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Differential roles of KLF4 in the development and differentiation of CD8⁺ T cells

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Abstract

The transcription factor Krüppel-like factor 4 (KLF4) can activate or repress gene expression in a cell-context dependent manner. We have previously shown that KLF4 inhibits the proliferation of naïve CD8⁺ T cells *in vitro* downstream of the transcription factor ELF4. In this work, we describe a novel role of KLF4 in the differentiation of CD8⁺ T cells upon infection. Loss of KLF4 had minimal effect on thymic T cell development and distribution of mature T cells in the spleen, blood, and lymph nodes. KLF4-deficient naïve CD8⁺ T cells also displayed normal homeostatic proliferation upon adoptive transfer into lymphopenic hosts. However, activation of KLF4-deficient naïve CD8⁺ T cells by *in vitro* TCR crosslink and co-stimulation resulted in increased proliferation. Furthermore, naïve KLF4-deficient OT-I CD8⁺ T cells generated increased numbers of functional memory CD8⁺ T cells compared to wild type OT-I CD8⁺ T cells co-injected in the same recipient in both primary and recall responses to *Listeria monocytogenes*-OVA. Collectively, our data demonstrate that KLF4 regulates differentiation of functional memory CD8⁺ T cells while sparing development and homeostasis of naïve CD8⁺ T cells.

Keywords

KLF4; CD8⁺ T cells; proliferation; differentiation; memory

1. Introduction

The Krüppel-like factor family is comprised of highly conserved transcription factors that control proliferation, development, and differentiation [1]. The Krüppel-like factor 4 (KLF4) promotes re-programming of somatic cells into pluripotent stem cells [2,3]. Depending on the cellular context, KLF4 can act as a tumor suppressor or an oncogene [4–7] by either enhancing or inhibiting proliferation [8–10]. This duality in function can be attributed to the presence of both activating and inhibitory regulatory domains within the KLF4 protein [11,12]. In the immune system, KLF4 regulates the development of monocytes, maintenance of splenic dendritic cells, and differentiation of T cells [10,13–16]. In addition, KLF4

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inhibits proliferation of B cells downstream of FOXO proteins [17]. We have previously shown that *in vitro* TCR stimulation of CD8⁺ T cells leads to a rapid downregulation of the transcription factor E74-like Factor 4(ELF4) followed by suppression of KLF4, a direct target of ELF4 [9,18]. In addition to the linear model ELF4→KLF4, differential target genes may lead to unique functions of KLF4 in lymphocytes. Increased *in vitro* proliferation of KLF4-deficient CD8⁺ T cells suggested a role of KLF4 in the quiescence of lymphocytes [9]. However, the role of KLF4 in the differentiation of CD8⁺ T cells has not been studied *in vivo*.

Naïve CD8⁺ T cells originate from bone marrow derived progenitor cells, which undergo sequential expansion, differentiation, and selection processes in the thymus to become functional and mono-specific T cells [19,20]. Once in peripheral tissues, naïve CD8⁺ T cells rely on TCR signaling for their maintenance and differentiation [21]. Low affinity self peptide–MHC recognitions, in synergy with IL-7 and IL-15, trigger rare events of cell divisions in naïve CD8⁺ T cells that result in a slow accumulation of CD8⁺ T cells with a memory phenotype [21]. On the opposite end of the spectrum, acute infections cause vigorous activation and differentiation of pathogen-specific CD8⁺ T cells by providing strong TCR stimulation and co-stimulation (signals 1 and 2) and eliciting high levels of inflammatory cytokines (signal 3) [22,23]. Immediate and long-term protection of the host is ensured by the generation of short-lived effector and longlived memory CD8⁺ T cells. Even though the conventional *in vitro* activation of naïve CD8⁺ T cells by plate bound anti-CD3 and anti-CD28 antibodies also efficiently triggers proliferation, this system does not accurately resemble the strength of stimulation and effect of the microenvironment to ensure a physiological differentiation program. Therefore, we decided to investigate the role of KLF4 in homeostatic processes of development and differentiation of CD8⁺ T cells in response to infectious agents because of the capacity of KLF4 to inhibit *in vitro* proliferation of CD8⁺ T cells [9].

In the present study, we show that KLF4 does not have a significant role in the homeostatic proliferation of CD8⁺ T cells and thymic differentiation but lessens the formation of memory CD8⁺ T cells after infection with *Listeria monocytogenes*. Our findings revealed a novel function of KLF4 in the regulation of memory CD8⁺ T cell development in response to infection.

2. Material and Methods

2.1. Mice

Klf4^{fl/fl} mice were obtained from Dr. K. Kaestner and bred with Vav-Cre and CD4-Cre mice as previously described [24,25]. For experiments with *Lm*-OVA, *Klf4^{fl/fl}* CD4-Cre and B6.SJL mice were bred with the OT-I transgenic mice to obtain *Klf4^{fl/fl}* CD4-Cre OT-I (CD45.2⁺) and B6.SJL OT-I (CD45.1⁺) mice, respectively. C57BL/6 mice were crossed with B6.SJL to obtain B6xB6.SJL (CD45.1⁺ CD45.2⁺) recipients. Mice were maintained in specific pathogen-free conditions at Baylor College of Medicine. All experiments and procedures were done in compliance with the Institutional Animal Care and Usage Committee of Baylor College of Medicine.

2.2. T cell proliferation

Naïve CD44^{low} CD8⁺ T cells were purified from the spleen of *Klf4^{fl/fl}* and *Klf4^{fl/fl}* Vav-Cre mice and labeled with 4 μM CFSE (Invitrogen) according to the manufacturer's instructions. For *in vitro* proliferation assay, CD8⁺ T cells were then cultured in 96-well plates (10⁵ cells per well) coated with 2 μg/ml or 10 μg/ml anti-CD3ε (Bio × Cell) in RPMI media (Lonza) containing 10% FBS in the presence of 2 μg/ml anti-CD28 (BD Biosciences). Dilution of the

CFSE dye was analyzed by flow cytometry on days 2.5 and 3 after plating. For *in vivo* homeostatic proliferation assay, purified naïve CD8⁺ T cells were labeled with CFSE and adoptively transferred (4×10^5 per mouse i.v.) into congenic B6.SJL recipient mice sublethally irradiated (4.5 Gy) 15 h before to induce lymphopenia. CFSE dilution was analyzed by flow cytometry in donor-derived CD8⁺ T cells isolated from the spleen on day 9 after transfer. For thymocyte proliferation assay, BrdU (10mg/ml) was injected i.p. to 3 month-old mice and thymi were collected 3 h later. BrdU detection was performed using BrdU Detection Kit (BD Biosciences) by flow cytometry.

2.3. Infection with Lm-OVA

Naïve splenic OT-I CD8⁺ T cells from wild type (WT) B6.SJL OT-I or *Klf4^{fl/fl}* CD4-Cre OT-I mice were negatively purified with BD-Imag magnetic separation system (BD Biosciences). Biotinylated anti-CD44 antibodies (BD Biosciences) were added to the antibody cocktail to enrich for CD44^{low} CD8⁺ T cells (90–95% purity). CD8⁺ T cells were adoptively transferred by i.v. injection into sex- and age-matched B6xB6.SJL recipients (1×10^3 OT-I cells per mouse). Recipient mice were infected 24 h later with a recombinant OVA-expressing *Listeria monocytogenes* strain (*Lm*-OVA, 4×10^3 CFU i.v.). To study secondary responses, mice were re-challenged with *Lm*-OVA (200×10^3 CFU, i.v.) 60 d after initial infection. The expansion of OT-I T cells was monitored in peripheral blood by serial tail vein bleeding.

2.4. Flow cytometry and cell sorting

Cells were pre-incubated with anti-CD16/CD32 (BioLegend) to block Fc receptors prior to cell surface staining. The following antibodies were used for surface staining: allophycocyanin-labeled anti-CD45.1, PerCP-Cy5.5-labeled anti-CD45.2, FITC-labeled anti-CD62L, Alexa Fluor® 488 labeled anti-CD44, PE-Cy7-labeled anti-CD8, PerCP-Cy5.5-labeled anti-CD4, PerCP-Cy5.5-labeled anti-CD8, PE-Cy7-labeled anti-c-kit, Alexa Fluor® 647-labeled anti-CD25 (BioLegend); PE-labeled anti-CD4, PerCP-Cy5.5-labeled streptavidin, FITC-labeled anti-Sca1, APC-labeled anti-c-kit, and PE-labeled anti-IL7R α (BD Biosciences); PerCP-Cy5.5-labeled anti-CD25, APC-labeled anti-CD8, and APC-eFluor® 780-labeled anti-CD24 (eBioscience). For lineage depletion we used the hematopoietic progenitor cell enrichment set (BD Biosciences). Thymocyte subsets were sorted on a MoFlo cell sorter (Cytomation, 97–99% purity). To study apoptosis, thymocytes were first stained with surface marker and then stained for Annexin V by using the Apoptosis Detection Kit from BD Biosciences. Effector functions of OT-I CD8⁺ T cells were evaluated by activating splenocytes for 5 hours with 1 μ g/ml OVA(257–264) peptide (AnaSpec) in RPMI medium containing 10% FBS at 37°C in the presence of 1 μ l/ml Golgi Plug and 0.8 μ l/ml Golgi Stop (BD Biosciences). Cells were then stained for CD8, CD45.1 and CD45.2, fixed and permeabilized using BD Cytotfix/Cytoperm buffer (BD Biosciences), and finally stained with FITC-labeled anti-IFN γ , allophycocyanin-labeled anti-TNF α (BD Biosciences), PE-labeled anti-perforin, or FITC-labeled anti-granzyme B (eBioscience).

2.5. Quantitative real-time and genomic PCR

Total RNA was extracted using RNeasy Micro kit (Qiagen) and cDNA was synthesized using a SuperScript III kit (Invitrogen) with random hexamer primers. Real-time PCR was performed on the Mx3005P QPCR System (Agilent Technologies) using LightCycler FastStart DNA Master SYBR Green I kit (Roche). The following primers were used: Actin forward, 5'-GTGGGCCGCTCTAGGCACCA-3', and reverse, 5'-CGGTTGGCCTTAGGGTTCAGGGG-3'; KLF4 forward, 5'-CTGAACAGCAGGGACTGTCA-3', and reverse, 5'-GTGTGGGTGGCTGTTCTTTT-3'. To assess deletion of the KLF4 gene, genomic DNA was extracted from bone marrow, thymus and purified splenic CD8⁺ T cells using

DNeasy Blood and Tissue kit (QIAGEN). The following PCR primers were used: forward-1, 5'-CTGGGCCCCACATTAATGAG-3'; forward-2, 5'-CGCTGACAGCCATGTCAGACT-3'; reverse, 5'-CAGAGCCGTTCTGGCTGTTTT-3'.

2.6. Statistical analysis

Statistical significance was determined by two-tailed Student *t*-tests calculated with GraphPad Prism 5 software as indicated in the legend to figures.

3. Results

3.1. T cell development in Klf4-deficient mice

We first assessed the relative expression of KLF4 at different stages of thymic development before studying immune response of mature CD8⁺ T cells. Double-negative (DN; CD4⁻CD8⁻), double-positive (DP; CD4⁺CD8⁺) and single-positive (SP) CD4⁺ and CD8⁺ T cells were purified from the thymi of wild-type CD57BL/6 mice by cell sorting. KLF4 transcripts were elevated in DN thymocytes and decreased in DP thymocytes and mature SP T cells (Fig. 1A), suggesting that KLF4 may play a role in early stages of T cell development. We used *Klf4^{fl/fl}* Vav-Cre⁺ mice to delineate the role of KLF4 in thymocytes [24]. In this model, KLF4 was deleted in all hematopoietic cells including the bone marrow-derived T cell progenitor cells that emigrate to thymus for lineage commitment and development of functional mono-specific T cells. KLF4 deletion was confirmed by PCR with genomic DNA from bone marrow cells, thymocytes, and splenic CD8⁺ T cells (Fig. 1B). Loss of KLF4 did not alter frequency of major subsets based on the expression of CD4 and CD8 in *Klf4^{fl/fl}* Vav-Cre (Δ/Δ) and *Klf4^{fl/fl}* (fl/fl) thymocytes (Fig. 1C). The frequencies of DN subsets defined by the expression of CD44 and CD25 (DN1- DN4) were also similar to controls (Fig. 1D). However, a reduction in the thymic cellularity of KLF4-deficient mice resulted in lower numbers of DN1-DN4, DP, and SP cells (Fig. 1C, D). A previous report demonstrated that deletion of KLF4 at the DN3-DN4 stage with CD4-Cre lowered thymic cellularity by reducing proliferation of DN thymocytes [10].

In our model, the reduction of KLF4-deficient thymocyte numbers was not due to the altered proliferation, as evidenced by similar frequencies of BrdU-positive cells compared to the control group (Fig. 2A). Of interest, we also did not observe differences in survival since the frequency of Annexin V-positive thymocytes was similar in both groups (Fig. 2B). Survival was further examined by *in vitro* culture of thymocytes from wild type and KLF4-deficient mice and analyzed by the expression of Annexin V. Loss of KLF4 did not affect the number of viable thymocytes (Annexin V negative), suggesting a similar cell intrinsic predisposition to undergo cell death (Fig. 2C). Therefore, it is possible that a lower thymic cellularity was caused by a paucity of thymic immigrants. However, we found a similar number of common lymphoid progenitor cells (Lin⁻ Sca-1^{int} c-kit^{int} IL7R α ⁺) in bone marrow (Fig. 2D) and early thymic progenitor cells (Lin⁻ CD4⁻ CD8⁻ CD44⁺ CD25⁻ c-kit⁺ CD24^{lo}) in thymus (Fig. 2E) [19,26]. Despite reduced numbers of CD4⁺ and CD8⁺ SP in KLF4-deficient thymi, we did not detect significant differences in the numbers of mature CD4⁺ and CD8⁺ T cells in the spleen and inguinal lymph nodes, and frequency in peripheral blood (Appendix).

3.2. Dispensable role of KLF4 in homeostatic proliferation of naïve CD8⁺ T cells

T cell development is regulated by sequential processes of proliferation and differentiation. A lower thymic cellularity with normal T cell numbers in the spleen of *Klf4^{fl/fl}* Vav-Cre mice suggests compensation with mechanisms of T cell maintenance. To further dissect the role of KLF4 in homeostatic proliferation of CD8⁺ T cells, we analyzed lymphopenia-induced proliferation of KLF4-deficient CD8⁺ T cells, which is largely driven by weak interactions with self peptide-MHC complexes in concert with IL-7 [21]. We adoptively

transferred naïve CFSE-labeled CD44^{low} CD8⁺ T cells purified from *Klf4^{fl/fl}* Vav-Cre⁺ or *Klf4^{fl/fl}* mice into lymphopenic mice, generated by sub-lethal irradiation, and analyzed CFSE dilution in the spleen 9 days after adoptive transfer. No significant differences were observed in T cell activation, based on the expression of CD44 and proliferation of KLF4-deficient CD8⁺ T cells (Fig. 3). These findings suggest that KLF4 plays a redundant role in the homeostatic proliferation of CD8⁺ T cells and thus previously reported enhanced proliferation likely takes place under stronger stimulation [9].

3.3. Loss of KLF4 increases *in vitro* proliferation of CD8⁺ T cells

The transcription factor ELF4 controls proliferation of naïve CD8⁺ T cells in part by activating the KLF4 gene [9]. Somatic deletion of the KLF4 gene using the *Klf4^{fl/fl}* Mx1-Cre system led to increased *in vitro* proliferation of CD8⁺ T cells [9]. However, in this model systemic deletion of KLF4 was induced by type I interferons secreted in response to poly-I:C administration and CD8⁺ T cells were isolated for analysis 9 months later, which may have affected the function of naïve T cells [27]. To rule out artifacts of inducible deletion, naïve CD44^{low} CD8⁺ T cells isolated from the spleen of *Klf4^{fl/fl}* Vav-Cre⁺ mice were labeled with CFSE and activated *in vitro* on plates coated with anti-CD3 (2 or 10 µg/ml) in the presence of anti-CD28 (2 µg/ml). Consistent with our previous observations, KLF4-deficient CD8⁺ T cells showed increased proliferation in both conditions (Fig. 4). By increasing the density of crosslinking antibody and degree of stimulation, we observed a more pronounced increase in the proliferative response of KLF4-deficient CD8⁺ T cells suggesting that KLF4 may restrict response to strong stimulations, such as bacterial infection.

3.4. KLF4 restricts generation of memory CD8⁺ T cells after *Lm*-OVA infection

Systemic infection with *Lm*-OVA results in a strong activation, proliferation and differentiation of naïve CD8⁺ T cells to effector and memory T cells [23]. We therefore studied the response to *Lm*-OVA infection of KLF4-deficient CD8⁺ T cells expressing transgenic OVA-specific TCR (OT-I) in an adoptive co-transfer model to compare to wild type CD8⁺ T cells in same recipient mice. We purified naïve CD44^{low} CD8⁺ T cells from *Klf4^{fl/fl}* CD4-Cre OT-I and WT OT-I mice and adoptively transferred a 1:1 mixture to congenic recipient hosts followed by infection with *Lm*-OVA. Then, donor WT and KLF4-deficient CD8⁺ T cells were monitored in peripheral blood by flow cytometry. Although the expansion of KLF4-deficient CD8⁺ T cells was comparable with WT CD8⁺ T cells at the peak of response (day 6), we observed 40–60% increase of KLF4-deficient memory CD8⁺ T cells at the end of immune response (Fig. 5A).

Since KLF2 regulates the expression of CD62L and trafficking of T cells, a member of the KLF family with the high homology to KLF4 [1,28], we next investigated differentiation and tissue distribution of KLF4-deficient CD8⁺ T cells. In contrast to KLF2, loss of KLF4 did not affect acquisition of effector functions and polarization to CD62L^{high} central and CD62L^{low} effector memory CD8⁺ T cells (Fig. 5B,C). At day 150 post-infection, we detected a similar distribution of KLF4-deficient and WT OT-I CD8⁺ T cells in the spleen, blood and lymph nodes, indicating that loss of KLF4 enhanced memory development without altering their homing capacity to these tissues (Fig. 5D).

An important feature of memory T cells is their capacity to rapidly respond to a secondary encounter with same antigen/pathogen. Thus, we re-challenged mice that received 60 days earlier a 1:1 mixture of naïve WT and KLF4-deficient OT-I CD8⁺ T cells followed by infection with *Lm*-OVA to test the role of KLF4 in the function of memory CD8⁺ T cells. Wild type and KLF4-deficient memory CD8⁺ T cells showed a comparable expansion 5 days after rechallenge whereas the number of KLF4-deficient memory T cells left at the end

of the recall response was significantly increased (Fig. 6A). Similar to primary response, the ratio of WT and KLF4-deficient memory CD8⁺ T cells increased uniformly in blood, spleen and lymph nodes and reached 2:1 on day 90 after re-challenge (Fig. 6B). Altogether, our findings show that KLF4 regulates the formation of memory CD8⁺ T cells following *Lm*-OVA infection.

4. Discussion

The transcriptional programs that regulate the development of effector and memory T cells to efficiently eliminate pathogens and ensure long-lasting protection have not been completely elucidated. In this work, we show that loss of KLF4 increased formation of memory CD8⁺ T cells in both primary and secondary responses to *Lm*-OVA in addition to restrain proliferation of CD8⁺ T cells activated by *in vitro* TCR stimulation. KLF4 had a minimal role in regulating differentiation of thymocytes and homeostatic proliferation of mature CD8⁺ T cells.

The total number of cells in the KLF4-deficient thymus was significantly reduced despite a normal phenotypic distribution of thymocytes. Loss of KLF4 did not affect survival and proliferation of thymocytes and the numbers of bone marrow-derived early thymic precursors (ETP). The numbers of common lymphoid progenitors –direct precursors of DN1 thymocytes– were also normal in KLF4-deficient bone marrow. Therefore, it is possible that KLF4 could modulate function of ETPs or control hematopoietic derived “niches” in the thymus for early immigrants. Despite a reduced thymic cellularity, peripheral CD4⁺ and CD8⁺ T cell counts were normal, suggesting that KLF4 is not required for the maintenance of mature T cell subsets.

We previously demonstrated that KLF4 is directly activated by ELF4 in CD8⁺ T cells [9]. KLF4-deficient CD8⁺ T cells shared some functional traits with ELF4-null CD8⁺ T cells including increased *in vitro* proliferation and homeostatic accumulation of memory-like CD8⁺ T cells [9]. Since ELF4 also regulates T cell activation by maintaining the pool of DUSP1 and DUSP5 [18], which are not regulated by KLF4, deletion of either ELF4 or KLF4 would likely reveal overlapping and unique functions. While deletion of ELF4 decreased threshold of TCR activation triggered by suboptimal stimulation, KLF4-deficient CD8⁺ T cells responded better to a stronger TCR stimulation. In contrast to ELF4, loss of KLF4 did not affect activation (upregulation of CD44) and rate of *in vivo* homeostatic proliferation of CD8⁺ T cells, indicating that the accumulation of CD44^{high} memory-like CD8⁺ T cells in KLF4-deficient mice was likely driven by cell-extrinsic mechanisms [9].

Krüppel-like factors have been reported to control cellular quiescence, a reversible cell-cycle arrest actively regulated by cell extrinsic and intrinsic factors. Early work largely based on *in vitro* studies with Jurkat cells suggested that KLF2 induces quiescence in lymphocytes by activating p21 [29]. However, it was later shown that KLF2 is dispensable during *in vivo* responses to antigen, demonstrating a disparity between *in vitro* and *in vivo* assays [30]. Similarly, we found KLF4 controls proliferation of CD8⁺ T cells in a context dependent manner, which depended on the intensity and quality of signals. The expansion of KLF4-deficient effector CD8⁺ T cells following infection with *Lm*-OVA was similar to wild type, indicating that additional co-stimulation and cytokine signals present at the priming site likely overruled the cell cycle regulation observed *in vitro*. However, KLF4 may alternatively promote expansion or survival of a small number of memory precursor cells, which would be undetectable in a bulk population.

T cell trafficking is in part regulated by the transcription factor FoxO1 that directly activates KLF2, which in turn controls expression of homing molecules CD62L, CCR7 and S1P₁

[28,31]. KLF4 is downregulated in FoxO1-null memory CD8⁺ T cells that displayed defective differentiation and low expression of CD62L and CCR7 [32,33]. These observations prompted us to examine the role of KLF4 in regulating differentiation and tissue distribution of CD8⁺ T cells after infection. No significant alterations were observed in the expression of CD62L (L-selectin) and tissue distribution of KLF4 deficient CD8⁺ T cells. However, KLF4-deficient CD8⁺ T cells produced more memory cells without affecting the central-to-effector memory ratio and tissue distribution. A larger pool of memory CD8⁺ T cells from primary response was further enhanced upon secondary challenge with same pathogen leading to 2-fold increase of memory CD8⁺ T cells, suggesting that KLF4-deficient memory T cells are functional and have increased proliferative capacity than controls. Collectively, this study described a new role for KLF4 in the proliferation of CD8⁺ T cells and their differentiation to immunological memory in response to infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ELF4	E74-like Factor 4
KLF4	Krüppel-like factor 4
<i>Lm</i>-OVA	<i>Listeria monocytogenes</i> -OVA

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HIGHLIGHTS

- KLF4 has minimal role in the development and homeostatic proliferation of CD8⁺ T cells.
- KLF4 restrains proliferation of CD8⁺ T cells in response to *in vitro* TCR stimulation.
- Loss of KLF4 augments development of CD8⁺ T cell memory after *Listeria* infection.

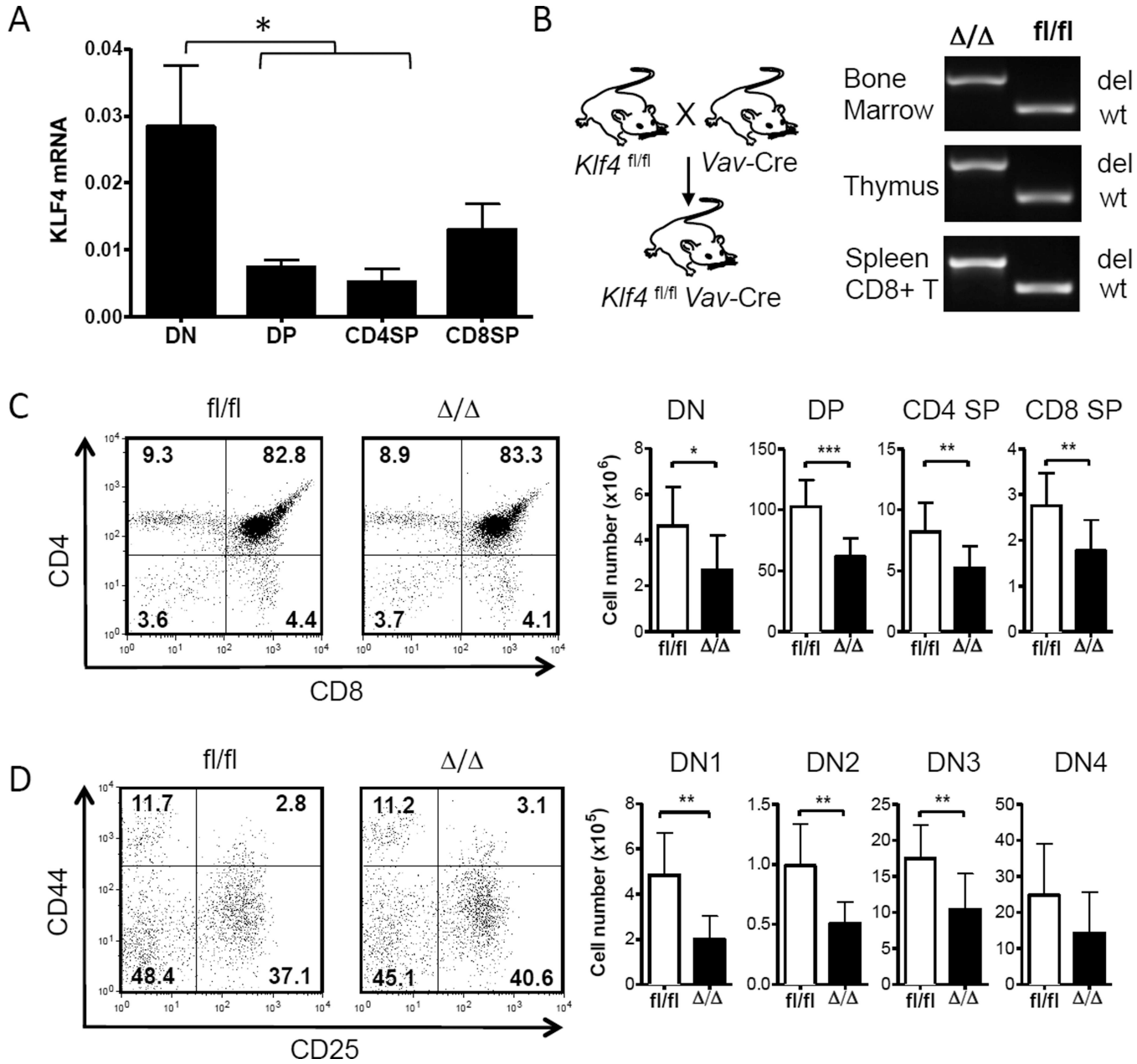


Fig. 1. Redundant role of KLF4 in T cell development

(A) Relative expression of KLF4 transcripts was measured in double-negative (DN; CD4⁻CD8⁻), double-positive (DP; CD4⁺CD8⁺), and CD4⁺ and CD8⁺ single-positive (SP) thymocytes purified from WT thymi by cell sorting (mean ± SD, n=3). (B) Diagram depicting deletion of KLF4 gene in genomic DNA extracted from bone marrow, thymus, and purified splenic CD8⁺ T cells of *Klf4*^{fl/fl} Vav-Cre (Δ/Δ) and Cre-negative control (*fl/fl*) CD8⁺ T cells was assessed by PCR (del = deleted allele; wt = wild-type allele). (C) Representative dot plots showing expression of CD4 and CD8 on thymocytes. Numbers indicate percentage of each population. Corresponding cell counts are shown as bar graphs (mean ± SD, n=9). (D) Expression of CD25 and CD44 on DN thymocytes with indicated relative frequency of each sub-population. Total cell counts of DN1 (CD44^{high}CD25^{low}), DN2 (CD44^{high}CD25^{high}), DN3 (CD44^{low}CD25^{high}) and DN4 (CD44^{low}CD25^{low}) sub-

populations are shown as bar graphs (mean \pm SD, n=9). *, P<0.05; **, P<0.01; ***, P<0.005 (unpaired two-tailed Student *t*-test).

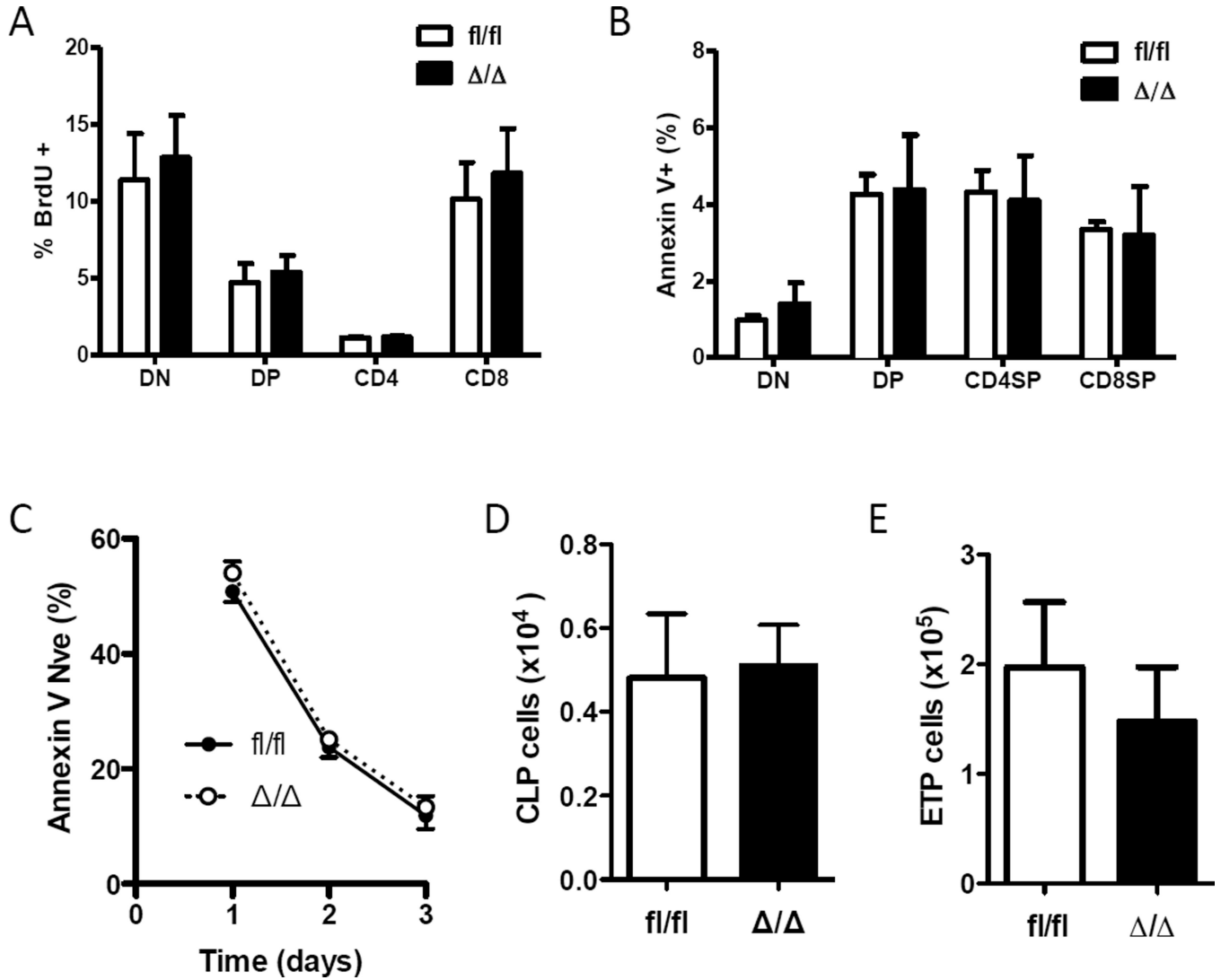


Fig. 2. Normal seeding, proliferation, and survival of KLF4-deficient thymocytes
 (A) Percent of BrdU-positive cells in each thymic population was determined by flow cytometry 3 h after BrdU injection (mean \pm SD, n=4). (B) Percentage of Annexin V-positive cells in DN, DP and single-positive thymocytes are shown as bar graphs (mean \pm SD, n=3). (C) Survival of thymocytes to *in vitro* culture was examined by flow cytometric detection of Annexin V. Live thymocytes (Annexin V negative) were determined at each time (mean \pm SD, n=4). (D) Number of common lymphoid progenitors (CLP) in bone marrow was determined by flow cytometry (Lin⁻ Sca-1^{int} c-kit^{int} IL7R α ⁺) (mean \pm SD, n=5). (E) Number of early thymic progenitor cells (Lin⁻ CD4⁻ CD8⁻ CD44⁺ CD25⁻ c-kit⁺ CD24^{lo}) was determined by flow cytometry (mean \pm SD, n=4).

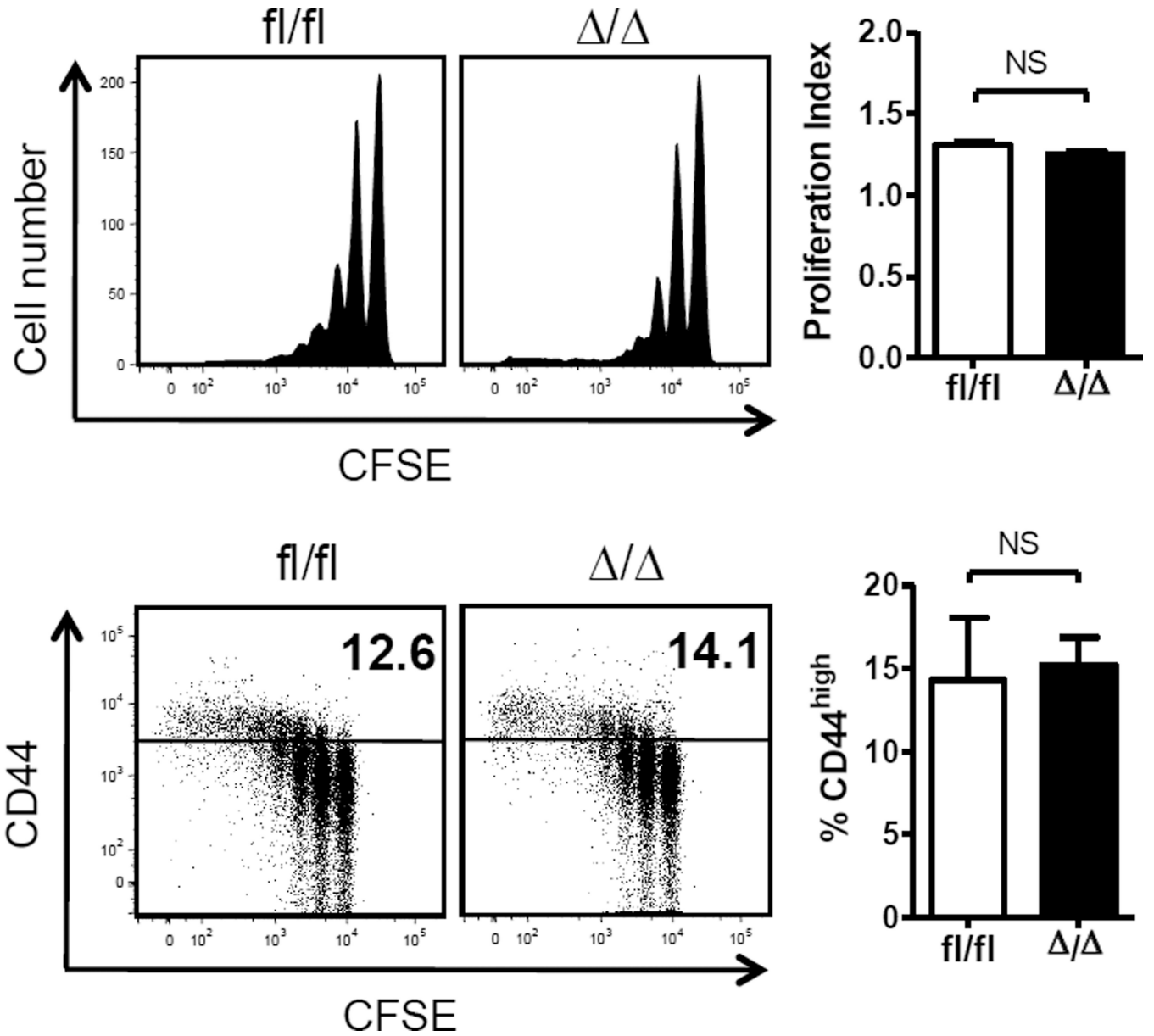


Fig. 3. KLF4 deletion in CD8⁺ T cells does not affect homeostatic proliferation
 Homeostatic proliferation of splenic naive CD8⁺ T cells after adoptive transfer into lymphopenic recipient mice. Proliferation index for each group is plotted as bar graph (mean \pm SD; n=3). Representative dot plots on the bottom show upregulation of CD44 in proliferating cells. Percentage of CD44^{high} cells is shown on each dot plot and plotted as bar graph (mean \pm SD; n=3). Data represent 2 independent experiments.

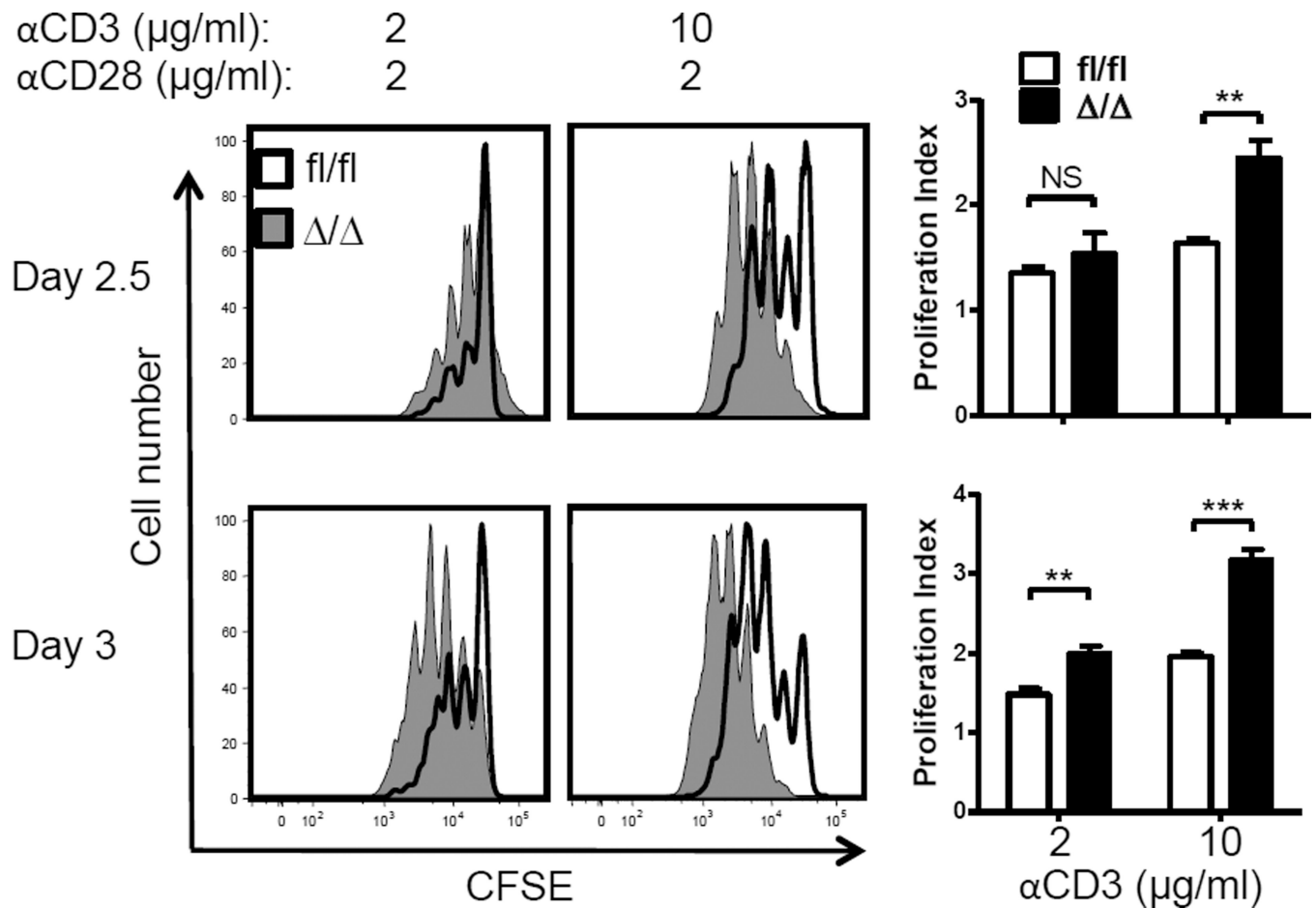


Fig. 4. KLF4 restrains CD8⁺ T cell proliferation in response to TCR stimulation

CFSE dilution profiles of purified CD8⁺ T cells cultured on plates coated with either 2 μ g/ml or 10 μ g/ml of anti-CD3 antibody. Corresponding proliferation indexes were plotted as bar graphs (mean \pm SD, n=3). Data represent 3 independent experiments. **, P<0.01; ***, P<0.005 (unpaired two-tailed Student *t*-test)

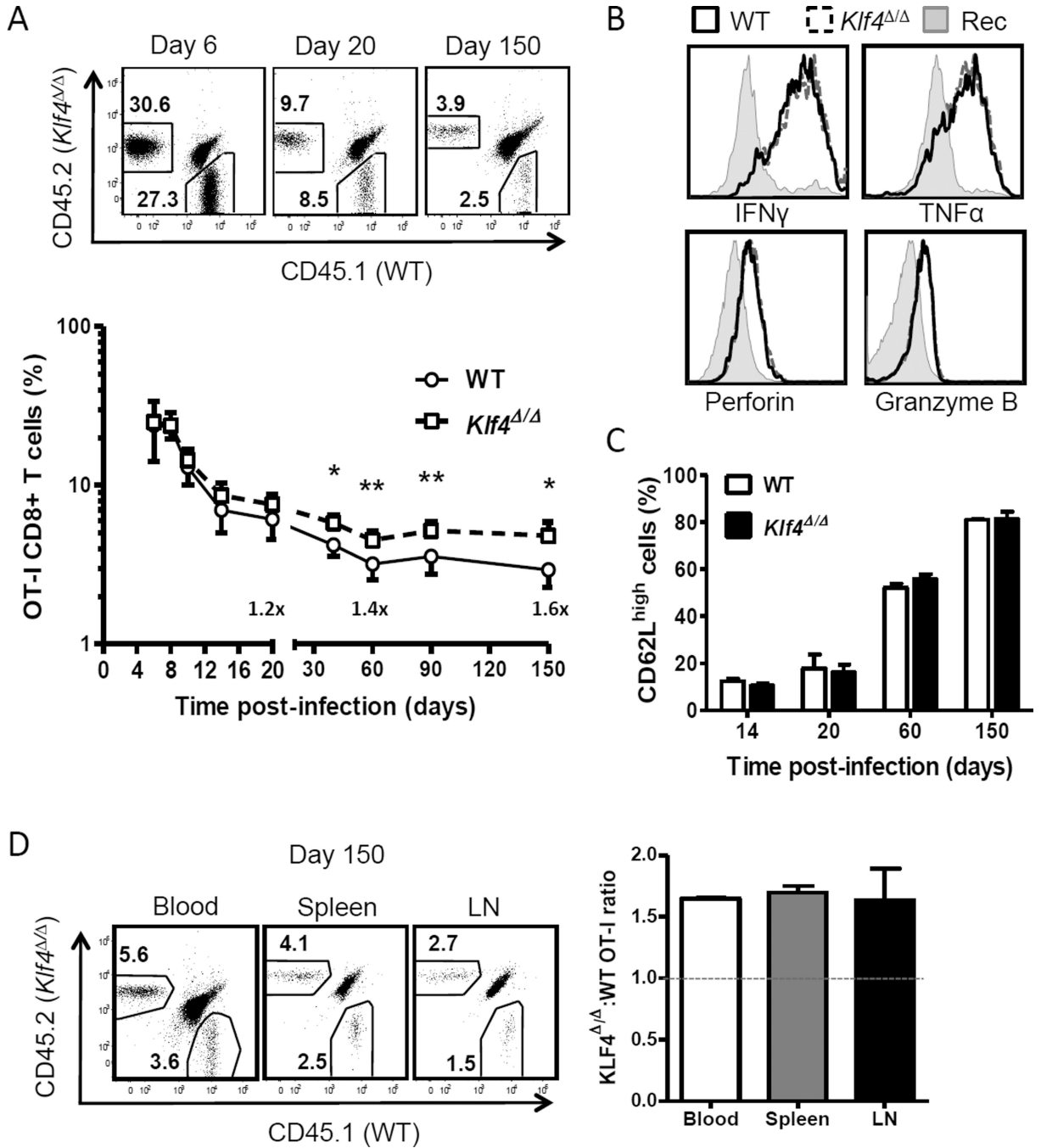


Fig. 5. KLF4 regulates formation of memory CD8⁺ T cells upon *Lm*-OVA infection

(A) Expansion of co-transferred WT (CD45.1⁺) and KLF4^{fl/fl} CD4-Cre (*Klf4^{Δ/Δ}*) (CD45.2⁺) OT-I CD8⁺ T cells in peripheral blood of congenic recipient mice infected with *Lm*-OVA. Representative dot plots and total kinetics are shown for days 6, 20 and 150 post-infection. (B) Expression of IFN γ , TNF α , perforin and granzyme B was assessed in WT and *Klf4^{Δ/Δ}* effector OT-I CD8⁺ T cells by intracellular staining. Solid and dashed histograms represent WT and *Klf4^{Δ/Δ}* OT-I effector CD8⁺ T cells, respectively; gray filled histograms show corresponding profiles of recipient CD8⁺ T cells used as a control. (C) Percentages of CD62L^{high} OT-I CD8⁺ T cells in each donor-derived population is plotted as bar graphs

throughout the response. (D) Relative frequencies of WT and *Klf4*^{Δ/Δ} OT-I memory CD8⁺ T cells in blood, the spleen and inguinal lymph nodes (LN) are shown in representative dot plots. The ratio of *Klf4*^{Δ/Δ} to WT OT-I CD8⁺ T cells in each tissue was calculated and plotted as bar graphs. Dashed line indicates initial ratio upon infection. The data are representative of 2 independent experiments (mean ± SD, n=3). *, P<0.05; **, P<0.01; (paired two-tailed Student *t*-test).

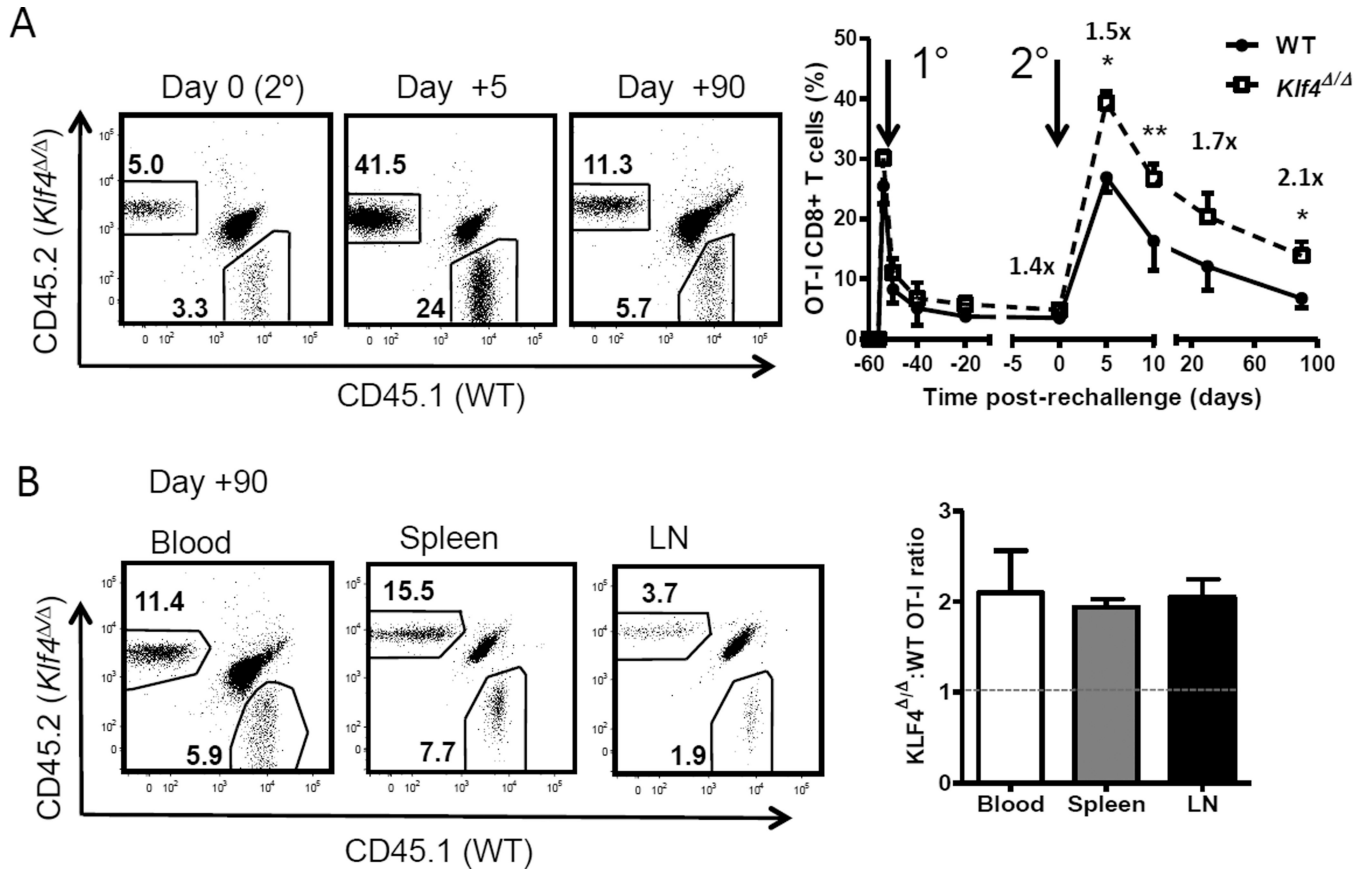


Fig. 6. Loss of KLF4 augments secondary response of memory CD8⁺ T cells
 (A) Secondary expansion of WT and *Klf4*^{Δ/Δ} OT-I memory CD8⁺ T cells in blood. Naive WT and *Klf4*^{Δ/Δ} OT-I CD8⁺ T cells were mixed at 1:1 ratio, adoptively transferred into congenic recipients and activated with *Lm*-OVA infection as shown in Fig. 4. Sixty days later, recipient mice were re-challenged with a lethal dose of *Lm*-OVA. Representative dot plots show total blood CD8⁺ T cells with relative frequencies of WT and *Klf4*^{Δ/Δ} OT-I CD8⁺ T cells before re-challenge (day 0), at the peak of secondary response (day +5) and 90 days after re-challenge (day +90). Total kinetics of primary and secondary response is plotted as percentage of total CD8⁺ T cells in blood. (B) Relative frequencies of secondary memory WT and *Klf4*^{Δ/Δ} OT-I CD8⁺ T cells in the spleen, blood and inguinal lymph nodes (LN) are shown in representative dot plots. The ratio of *Klf4*^{Δ/Δ} to WT OT-I CD8⁺ T cells in each tissue plotted as bar graphs. Dashed line indicates the ratio in blood before rechallenge. The data are representative of 2 independent experiments (mean ± SD, n=3). *, P<0.05; **, P<0.01; (paired two-tailed Student *t*-test).