induces central memory-like cells (27–29). To begin assessing the role of KLF2 in regulating the differentiation status of peripheral T cells, we characterized how culture in IL-2, IL-7, or IL-15 affected mRNA expression of Klf2 and its proposed targets. Real-time RT-PCR analysis showed that Klf2 mRNA is abundantly expressed in naive OT-I T cells but significantly decreased upon stimulation with OVAap (data not shown), corresponding to the findings in previous reports (16, 18). Subsequent culture with IL-2 for six additional days maintained these low levels of Klf2 mRNA, whereas IL-7- or IL-15–treated cells displayed a substantial recovery of Klf2 mRNA expression (Fig. 1A), consistent with previous studies (16, 17). Differentiation of T cells from naive to effector and memory cells is characterized by changes in expression of the trafficking molecules S1P1 and CD62L. These are expressed in naive and central memory cells but not in effector cells, and their expression was shown to be KLF2 dependent, at least in part (10, 11). Paralleling the expression of KLF2, transcripts of genes encoding S1P1 and CD62L were low in activated T cells and were induced by culture with IL-7 or IL-15 but not with IL-2 (Fig. 1A). As expected based on previous studies (27, 28), CD62L expression correlated with CD62L transcription (Fig. 1B). Elevated expression of CD69, which is generally inversely expressed with cell surface S1P1 (30, 31), was also observed for cells cultured in IL-2 but not for those cultured in IL-7 or IL-15 (Fig. 1B).

We next assessed expression of cell cycle-regulatory genes. Forced expression of KLF2 was reported to induce exit from the cell cycle by inhibiting the transcription of c-myc and increasing expression of the negative regulator p21Cip1 (7, 8). Despite the rapid initial proliferation of IL-2–cultured cells, expression of c-myc mRNA was lower in this population than in IL-7– or IL-15–treated cells (Fig. 1A). Similarly, mRNA expression of p21Cip1, a cell-growth inhibitor, was significantly increased with IL-2 treatment but not with IL-7 or IL-15. These results are consistent with other studies showing that activated CD8 T cells began to exit the cell cycle at ~6–8 d of culture in IL-2, whereas proliferation was sustained in IL-15–containing media (29). Importantly, these data did not correspond to the model previously suggested, which predicted that KLF2 re-expression would lead to reduced c-myc transcription and elevated p21Cip1 transcription (7, 8). Overall, KLF2 expression in postactivated CD8 T cell populations correlated with transcriptional regulation of genes controlling lymphocyte trafficking but not cellular quiescence.

**KLF2-deficient thymocytes are functionally mature and have normal proliferative capacity**

We next wished to test the impact of KLF2 deficiency on postactivation T cell gene expression. However, it was unclear whether the function of KLF2-deficient T cells may be compromised. When CD4 or CD8 single-positive (SP) thymocytes are produced in the thymus they are not fully mature, rather, they continue to functionally develop. For example, semi-mature CD4 SP thymocytes remain susceptible to apoptosis when stimulated via TCR, whereas fully mature CD4 SP thymocytes are competent to proliferate (32). This delay in maturation presumably facilitates negative selection by tissue-specific Ags encountered in the medulla. The change from apoptosis susceptibility to resistance is associated with cell-surface phenotype changes, such as downregulation of CD24 and CD69 and upregulation of CD62L and Qa2 (33). We previously reported that KLF2-deficient SP thymocytes do not have a profound survival defect in vivo, but it was unclear whether these cells achieved full maturity, because they fail to downregulate CD69 and upregulate CD62L (10). However, KLF2 deficiency did not prevent downregulation of CD24 or upregulation of Qa2 on SP thymocytes (Fig. 2A). Indeed, the regulation of these markers is even more dramatic in KLF2-deficient SP thymocytes than in WT SP thymocytes, suggesting that KLF2-deficient SP thymocytes are older than their WT counterparts, consistent with their thymic retention (10, 33). Given this mixed phenotype, we sought to test directly whether KLF2 was required for complete functional maturation of thymocytes.

To address functional competence, we stimulated CD4 SP thymocytes obtained from Klf2<sup>fl/flCD4Cre</sup> and control mice with anti-CD3 and anti-CD28. Apoptosis was measured by Annexin V binding at 18 h, and proliferation was measured by CFSE dye dilution at 48 h. In the control cultures, semimature (Qa2<sup>low</sup>) SP thymocytes were most susceptible to apoptosis (Fig. 2B), whereas proliferation was detected only in the mature (Qa2 hi) SP subset (Fig. 2C). This distinction was maintained in KLF2-deficient cells (Fig. 2B, 2C). Although Klf2 mutant thymocytes contained a smaller proportion of Qa2<sup>low</sup> CD4 SP cells, such cells preferentially underwent apoptosis (Fig. 2B, bar graph). In addition, the prominent pool of KLF2-deficient Qa2 hi cells seemed to be functionally competent, because they divided and underwent minimal apoptosis following activation (Fig. 2B, 2C). We saw similar responses by WT and Klf2<sup>−/−</sup> CD8 SP thymocytes in these assays, although there was considerable variability in the frequency and susceptibility to spontaneous apoptosis of semimature (Qa2<sup>{low}</sup>, CD24<sup>high</sup>) CD8 SP thymocytes (data not shown), making this analysis more difficult to interpret. Importantly, we found that KLF2 deficiency did not lead to spontaneous proliferation in the CD4 or CD8 SP pools (Fig. 2C, data not shown), in contrast to the dysregulated proliferation suggested following analysis of T cells isolated from the periphery of KLF2-deficient mice (6). It is possible that the few KLF2-deficient T cells that are found in peripheral tissues are abnormal, as a result of their impaired thymic egress and/or the profound T cell lymphopenia in such animals.

**FIGURE 1.** Differential cytokine effects on postactivated CD8<sup>+</sup> T cells. Spleen cells from Rag<sup>−/−</sup> OT-I mice were stimulated with OVAap for 2 d. Viable cells were subsequently cultured in the presence of IL-2, IL-7, or IL-15 for another 6 d. A, RNA samples were obtained at day 2 or 8 of the stimulation culture and subjected to real-time PCR analysis of indicated genes. B, Expression of CD62L and CD69 on CD8<sup>+</sup> T cells at the end of culture (day 8). Data are representative of two to four independent experiments with similar results.
FIGURE 2. KLF2-deficient cells are functionally mature. A, Phenotype of naive CD8SP thymocytes from Klf2<sup>−/−</sup>CD4Cre (KO) or control (WT) thymocytes were transferred into congenic recipients that were then infected with a vaccinia virus expressing OVA. The total expansion of OT-I T cells at 5 d postinfection. Data are representative of two or three independent experiments with similar results.

Next, we tested the ability of KLF2-deficient T cells to undergo activation and expansion in vivo. Klf2<sup>−/−</sup> and Klf2<sup>−/−</sup> OT-I TCR Tg animals were generated and used to make fetal liver radiation chimeras (10). Thymocytes from these chimeras were transferred into congenic recipients that were then infected with a vaccinia virus strain expressing OVA. The total expansion of OT-I T cells at the peak of the response (day 5) was similar between KLF2-deficient and control cells with regard to the expression of CD25 and CD44 (Fig. 2D). Notably, however, the distribution of Klf2<sup>−/−</sup> T cells in recipients was distinct, being relatively low in blood and lymph nodes, but similar to controls in the spleen. These data suggested that the KLF2-deficient T cells exhibited a normal initial proliferative response to Ag in vivo but showed defective trafficking, similar to that observed for naive T cells (10).

KLF2 is required for expression of S1P<sub>1</sub> and CD62L but not c-myc or p21<sup>Cip1</sup> in postactivated T cells

Our studies on gene expression by in vitro activated CD8 T cells suggested a correlation between KLF2 re-expression and the induction of suitable trafficking (but not cell cycle) regulatory genes (Fig. 1). However, these studies did not determine whether KLF2 re-expression was required for this expression of trafficking molecules. Furthermore, it was possible that KLF2 did control transcription of c-myc or p21<sup>Cip1</sup>, but not in the way initially predicted. Having established that KLF2-deficient T cells are functionally competent, we sought to explore these issues by analyzing gene expression by in vitro activated control and KLF2-deficient T cells. Because mature KLF2 T cells cannot exit the thymus, we again used thymocytes from Klf2<sup>−/−</sup>CD4Cre and Klf2<sup>−/−</sup> control mice, subjecting these cells to in vitro activation and subsequent culture with IL-2 or IL-15. Two days after stimulation, KLF2-deficient and control CD8SP cells were similar phenotypically, showing typical activation characteristics, including upregulation of CD44, CD25, and CD69. Expression of CD62L was still lower in KLF2-deficient cells than in control cells, but the difference between the two groups was smaller after activation compared with the naive populations. The two groups were also similar following subsequent IL-2 treatment, with both pools exhibiting a CD62L<sup>lo</sup>, CD69<sup>hi</sup> phenotype. In contrast, following IL-15 culture, KLF2-deficient CD8SP cells showed defective upregulation of CD62L and sustained CD69 expression, resulting in reduced frequency of (central) memory-like cells compared with control cells. After these cytokine treatments, expression of CD25 and CD44 were similar in both groups.

These phenotypic differences in CD69 and CD62L expression after IL-15 treatment were in accordance with real-time PCR results (Fig. 3B). As seen earlier, Klf2 mRNA was induced by IL-15 treatment, and this correlated with S1P<sub>1</sub> and CD62L mRNA expression (Fig. 3B). However, IL-15 treatment of KLF2-deficient cells resulted in minimal upregulation of S1P<sub>1</sub> and CD62L mRNA (Fig. 3B). In contrast, we observed no substantial differences between KLF2-deficient and control cells with regard to the expression of c-myc and p21<sup>Cip1</sup> mRNA (Fig. 3B).

In recent studies, we reported that some trafficking molecules, including β7-integrin and CXCR3, are dysregulated in KLF2-deficient animals as a result of nonautonomous, bystander effects in the thymus (13, 14). Hence, we might expect expression of these molecules in postactivated T cells to be independent of...
KLF2. Indeed, our analysis showed that CXCR3 and β7-integrin expression was similar on Klf2<sup>−/−</sup> and WT cells following in vitro stimulation and irradiated splenocytes for 2 d. Viable cells were cultured in the presence of IL-2 or IL-15 for an additional 6 d. A, Changes in CD8<sup>+</sup> T cell phenotype at various time points after activation. B, At the end of culture (day 8), CD8<sup>+</sup> cells were enriched by magnetic beads, and RNA was extracted. Expression of indicated genes was evaluated by real-time PCR. Values are relative to the level of the IL-2–treated WT group, which was defined as 1.0. C, At day 8, cells were labeled with CFSE and further cultured with IL-15 for an additional 24 or 72 h. Graphs show the CFSE dilution in CD8SP gate. D, At day 8, viable cells were enriched, and Annexin V staining was examined before (0 h) and after the 24-h culture in the presence of IL-15. Apoptosis during these 24 h was calculated as described in Materials and Methods. Data are representative of two to four independent experiments with similar results.
their prolonged thymic retention might influence gene expression following activation. Furthermore, as we recently reported, IL-4 produced by the KLF2-deficient thymocytes can alter the phenotype and potentially the function of bystander cells (13, 14). Therefore, we chose to use an inducible \( \text{Klf2}^{\text{fl/fl}} \) system, allowing us to study naive peripheral T cells acutely deleted of the \( \text{Klf2} \) gene. Splenocytes were obtained from \( \text{Klf2}^{\text{fl/fl}} \) and control \( \text{Klf2}^{+/+} \) mice and following in vitro activation for 48 h, the cells were treated with Tat-Cre, which penetrates into the cell nuclei in a dose-dependent manner (35, 36). These cells were also Tg for a YFP reporter locus (in which YFP expression is induced by Cre-mediated elimination of a floxed STOP cassette), allowing flow cytometric identification of cells that had acquired Cre during this in vitro culture. Following activation, the T cells were cultured in the presence of IL-2 or IL-15 to induce effector-like or memory-like CD8 T cells, respectively. As reported in similar Tat-Cre systems (37), we saw expression of the YFP reporter on 40–60% of treated CD8\(^{+}\) T cells during our cultures, and this correlated with loss of KLF2 (see later discussion).

As expected, YFP\(^{+}\)CD8\(^{+}\) T cells showed a reduction in CD62L and an increase in CD69 in the \( \text{Klf2}^{\text{fl/fl}} \) group compared with the \( \text{Klf2}^{+/+} \) group following culture with IL-15 but not IL-2 (Fig. 4A). However, the defect in CD62L cell surface expression by induced KLF2-deficient cells was not as profound as that seen on conditional KLF2-deficient thymocytes (Fig. 3A). It is not clear whether this difference is a consequence of the timing of KLF2 deletion (relative to initial expression of the \( \text{CD62L} \) gene) or the differentiation state of cell (thymocyte versus peripheral T cell). In contrast, expression of CD44 and CD25 was not affected by inducible \( \text{Klf2} \) deletion. We then sorted YFP\(^{+}\)CD8\(^{+}\) T cells from cells cultured with IL-15 and examined the mRNA expression by quantitative real-time PCR. As expected, KLF2 mRNA was lost in the YFP\(^{+}\) \( \text{Klf2}^{\text{fl/fl}} \) cells but not in YFP\(^{+}\) \( \text{Klf2}^{+/+} \) cells, suggesting efficient inducible deletion of KLF2 in this system. In addition, mRNA levels of S1P1 and CD62L were considerably lower in the YFP\(^{+}\) \( \text{Klf2}^{\text{fl/fl}} \) cells compared with the \( \text{Klf2}^{+/+} \) group. Low S1P1 expression offers a likely explanation for the elevated CD69 protein-expression levels on \( \text{Klf2}^{\text{fl/fl}} \)-deleted T cells, because of the mutual antagonism of CD69 and S1P1 for surface expression (30, 31). In contrast, the effect of \( \text{Klf2} \) depletion on mRNA expression of the cell cycle regulators c-myc and p21 was moderate, at best. These results were similar to those in KLF2-

![FIGURE 4. Inducible loss of KLF2 after activation impairs the expression of trafficking- but not quiescence-associated molecules. Spleen cells from \( \text{Klf2}^{\text{fl/fl}} \) (F/F) or \( \text{Klf2}^{+/+} \) (+/+)) mice bearing \text{ROSA26-flox-STOP-YFP} allele were stimulated with plate-bound anti-CD3 for 2 d and then cultured with Tat-Cre for 45 min. Viable cells were cultured in the presence of IL-2 or IL-15 for an additional 6 d. A. Phenotype of CD8\(^{+}\)YFP\(^{+}\) cells at the end of the culture. B, CD8\(^{+}\)YFP\(^{+}\)CD44\(^{+}\) cells were sorted from the IL-15–treated group. RNA was extracted and used for the template of the real-time PCR analysis for the indicated genes. Expression levels are shown relative to WT group, which was defined as 1.0. C, IL-15–treated F/F or \( \text{Klf2}^{\text{fl/fl}} \) (Thy1.2\(^{-}\)) cells were mixed with an equal number (5 \( \times \) 10\(^{6}\)) of naive splenocytes from B6. PL mice (CD45.2\(^{+}\)Thy1.1\(^{-}\)) and injected i.v. into B6. SJL mice (CD45.1\(^{+}\)Thy1.2\(^{-}\)). Twenty-four hours after adoptive transfer, the blood, spleen (SPL), peripheral lymph nodes (PLN), and mesenteric lymph nodes (MLN) from recipients were analyzed for the frequency of CD45.2\(^{+}\)Thy1.1\(^{-}\)-YFP\(^{+}\)CD8\(^{+}\) cells (test cells) and CD45.2\(^{+}\)Thy1.1\(^{-}\)-CD8\(^{+}\) cells (internal control). Homing index was calculated as described in Materials and Methods. Data are representative of two or three independent experiments with similar results.](http://www.jimmunol.org/)

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deficient mature thymocytes from conditional KO mice (Fig. 3A, 3B), supporting the relevance of former experiments to the function of KLF2 in peripheral T cells.

To examine the physiological significance of phenotypic changes caused by acute Klf2 deletion, we treated Klf2fl/fl and Klf2−/− cells with Tat-Cre and cultured them with IL-15 before testing their homing capacity in vivo. In this experiment, IL-15–cultured memory-like CD8+ T cells were adoptively cotransferred with naive CD8+ T cells from the spleen of congenic normal mice, which provide an internal control. Migration efficiency of these internal control cells was defined as 1.0 (Fig. 4C). Twenty-four hours after transfer to normal mice, migration to the blood was similar between Klf2fl/fl and Klf2−/− cells. In contrast, Klf2fl/fl cells migrated less efficiently than did Klf2−/− cells to secondary lymphoid organs, including the spleen and lymph nodes. In lymph nodes, migration of Klf2−/− memory-like cells was similar to that of internal control naive CD8+ T cells, as indicated by the homing index near 1.0 (Fig. 4C). In contrast, Klf2−/− memory-like cells migrated to the spleen 3-fold more efficiently than did internal control cells, whereas Klf2fl/fl memory-like cells showed a splenic homing capability equivalent to control cells. Thus, KLF2 seemed to be important for normal homing of postactivated T cells to secondary lymphoid organs, whereas the quiescence of cells did not seem to be perturbed. The presence of KLF2-deficient cells in the blood was surprising but could relate to the fact that these cells (unlike their WT counterparts) are denied access to lymph nodes; therefore, they may remain in the circulation after i.v. transfer for the short duration of these studies.

These studies, using an inducible Klf2−/− deletion approach, argue that the requirement for KLF2 in induction of S1P1 and CD62L (but not c-myc or p21Cip1) gene expression is not related to possible effects of KLF2 loss during thymic development.

KLF2 deficiency affects cell distribution but not number postinfection

To investigate the involvement of endogenous KLF2 in T cell quiescence and migration in vivo, we next examined the immune response of KLF2-deficient CD8+ T cells against infection and subsequent contraction and differentiation into memory cells. Although some of the phenotypic abnormalities of thymocytes in polyclonal KLF2-deficient mice are due to nonautonomous bystander effects (13, 14), OT-I TCR Tg cells were spared from this bystander effect, providing a good tool to examine the direct effects of KLF2 deficiency (14). Equivalent numbers of KLF2-deficient and control OT-I Tg thymocytes (distinguished by congenic markers) were cotransferred into B6 mice, and the recipients were infected with L. monocytogenes-expressing OVA. As shown in Fig. 5A, in terms of total cell number, the kinetics of T cell response were very similar between the WT and KLF2-deficient groups, including the initial expansion, subsequent contraction, and survival for memory differentiation. Once again, these data suggest that a loss of KLF2 does not lead to a dramatic defect in cell cycle regulation. However, KLF2-deficient cells were underrepresented compared with control cells in the lymph nodes and the blood, although statistical significance between the two groups was not obtained for blood (Fig. 5B). Similar to our in vitro studies (Figs. 3A, 4A), we found that KLF2-deficient donor cells showed decreased expression of CD62L and increased expression of CD69 at the memory stage (Fig. 5C). Thus, although there is no significant abnormality in the total cell number upon infection, tissue distribution of memory T cells was strikingly affected by KLF2 deficiency. A potential caveat for these studies is that the development of WT versus Klf2−/− OT-I thymocytes is different as a result of the retention of Klf2−/− cells in the thymus. In preliminary studies, we sorted CD24high, Qa-2low CD8 SP cells from WT and Klf2−/− OT-I thymocytes prior to adoptive transfer and infection with L. monocytogenes-expressing OVA, and we observed results similar to those obtained with bulk OT-I CD8 SP thymocytes (data not shown).

Discussion

KLF2 has been suggested to be a prototypical quiescence factor for T cells, affecting the expression of cell cycle regulators, including c-myc and p21Cip1 (6–8, 38). However, this model largely relies on the findings from retroviral transfection into T cell lines, in which the resulting halt of autonomous tumor cell growth suggests that KLF2 can work as a quiescence factor when overexpressed (7, 8, 11). However, our previous study showed that KLF2-deficient mature thymocytes, which cannot leave the thymus, survive and persist after transfer to normal recipients although they exhibit a trafficking defect (10). In addition, the proliferative response of these naive thymocytes against TCR stimulation was quite normal in vivo and in vitro (Figs. 2, 5), suggesting the dispensability of KLF2 in naive T cell quiescence.

In the current study, we examined the role of endogenous KLF2 in postactivated CD8+ T cells cultured with IL-2 or IL-15, because...
these cytokines have strikingly different effects on KLF2 re-expression in postactivated T cells (16, 17) (Fig. 1A). Expression of c-myc and p21CIP1 in postactivated CD8+ T cells was also strongly influenced by the specific cytokines used for culture; however, in contrast to the conclusion from previous studies (7, 8), this regulation was entirely independent of endogenous KLF2 (Fig. 1A, 3B, 4B). In contrast, expression of the trafficking molecules S1P1 and CD62L was dramatically impaired by KLF2 deficiency in postactivated CD8 T cells (Figs. 3, 4). Furthermore, the in vivo Ag-specific response of KLF2-deficient CD8 T cells was characterized by defective trafficking but no change in expansion or contraction dynamics (Fig. 5). These findings reinforce the model that KLF2 is pivotal for normal T cell migration but is dispensable for T cell quiescence.

Recent studies reported the involvement of signaling through the PI3K–Akt-signaling pathway in negatively regulating KLF2 expression in T cells. One group showed that mammalian target of rapamycin suppresses KLF2 expression downstream of PI3K and Akt (17), whereas another group suggested that the Akt-regulated transcription factor Foxo induces KLF2 transcription (39). In either case, differential expression of KLF2 by IL-2 versus IL-15 in postactivated T cells could be attributed to the fact that IL-2, but not IL-15, sustains high PI3K–Akt signaling (17). Cantrell and colleagues recently reported that optimal activation of the PI3K–Akt pathway with functional phosphoinositide-dependent kinase 1 (PDK1) is required for the downregulation of KLF2, CD62L, and S1P1 (40). In contrast, proliferation of the T cells after TCR stimulation was not affected, even with the suboptimal activation of the PI3K–Akt pathway with PDK1 mutation, indicating that the proliferation in primary Ag stimulation is independent of KLF2 loss (40). This is in accordance with our overall observation that the expression of trafficking molecules, but not cell cycle-regulatory genes, was influenced by endogenous KLF2 expression.

The molecular mechanism of homing to the spleen remains unclear, whereas migration into the lymph nodes is quite well defined. It was reported that memory-like cells induced in vitro by Ag stimulation and subsequent IL-15 treatment show enhanced migration to the spleen compared with naive T cells and IL-2–treated effector-like cells (25). Consistent with this finding, we observed that WT IL-15–treated memory-like CD8+ T cells accumulated in the spleen 3-fold more efficiently than did naive CD8+ T cells 24 h after transfer into normal recipient mice (Fig. 4C). In contrast, KLF2-deficient memory-like cells migrated to the spleen only as well as did naive CD8+ T cells, suggesting that efficient migration of WT IL-15–treated memory-like cells to the spleen is dependent on KLF2 (Fig. 4C). As has been reported, normal memory cells migrate to the spleen similarly to or slightly less efficiently than do naive cells (41–44); indeed, KLF2-deficient and KLF2-sufficient memory cells generated in vivo postinfection were present in the spleen at similar frequencies (Fig. 5B). Further analysis of KLF2-sufficient and -deficient IL-15–treated cells will be useful to better understand the mechanisms regulating splenic migration.

It is possible that regulation of T cell quiescence is redundantly controlled by multiple KLF family members, of which there are many (3, 45–47), in addition to KLF2. KLF4 is closely related to KLF2; a recent study found that inducible deletion of KLF4 led to enhanced proliferation of CD8+ T cells in response to primary TCR stimulation (48), although T cell function was evaluated at 9 mo following the induction of KLF4 gene deletion in those studies, and it is not clear whether the effects were mediated primarily on naive T cells. To further explore this issue, we analyzed gene expression following activation of T cells deficient for KLF2 and KLF4 (data not shown), but we observed minimal differences compared with T cells deficient for KLF2 alone (data not shown). Hence, these data further argue against a key role for KLF2 (and similar factors) in the physiological regulation of c-myc and p21CIP1 expression in postactivated T cells.

At the same time, our data do not exclude a model that KLF2 (and/or KLF4) may be capable of repressing c-myc and inducing p21CIP1 under some situations of activation or during T cell development. Our studies focused on CD8 T cells stimulated with cognate Ag; however, other stimuli, such as lymphopenia-driven proliferation, also induce naive T cell expansion. Furthermore, c-myc is induced at key proliferative steps of T cell development (49), and it is possible that KLF2 expression during T cell differentiation is important for control of cell cycle progression. Nevertheless, our data suggest that the expression of KLF2 is not critical for cell cycle restraint following mature CD8 T cell activation.

In conclusion, we showed that endogenous KLF2 expression plays a nonredundant function in regulating the expression of trafficking molecules and controlling the migration of postactivated CD8+ T cells, yet we were unable to demonstrate a critical role for KLF2 in regulating key cell cycle T cell proliferation. Our data might also argue that the documented impact of forced KLF2 expression on the induction of cellular quiescence is a result of nonphysiologically high expression levels of these transcription factors. Further studies are needed to explore this finding further.

Acknowledgments
We thank Dr. Donna Farber (University of Maryland) for the generous gift of Tat-Cre, Dr. Frank Costantini (Columbia University) for kindly providing key mouse strains, and Drs. John Harty (University of Iowa) and Hao Shen (University of Pennsylvania) for ActA¬L. monocytogenes-expressing OVA. We appreciate the input of all of the Jamequst laboratory members during the design and analysis of these studies.

Disclosures
The authors have no financial conflicts of interest.

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Supplementary Figure 1

Wild type or KLF2⁻/⁻ thymocytes were isolated and cultured as in Figure 3. (A) Shows expression of indicated cell surface markers on CD8 T cells at various times points during in vitro activation and cytokine culture, while (B) shows real-time PCR data for mRNA expression of the genes indicated, assayed on day 8 of the in vitro culture in IL-2 or IL-15.
Supplementary Figure 1A
Supplementary Figure 1B