The transcription factor KLF2 restrains CD4+ T follicular helper cell differentiation

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Summary

T follicular helper (Tfh) cells are essential for efficient B cell responses, yet the factors that regulate differentiation of this CD4+ T cell subset are incompletely understood. Here we found that the KLF2 transcription factor serves to restrain Tfh cell generation. Induced KLF2 deficiency in activated CD4+ T cells led to increased Tfh cell generation and B cell priming, while KLF2 overexpression prevented Tfh cell production. KLF2 promotes expression of the trafficking receptor S1PR1, and S1PR1 downregulation is essential for efficient Tfh cell production. However, KLF2 also induced expression of the transcription factor Blimp-1, which repressed transcription factor Bcl-6 and thereby impaired Tfh cell differentiation. Furthermore, KLF2 induced expression of the transcription factors T-bet and GATA3 and enhanced Th1 differentiation. Hence, our data indicate KLF2 is pivotal for coordinating CD4+ T cell differentiation through two distinct and complementary mechanisms: via control of T cell localization, and by regulation of lineage-defining transcription factors.

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Introduction

During the immune response toward foreign antigens, the germinal center (GC) reaction represents a central mechanism for generating high affinity antibodies of diverse isotypes (Victora and Nussenzweig, 2012). Fundamental in this process is the activity of CD4^{+} T follicular helper (Tfh) cells, which coordinate generation of the germinal center, initiate help for antigen specific B cells, and promote selection of germinal center B cell clones that have developed enhanced antigen recognition through somatic hypermutation (Crotty, 2011; Liu et al., 2013; Victora and Nussenzweig, 2012; Vinuesa and Cyster, 2011). Characteristic features of Tfh cells include expression of inducible costimulatory (ICOS), programmed death 1 (PD-1), the chemokine receptor CXCR5 and the cytokine interleukin-21 (IL-21), and these molecules are key for Tfh cell generation and function (Crotty, 2011; Liu et al., 2013; Victora and Nussenzweig, 2012; Vinuesa and Cyster, 2011). Cells with a Tfh cell phenotype accumulate around and enter B cell follicles, while cells that localize within GC are characterized by high expression of CXCR5, PD-1 and Bcl-6 (Crotty, 2011; Liu et al., 2013; Victora and Nussenzweig, 2012; Vinuesa and Cyster, 2011). Migration and retention of Tfh in the GC depends on CXCR5 and the sphingosine-1-phosphate receptor S1PR2 (Moriyama et al., 2014) Downregulation of CCR7 is also critical for Tfh cell accumulation in the follicle and normal GC responses (Haynes et al., 2007), however other factors that negatively regulate Tfh cell trafficking are not well defined.

Multiple transcription factors, including c-Maf, Batf, Irf4, STAT1, STAT3 and Ascl2 are involved in development and function of Tfh cells (Crotty, 2011; Liu et al., 2014; Liu et al., 2013), but maintenance and full differentiation of Tfh critically requires expression of Bcl-6 (Choi et al., 2011; Crotty, 2011; Liu et al., 2014; Liu et al., 2013; Liu et al., 2012; Vinuesa and Cyster, 2011). The Tfh differentiation pathway is opposed by other factors, the best studied of which is Blimp-1. Bcl-6 and Blimp-1 are mutually antagonistic, making the balance in expression of these two factors a critical element in determining helper T cell fate. IL-2R signaling impairs Tfh generation in a mechanism involving Blimp-1 and STAT5 (Ballesteros-Tato et al., 2012; Johnston et al., 2012; Oestreich et al., 2012; Pepper et al., 2011). Furthermore, the transcription factors Foxo1 and Foxp1 both restrain Tfh cell generation, although the mechanisms involved are not fully defined (Kerdiles et al., 2010; Wang et al., 2014; Xiao et al., 2014). Activated CD4^{+} T cells that do not mature into Tfh cells may join one of several alternative "non-Tfh" subsets (including T helper 1 (Th1), Th2, Th17 and Treg cells) that are thought to not localize into the germinal center. Key transcription factors for several of these alternative fates are blocked by Bcl-6 (Crotty, 2011; Liu et al., 2013; Nurieva et al., 2009), further establishing this factor as central to reinforcing Tfh differentiation.

Hence, in order to effectively participate in the germinal center response, Tfh must: a) migrate into the B cell follicle and reside in the GC; b) acquire specific functional properties needed for effective B cell help; and c) exclude alternative differentiation fates. It is unclear, however, whether these three aspects are coordinately regulated, and if so what factors are involved in that control.

The transcription factor KLF2 is essential for naïve T cell trafficking, in part through promoting expression of CD62L (L-selectin) and S1PR1, which are critical for lymphocyte entry and egress, respectively, in secondary lymphoid tissues (Bai et al., 2007; Carlson et al., 2006; Hart et al., 2012; Takada et al., 2011). More recently, we reported that low expression of KLF2 and S1PR1 were prerequisite for effective generation of tissue-resident memory CD8^{+} T (Trm) cells – a population that is prominent in non-lymphoid tissues and does not recirculate via the blood and lymph (Skon et al., 2013). Those studies suggested that T lymphocyte residence and recirculation were characterized by low and high expression of KLF2, respectively. Similarly, in order to function in sustained B cell help, Tfh must become a resident population, within the active germinal center. Hence, in this report we explore whether KLF2 impacts the capacity of activated CD4^{+} T cells to become Tfh cells. We found that KLF2 expression impairs Tfh differentiation, while KLF2 deficiency enhances Tfh cell generation. These effects relate to the capacity of KLF2 to induce expression of Blimp-1: however, we also showed that KLF2 promotes expression of T-bet and GATA3, indicating that KLF2 controls various aspects of Th differentiation.

Results

Tfh exhibit a KLF2$_{lo}$ phenotype

We initially studied KLF2 expression in antigen specific CD4^{+} and CD8^{+} T cells responding to lymphocytic choriomeningitis virus (LCMV), using a previously described KLF2-GFP reporter mouse strain (Skon et al., 2013; Weinreich et al., 2009). In keeping with our earlier findings (Skon et al., 2013), the vast majority of effector CD8^{+} T cells in lymphoid tissues express KLF2 (Figure 1A), yet we noted that KLF2 expression in effector CD4^{+} T cells was bimodal, with some cells expressing similar amounts of KLF2 as observed in the CD8^{+} T cell population, while other cells exhibited substantially reduced KLF2 expression (Figure 1A). We have reported that KLF2 downregulation characterized non-recirculating Trm (Skon et al., 2013) and the B cell helper function of Tfh cells obliges them to be retained within the priming lymphoid tissue (Crotty, 2011; Victora and Nussenzweig, 2012). Hence we investigated whether the amount of KLF2 expressed correlated with the Tfh cell subset. Indeed, we found that the KLF2^{lo} subset was highly enriched for cells expressing a Tfh cell phenotype (CXCR5^{hi}, PD-1^{hi} and Bcl-6^{hi}), while non-Tfh cells were more prevalent in the KLF2^{hi} population (Figure 1B). Further analysis showed that cells with Tfh cell phenotype were KLF2^{lo}, while non-Tfh cell populations expressed higher amounts of KLF2 (Figures 1B and 1C). Cells in the CXCR5^{int}, PD-1^{lo} population are likely to be a mixture of developing Tfh cells and precursors for central memory CD4^{+} T cells (Crotty, 2011; Pepper and Jenkins, 2011), and hence are not further discussed. This expression pattern was not limited to CD4^{+} T cells responding to LCMV, since we observed similar profiles for polyclonal CD4^{+} T cells responding to distinct epitopes during acute infection with the bacteria *Listeria monocytogenes* (Figure 1D).

To extend these findings and visualize differences in KLF2 expression in the context of lymphoid tissue architecture, we used immunohistochemistry to determine KLF2-GFP expression *in situ* in the draining lymph node following immunization with the protein Phycoerythrin (Figure S1). CD4^{+} T cells that were physically localized to the GC had

significantly lower KLF2 expression than CD4^{+} T cells located in the T cell zone (Figures 2A and 2B). Indeed, GFP expression was clearly lower in the GC as a whole indicating that both CD4^{+} T cells and B cells in this zone were KLF2^{lo} (Figures 2B and 2C). Collectively, these data suggested that the reduced KLF2 expression is a signature feature of the Tfh cell population.

Dynamic changes in KLF2 and S1PR1 expression during the CD4^{+} T cell response

To further investigate the regulation and function of KLF2 during CD4^{+} T cell lineage commitment, we developed an adoptive transfer system using TCR transgenic CD4^{+} T cells (TEa), specific for Eα/I-A[b] (Grubin et al., 1997). To enhance antigen-specific B cell interactions and optimize Tfh cell differentiation (Crotty, 2011; Victora and Nussenzweig, 2012), we co-transferred MD4 BCR transgenic B cells specific for hen/duck egg lysozyme (HEL/DEL) (Hartley et al., 1991), and immunized the recipient mice with a conjugate antigen (Eα-DEL) bearing antigens for both TEa and MD4 cells (Figures S2A–S2C). We used this system to define the kinetics of KLF2 and S1PR1 expression *in vivo*, through adoptive transfer of KLF2-GFP (Skon et al., 2013; Weinreich et al., 2009) or S1PR1-GFP reporter (Cahalan et al., 2011) TEa CD4^{+} T cells. At an early activation stage (day 2), primed TEa cells uniformly reduced KLF2 and S1PR1 reporter expression in secondary lymphoid organs (Figure 3A), in keeping with previous data on KLF2 and S1PR1 downregulation following TCR engagement (Cahalan et al., 2011; Cyster and Schwab, 2012; Skon et al., 2013). From day 5 of the response, however, non-Tfh phenotype cells showed sustained KLF2 re-expression, whereas Tfh phenotype cells maintained low KLF2 reporter expression well into the memory phase (day 30). S1PR1 reporter expression was also markedly lower in Tfh cells than the non-Tfh population (Figure 3A), consistent with the very low KLF2 expression by that population.

We also observed higher expression of the activation marker CD69 in Tfh compared to non-Tfh effector populations (Figure 3B). Studies suggest TCR engagement is required for sustaining Tfh proliferation and maintenance (Choi et al., 2013), and it has been proposed that CD69 expression on Tfh cells is an indication of TCR stimulation (Fazilleau et al., 2009). This is relevant because TCR signals cause KLF2 downregulation. However, CD69 and S1PR1 inhibit each other's cell surface expression, and loss of S1PR1 results in elevated basal CD69 expression (Bankovich et al., 2010; Pham et al., 2008; Skon et al., 2013), complicating interpretation of the CD69^{+} phenotype. Hence we also looked for evidence of recent TCR signaling using the Nur77-GFP reporter transgenic system (Moran et al., 2011). By day 14 of the response, we saw evidence for sustained TCR signaling in the Tfh cell pool (consistent with the conclusions of previous studies (Fazilleau et al., 2009; Tubo et al., 2013)), while Nur77-GFP levels were declining in non-Tfh phenotype cells. However, at an earlier time point (day 7), Nur77-GFP expression was similar in Tfh and non-Tfh cells (Figure 3C), suggesting other factors may contribute to KLF2 downregulation. Tfh differentiation and maintenance also requires ICOS signaling, typically induced as T cells encounter ICOS-L expressed on follicular B cells (Crotty, 2011; Liu et al., 2013; Victora and Nussenzweig, 2012; Vinuesa and Cyster, 2011). ICOS activates the phosphoinositide 3-kinase (PI3K) pathway which is known to extinguish KLF2 expression (Fabre et al., 2008; Sinclair et al., 2008; Skon et al., 2013). Hence, we tested how ICOS-L blockade, initiated at

day 7 of immunization, would impact KLF2 expression and Tfh differentiation (Figure S2D). Five days of ICOS-L blockade caused substantial loss of Tfh phenotype cells (Figure S2E), confirming the relevance of this pathway for Tfh cell maintenance, but limiting the capacity to assess changes in KLF2 expression in the Tfh cell pool. However, after short-term ICOS-L blockade a substantial Tfh cell population remained (Figure S2E), and those cells showed a marked increase in KLF2 expression, compared to controls (Fig 3D,E). No change in KLF2 expression was observed in the non-Tfh cell population. These data suggest ICOS engagement plays a critical role in repressing KLF2 expression during Tfh generation.

Loss of KLF2 enhances Tfh cell generation and the GC B cell response

While these results indicate that Tfh characteristically display reduced KLF2 and S1PR1 expression, the functional relevance of this expression pattern was unclear. Hence, we examined the consequences of dysregulated KLF2 expression. Analysis of KLF2-deficient naïve T cells is compromised by their altered trafficking (Bai et al., 2007; Carlson et al., 2006), hence we utilized an inducible knockout approach in which tamoxifen administration stimulates ERT2-Cre to mediate *Klf2* ablation (monitored through a Cre-induced YFP reporter: Figures S3A and S3B) (Liu et al., 2014; Wang et al., 2014). *Klf2* gene deletion in TEa T CD4^{+} T cells was initiated at day 2 of the response (when KLF2 expression level is already low – Figures 3A and S3A). Ablation of *Klf2* led to a striking increase in the frequency (Figure 4A) of Tfh phenotype CD4^{+} T cells compared to controls, consistent with the hypothesis that KLF2 acts to restrain Tfh cell differentiation.

To test whether KLF2-deficient Tfh CD4^{+} T cells were functional, we tested their capacity to mediate antigen-specific B cell priming and the GC reaction. *Klf2*-inducible knockout TEa T cells were transferred into TCRα (or TCRβ)-deficient recipients and primed with Eα-SA-DEL. In this way, the antigen-specific response of endogenous polyclonal B cells can be monitored, while T cell help is limited to the donor population. As expected, induced *Klf2* deletion increased the frequency and number of Tfh phenotype CD4^{+} T cells compared to controls (Figures S3C and S3D). Serum anti-Eα-SA-DEL IgM, IgG1, IgG2a, IgG2c, IgG3 as well as IgE titers were increased within 5 days of induced KLF2-knockout (Figures 4B and S3E). Moreover, inducible KLF2 deletion in donor TEa cells increased the number of both plasma cells (Figure 4C) and GC B cells (Figure 4D) within the Eα-SA specific polyclonal B cell population (Figure S3F). Similar effects were seen at day 14 of the response (data not shown). Furthermore, at later time points (days 14 and 30 post-immunization), the number of antigen-specific isotype-switched memory B cells was substantially increased when KLF2 deficient TEa cells (rather than WT TEa cells) were present (Figure 4E). Taken together, these results showed that deletion of KLF2 in early activated CD4^{+} T cells promoted polarization toward Tfh cells, and that those T cell populations were functional, providing help for robust antigen specific B cell priming, GC dependent isotype switching and generation of memory B cells.

Forced expression of KLF2 or S1PR1 in CD4^{+} T cells impairs generation of Tfh cells

As a complementary approach, we assessed the impact of increased KLF2 expression on GC Tfh differentiation *in vivo*, using a retroviral overexpression system (Skon et al., 2013). At day 7 p.i., forced expression of KLF2 in TEa CD4^{+} T cells resulted in a dramatic inhibition

of Tfh cell differentiation (compared to non-transduced and "empty" retroviral transduced controls) (Figures 5A and S4A), supporting the proposal that KLF2 plays a dominant negative regulatory role in Tfh cell differentiation. KLF2 is required for S1PR1 expression in T cells, and previous studies have suggested that induction of S1PR1 is sufficient to substitute for KLF2 in promoting thymocyte egress (Zachariah and Cyster, 2010) and inhibiting Trm generation (Skon et al., 2013). Indeed, ectopic expression of S1PR1, like KLF2, significantly decreased generation of Tfh CD4$^+$ T cells (Figures 5A and S4A). An important question was what Th cell subset was favored by KLF2 over-expression. To assess this, we analyzed expression of lineage-defining transcription factors in responding CD4$^+$ T cells using a sequential gating strategy (Figure S4B), revealing that forced KLF2 expression caused a substantial increase in the frequency of T-bet$^+$ cells (indicative of Th1 differentiation), and a reduction in the frequency of RORγt$^+$ cells (associated with the Th17 subset) (Figure 5B). A small increase in the frequency of T-bet$^-$, GATA3$^+$ cells was also observed. Forced expression of S1PR1 had minimal effect on the frequency of T-bet$^+$, GATA3$^+$ or RORγt$^+$ populations, but either KLF2 or S1PR1 overexpression resulted in a reduced frequency of Bcl-6$^+$ cells, in keeping with impaired Tfh differentiation (Figure 5B). These results suggest downregulation of KLF2 and its target S1PR1 are obligatory steps in the production of Tfh CD4$^+$ T cells, and that enforced KLF2 expression favors generation of cells expressing the canonical Th1 cell transcription factor T-bet.

KLF2 inhibits Tfh cell production independent of S1PR1 regulation

These findings might suggest the critical function of KLF2 in controlling Tfh cell production is through control of S1PR1 expression. To test this, we neutralized S1PR1 functional activity with the drug FTY720 (Cyster and Schwab, 2012). While S1PR1 overexpression led to reduced generation of Tfh, this effect was substantially reversed by treatment with FTY720 (Figures 5C and S4C), consistent with the hypothesis that S1PR1 expression blocks Tfh cell generation. Surprisingly, however, FTY720 treatment had no effect on the skewed Tfh cell differentiation induced by forced KLF2 expression (Figures 5C and S4C). This implied that the effects of forced KLF2 expression were not limited to induction of S1PR1. It is unlikely this is simply a consequence of insufficient FTY720 dosage, since mRNA expression for S1PR1 was lower in KLF2 transduced compared to S1PR1 transduced CD4$^+$ T cells (see Figure 6A). Furthermore, FTY720 treatment did not impact CD4$^+$ T cell differentiation in the control-transduced population (Figures 5C and S4C), suggesting that S1PR1 function alone was not regulating generation of the Tfh cell subset in normal cells. These data indicate that S1PR1 downregulation is necessary but not sufficient to permit Tfh differentiation, but that S1PR1 regulation was not the dominant pathway through which KLF2 regulates generation of the Tfh cell subset.

KLF2 induces Blimp-1 to reduce Bcl-6 expression

Our findings suggested that KLF2 might have additional downstream targets that affect Tfh cell generation. We assessed the impact of KLF2 overexpression or deletion on expression of known factors in the Tfh differentiation pathway using *in vitro* stimulated CD4$^+$ T cells maintained in non-polarizing culture conditions (Figure 6A–C, S5A). Quantitative RT-PCR data showed that KLF2 over-expression led to significantly increased expression of *Prdm1* (the gene encoding Blimp-1), and reduced expression of Bcl6 (Figure 6A, 6C). In contrast,

KLF2 ablation led to a reduction in Blimp-1 expression and induction of Bcl6 (Figure 6B). Hence, these data indicate KLF2 regulates the expression of transcription factors that dominantly regulate Tfh cell differentiation (Crotty, 2011; Johnston et al., 2009; Oestreich et al., 2012). In contrast, we did not observe effects of KLF2 manipulation on mRNA expression of Ascl2, CXCR5, ICOS or IL-21 in these *in vitro* cultured cells (Figures 6A, 6B and data not shown). Forced expression of S1PR1 had no effect on Blimp-1 or Bcl-6 expression (Figures 6A, 6C). Although these data showed that KLF2 impacts the balance of Blimp-1 and Bcl-6 expression, the transcriptional antagonism between those two factors complicates defining how KLF2 regulates this expression profile. To test whether KLF2 directly binds to promoters for these genes, we performed chromatin immunoprecipitation (ChIP) assays for KLF2-GFP on both naïve and activated CD4$^+$ T cells, using PCR probes for regions close to proposed transcriptional start sites (Figure S5B). As expected, KLF2 was found at the promoter of *S1pr1* in naïve and activated CD4$^+$ T cells (Figure 6D). In addition, we found that KLF2 bound the promoter region of *Prdm1*, the gene encoding Blimp-1, after T cell activation, but we did not observe a significant ChIP signal for KLF2 at the *Bcl6* promoter (Figure 6D). These findings are consistent with changes in *Bcl6* mRNA expression (Figures 6A and 6B) being secondary to KLF2 induction of the repressor Blimp-1. These findings raised the question of whether the ability of KLF2 overexpression to impair Tfh differentiation required Blimp-1. To explore this, we used a retroviral co-transduction system, allowing for both overexpression of KLF2 and expression of Cre recombinase in cells with floxed *Prdm1* alleles, to induce *Prdm1* deletion (Johnston et al., 2012). Transduction with KLF2 and Cre retroviruses was monitored by the markers Thy-1.1 and mAmetrine, respectively (Figure S5C). SMARTA TCR transgenic *Prdm1*$^{fl/fl}$ cells were used, and the response to LCMV infection monitored at day 7 post-infection (Figure S5C). Cells transduced with Cre alone showed increased Tfh differentiation, consistent with *Prdm1* deletion, while cells with forced KLF2 alone exhibited reduced Tfh generation, as expected (Figures 6E, S5C). However, the Tfh population frequency in cells transduced with both retroviruses was considerably higher than those transduced for KLF2 alone, indicating that Blimp-1 is critical for the effects of KLF2 overexpression (Figure 6E). Still, a trend toward lower Tfh frequency in dual transduced cells compared to those transduced with Cre alone, suggested that forced KLF2 expression may also operate through Blimp-1 independent pathways to impair Tfh generation.

KLF2 promotes expression of T-bet and Gata3 and Th1 generation

Our studies showed that KLF2 overexpression *in vivo* leads to an increased frequency of T-bet and GATA3 expressing CD4$^+$ T cells (Figure 5B), hence we also explored how expression of transcription factors that define other T helper (Th) cell subsets was affected by KLF2 manipulation (Figure 7A,B). Over-expression of KLF2 *in vitro* led to a substantial increase in expression of T-Bet (*Tbx21*) and *Gata3* genes and proteins, but did not affect expression of the gene for Rorγt (*Rorc*) or *Foxp3* (Figure 7A, C and data not shown). We saw an increase in the frequency of cells co-expressing T-bet and GATA3 (Figure 7D), and ChIP assays revealed that KLF2 occupies the regulatory regions of the genes for T-bet (*Tbx21*) and GATA3 (*Gata3*) following T cell activation (Figure 7E). However, induced KLF2 deficiency did not lead to reduced expression of *Tbx21* or *Gata3* (Figure 7B), in

contrast with the decline in *Prdm1* expression (Figure 6A), suggesting KLF2 is not required for *Tbx21* or *Gata3* expression.

Next we assessed whether KLF2 manipulation led to altered effector function. TEa CD4^{+} T cells were activated and transduced with either empty, KLF2 or S1Pr1 retroviruses, cultured in non-polarizing ("Th0") or Th1 polarizing conditions, and then assayed for production of IFN-γ or IL-4 after restimulation. Forced expression of KLF2 (but not of S1Pr1) led to a substantial increase in the frequency of IFN-γ producing cells from the Th0 cultures, and increased IFN-γ expression levels in cells from both Th0 and Th1 polarized cultures (Figure 7F). In contrast, induced deletion of KLF2 in Th1 polarized CD4+ T cells led to both a reduced frequency of IFN-γ producing cells and reduced IFN-γ expression levels in those cells (Figure 7F). IL-4 production was not detected from any groups under these culture conditions (Figure S6A,B). Forced KLF2 expression also led to enhanced Th1 differentiation *in vivo*, as indicated by the increased frequency of IFN-γ producing cells and elevated IFN-γ expression levels induced in cells recovered 7 days following immunization (Figure 7G).

KLF2 over-expression might induce non-physiological gene expression patterns. Hence we also evaluated whether endogenous KLF2 expression correlated with expression of lineage-defining transcription factors in differentiating TEa CD4^{+} T cells. KLF2 expression was lowest in the Bcl-6^{hi} pool, corresponding to Tfh cells (Figure 7H). In contrast, TEa CD4^{+} T cells co-expressing T-Bet and GATA3 exhibited high KLF2 expression (Figures 7H and S6E). Other transcription-factor-defined subsets showed intermediate expression of endogenous KLF2 (Figure 7H). These data support the hypothesis that physiological KLF2 expression levels correspond with expression of the Th lineage-defining transcription factors that are direct targets of KLF2.

Discussion

The factors regulating Tfh cell differentiation and localization within the GC are still being defined. In this report, we show that one transcription factor, KLF2, influences both activated CD4^{+} T cell trafficking (through regulation of S1PR1) and Th subset differentiation (through control of Blimp-1, T-bet and Gata3), such that KLF2 expression directs differentiating CD4^{+} T cells away from the Tfh fate. Our findings are consistent with studies showing low expression of *Klf2* and *S1pr1* transcripts in Tfh cells (Kitano et al., 2011; Xiao et al., 2014), although those reports did not explore the significance those findings. Furthermore, KLF2 does not simply block Tfh differentiation but, through regulation of multiple key transcription factors, serves to shape alternative Th differentiation choices. Indeed, we find that the amount of endogenous KLF2 expression correlates with expression of lineage-defining transcription factors, suggesting that KLF2 levels can tune the Th cell-subset differentiation fate.

We and others reported that KLF2 regulates expression of S1PR1, which is critical for lymphocyte recirculation (Bai et al., 2007; Carlson et al., 2006; Skon et al., 2013; Zachariah and Cyster, 2009). Indeed, for thymocyte egress and establishment of Trm, expression of S1PR1 largely accounts for the role of KLF2 (Skon et al., 2013; Zachariah and Cyster, 2009,

2010). We found that forced expression of S1PR1 in activated CD4$^+$ T cells led to a dramatic reduction in generation of Tfh cells. S1PR1 expression may impair migration of activated T cells into the B cell follicle, thereby blunting the signals that normally sustain Tfh differentiation. S1PR1 signals over-ride migration induced by CXCR5 in MZ B cells (Arnon et al., 2013), and counteract responses through a related chemokine receptor CCR7 in T cells (Cyster and Schwab, 2012; Pham et al., 2008). In addition, S1PR2 was shown to cooperate with CXCR5 for efficient Tfh generation (Moriyama et al., 2014) – since S1PR1 and S1PR2 signal through distinct G-protein complexes, they may have opposing effects on cell migration (Cyster and Schwab, 2012). Nevertheless, our data indicated that S1PR1 regulation is insufficient to explain the effects of KLF2 expression on Tfh differentiation.

Further studies showed that KLF2 affected several T cell lineage-defining transcription factors. Overexpression of KLF2 led to increased expression of Blimp-1, while induced ablation of the *klf2* gene led to the opposite outcome. Bcl-6 expression changed in the reciprocal direction, as expected from the known mutual repression exerted between Bcl-6 and Blimp-1 (Crotty, 2011; Johnston et al., 2009; Oestreich et al., 2012), but our data from ChIP suggested that Blimp-1 was a direct target for KLF2 binding. Furthermore, studies with inducible *Prdm1*–deficient cells demonstrated that the capacity of forced KLF2 to impair Tfh generation was largely dependent on Blimp-1. Previous studies on CD8$^+$ T cells showed that forced KLF2 caused elevated Blimp-1 expression (Hu and Chen, 2013; Preston et al., 2013), consistent with our results. However, recent studies on pre B-cells found that KLF2 dramatically repressed (rather than promoted) expression of Blimp-1 (Winkelmann et al., 2014), suggesting the nature of Blimp-1 regulation by KLF2 may be cell-type or developmental-stage specific. We did not note gene expression changes in Ascl-2, CXCR5, ICOS or IL-21 when KLF2 expression was manipulated, but it is possible that our *in vitro* studies would not reveal those changes, and analysis of KLF2 binding to other genes involved in Tfh cell differentiation and migration will be important. Nevertheless, our data on the impact of KLF2 on the balance between Blimp-1 and Bcl-6 expression provides a ready explanation for KLF2's ability to derail the Tfh differentiation pathway.

Surprisingly, we also observed that elevated KLF2 expression induced the T-bet and Gata3 transcription factors, and ChIP assays suggested KLF2 directly bound to the regulatory regions for the genes encoding these factors. Our data suggest this regulation is not simply an artifact of overexpression studies, since analysis of normal TEa cells responding *in vivo* showed that the populations expressing T-bet and Gata3 had significantly higher levels of endogenous KLF2. T-bet and Gata3 are frequently co-expressed in human Th1 cells (Paliard et al., 1988), but analysis in mouse T cells suggest these two factors are normally differentially expressed (Th1 cells expressing T-bet, Th2 cells expressing Gata3) (Zhu et al., 2010). While there have been studies suggesting that restimulating Th1 cells in Th2 conditions can provoke T-bet$^+$Gata3$^+$ cells with hybrid Th1 and Th2 properties (Hegazy et al., 2010), our studies found that KLF2 overexpressing CD4$^+$ T cells are potentiated for production of the Th1 cytokine IFN-γ, but did not exhibit detectable production of the Th2 cytokine IL-4. Studies on characteristics of T-bet$^+$Gata3$^+$ CD4$^+$ T cells suggest that T-bet typically co-opts Gata3 to support Th1 lineage gene expression (Kanhere et al., 2012), consistent with our findings. While Blimp-1 has been reported to directly repress expression

of T-bet and IFN-γ in activated CD4^{+} T cells (Cimmino et al., 2008), our studies indicate that, when induced by KLF2, Blimp-1 and T-bet can be co-expressed. Though the significance of KLF2^{hi} cells expressing T-bet and Gata3 will require further study, these findings suggest that KLF2 expression does not only restrain Tfh differentiation, but can foster differentiation into other Th lineages (Th1 and potentially Th2). We observed that the frequency of RORγt expressing cells was reduced by forced KLF2 expression (although whether this reflects active impairment of Th17 differentiation versus preferential skewing toward Th1 differentiation is not clear) and an intriguing recent study showed that KLF2 is also critical for effective induction of Foxp3 in induced Treg cells (Pabbisetty et al., 2014). Hence accumulating data suggest that KLF2 acts as a critical element in Th cell subset differentiation.

What factors inhibit KLF2 expression during Tfh differentiation? ICOS-ICOSL interactions are critical for Tfh generation, and our studies showed that ICOS-L blockade led to increased KLF2 expression in Tfh phenotype cells. ICOS signaling induces the PI3K pathway (Crotty, 2011; Gigoux et al., 2009), and several studies indicate that strong PI3K-Akt activation impairs KLF2 expression, at least in part due to degradation of the transcription factor Foxo1 (Fabre et al., 2008; Kerdiles et al., 2009; Kerdiles et al., 2010; Sinclair et al., 2008; Skon et al., 2013). Foxo1 ablation leads to substantially enhanced Tfh differentiation (Kerdiles et al., 2010; Xiao et al., 2014), and recent studies indicate that degradation of Foxo1 through action of the E3 ubiquitin ligase Itch was important for Tfh differentiation, and that Itch deficiency led to elevated expression of Foxo1 target genes (including KLF2) (Xiao et al., 2014). Although we find that ICOS engagement is important for repression of KLF2, this is unlikely to be the only relevant factor. TCR signaling also induces loss of KLF2 expression, and we find evidence of sustained TCR signaling in Tfh phenotype cells. Furthermore, studies with CD8^{+} T cells reveal that exposure to various cytokines - including TGF-β, IL-33, IL-12, IFN-I and TNF –impair KLF2 expression (Bai et al., 2007; Sinclair et al., 2008; Skon et al., 2013). Hence the specific cytokine milieu surrounding an activated CD4^{+} T cell may dictate its KLF2 expression. Defining how disparate signals coordinate to regulate KLF2 expression during Tfh differentiation will require further investigation.

Kruppel-like factors play diverse roles in multiple tissues, often related to late differentiation steps (Hart et al., 2012; Skon et al., 2013). The studies reported here demonstrate a significant impact of KLF2 expression on helper CD4^{+} T cell subset differentiation in two separable ways: Through trafficking (via S1PR1) and through regulation of three lineage-defining transcription factors (Blimp-1, T-bet and GATA3). Hence KLF2 serves a hitherto unsuspected function in dictating the lineage fate of CD4^{+} T cells.

Experimental procedures

(Further details are provided in Supplementary Experimental Procedures)

Mice

C57BL/6 (B6) and B6.SJL mice were purchased from the National Cancer Institute, and ERT2-Cre, Rosa26-YFP, Blimp1-YFP and *Tcra*^{−/−} mice were obtained from Jackson

Laboratories. TEa TCR transgenic mice (specific for a peptide from the I-Eα MHC II molecule (pEα) bound to I-A^b), and MD4 BCR transgenic mice (specific for hen/duck egg lysozyme (HEL/DEL)) were maintained at the University of Minnesota. Cells from prdm1$^{fl/fl}$ Bcl6$^{+/-}$ SMARTA mice were provided by Drs. Phil Nance and Shane Crotty (La Jolla Institute for Allergy and Immunology) (Johnston et al., 2012). The S1PR1-GFP, KLF2-GFP, Nur77-GFP and *Klf2*$^{fl/fl}$ mice have been previously described (Cahalan et al., 2011; Moran et al., 2011; Skon et al., 2013; Weinreich et al., 2010; Weinreich et al., 2009) and were crossed to TEa mice at the University of Minnesota. Animals were maintained under specific pathogen free conditions at the University of Minnesota. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Infections and MHC II-tetramer based cell enrichment

Mice were injected intravenously with 1×10^7 colony-forming units of ActA-deficient LM-2W1S bacteria or intraperitoneally (i.p.) with 2×10^5 plaque-forming units of the LCMV Armstrong strain. Tetramers composed of I-A^b and either 2W1S, LLO190-201, or LCMV glycoprotein (GP) 66–77 peptides were made as described previously (Moon et al., 2009; Tubo et al., 2013).

Adoptive transfer and Eα-SA-DEL immunization

For adoptive transfer experiments, 1×10^5 TEa CD4$^+$ T cells were typically co-transferred with 5×10^4 MD4 B cells into WT B6, B6.SJL, or *Tcra*$^{-/-}$ mice depending on the CD45 congenic marker expression of the donor cells.

Inducible KLF2 deletion and B cell germinal center reaction

In vivo KLF2 deletion of *Klf2*$^{fl/fl}$ (KLF2$^{fl/fl}$ ETR2^{-Cre} *Rosa26*-YFP TEa) cells in WT B6.SJL, *Tcra*$^{-/-}$ or *Tcrb*$^{-/-}$ mice was achieved by administering tamoxifen (10 mg/ml) in sunflower seed oil i.p. for 5 consecutive days from day 2 post- immunization. At day 7 post-immunization, the spleen and inguinal, axillary, brachial, cervical and mesenteric LNs were harvested and analyzed. Sera from immunized mice were collected at day 0, 2 and 7 post immunization, and antigen-specific antibodies were measured by ELISA as previously described (Pape et al., 2011).

Retroviral transduction approaches

Naive CD4$^+$ T cells from TEa or Blimp1-YFP mice were isolated and activated by plate-bound anti-CD3 and anti-CD28 with recombinant IL-2 (20ng/ml). 24 hours after activation, cells were spin-infected by retroviruses MiT-KLF2, MiT-S1PR1 or control empty vector (MiT-Empty) as described previously (Skon et al., 2013).

Chromatin immunoprecipitation (ChIP) and Quantitative RT-PCR

ChIP was performed as previously described (Li et al., 2013). Detailed procedure and primer information are described in Supplemental Information.

Immunohistochemistry

KLF2-GFP or WT B6 mice were subcutaneously immunized with PE (15μg in CFA) at the base of the tail and were sacrificed after 14 days. Draining LNs were fixed with 4% PFA and incubated in 30% sucrose solution. Five micrometer sections were cut and stained with anti-GFP antibody (Life technology). GFP fluorescence intensities were quantified in GC (GL7 and B220 abundant) or T cell zone (CD4$^+$ abundant and B220 negative) CD4$^+$ T cells using ImageJ software according to histocytometric algorithms as previously described (Gerner et al., 2012).

Statistical analysis

Data were analyzed with Prism software 4.0 (GraphPad). For standard data sets, an unpaired two-tailed Student's *t*-test was used. For values that differed by over tenfold, the data was log10-transformed before *t*-test analysis. When data were normalized (by the appropriate control samples), normalization involved division of all values by the overall mean of the control values to avoid type I and II errors during calculation of significance through the *t*-test. Data sets (in Prism format) are available on request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- KLF2 expression varies in distinct T helper cell subsets

- Downregulation of KLF2 and S1PR1 is required for Tfh cell differentiation

- KLF2 promotes expression of Blimp-1, T-bet and Gata-3

- KLF2 enhances differentiation of functional Th1 cells

The factors that control T follicular helper (Tfh) cell lineage choice are incompletely understood. Jameson and colleagues find that low expression of transcription factor KLF2 favors generation of Tfh cells, while high KLF2 expression impairs Tfh cell differentiation and promotes Th1 cell generation.
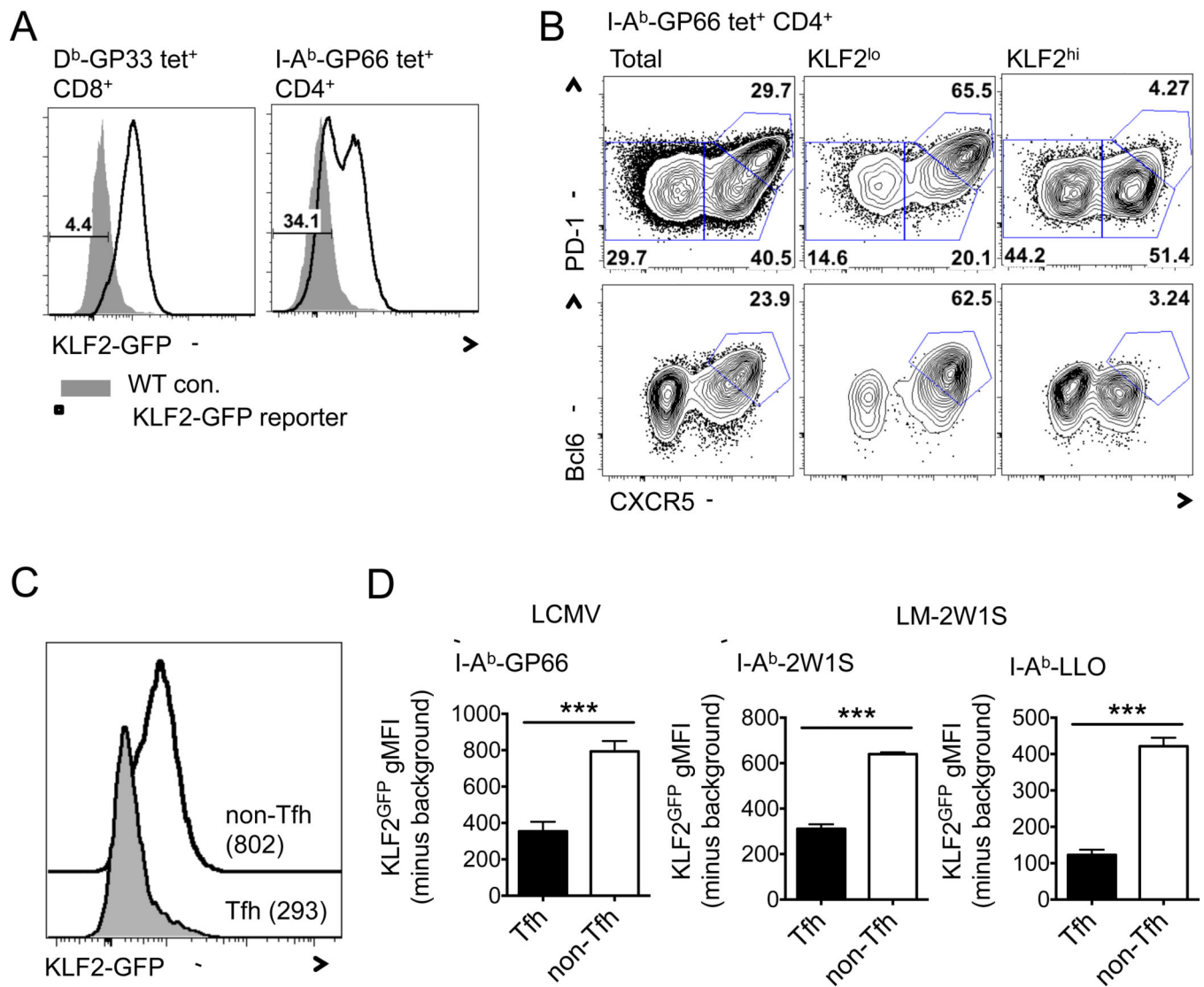
Figure 1. KLF2 is down-regulated in Tfh cells

KLF2-GFP reporter mice were infected with LCMV (A–D) or recombinant *Listeria monocytogenes* expressing the 2W1S epitope (LM-2W1S)(D), and analyzed 7 days later. (A) Antigen-specific splenic CD8+ and CD4+ T cells were enriched using Db-GP33 and I-Ab-GP66 tetramers, respectively, and monitored for KLF2-GFP expression. Shadow area shows background GFP signal in non-transgenic wild-type (WT) controls. (B) CXCR5, PD-1 and Bcl-6 expression is shown for total, KLF2-GFPlo and KLF2-GFPhi LCMV specific CD4+ T cells. (C) KLF2-GFP expression by Tfh (CXCR5hiPD-1+) and non-Tfh (CXCR5−PD-1−) phenotype cells in I-Ab-GP66 tetramer+ CD4+ T cells from LCMV infected mice. Numbers indicate geometric mean fluorescence intensity (gMFI) of KLF2-GFP expressed in each population. In (D), KLF2-GFP expression is shown for Tfh and non-Tfh phenotype cells in the LCMV specific CD4+ T cells (left), and in I-Ab-2W1S tetramer+ and I-Ab-LLO tetramer+ CD4+ T cells LM-2W1S infected mice (right). Data are shown as gMFI minus background gMFI of WT CD4+ T cells. Data are from at least three independent experiments with a total of 13 (A, B, C, D for LCMV infection) or 8 (D for

LM-2W1S infection) KLF2-GFP reporter mice, and are representative of (A, B, C) or accumulated (D) from the independent experiments as mean ±s.e.m. Statistical significance was determined using two-tailed t-test: ns, not significant (P > 0.05); and ***, P < 0.001.
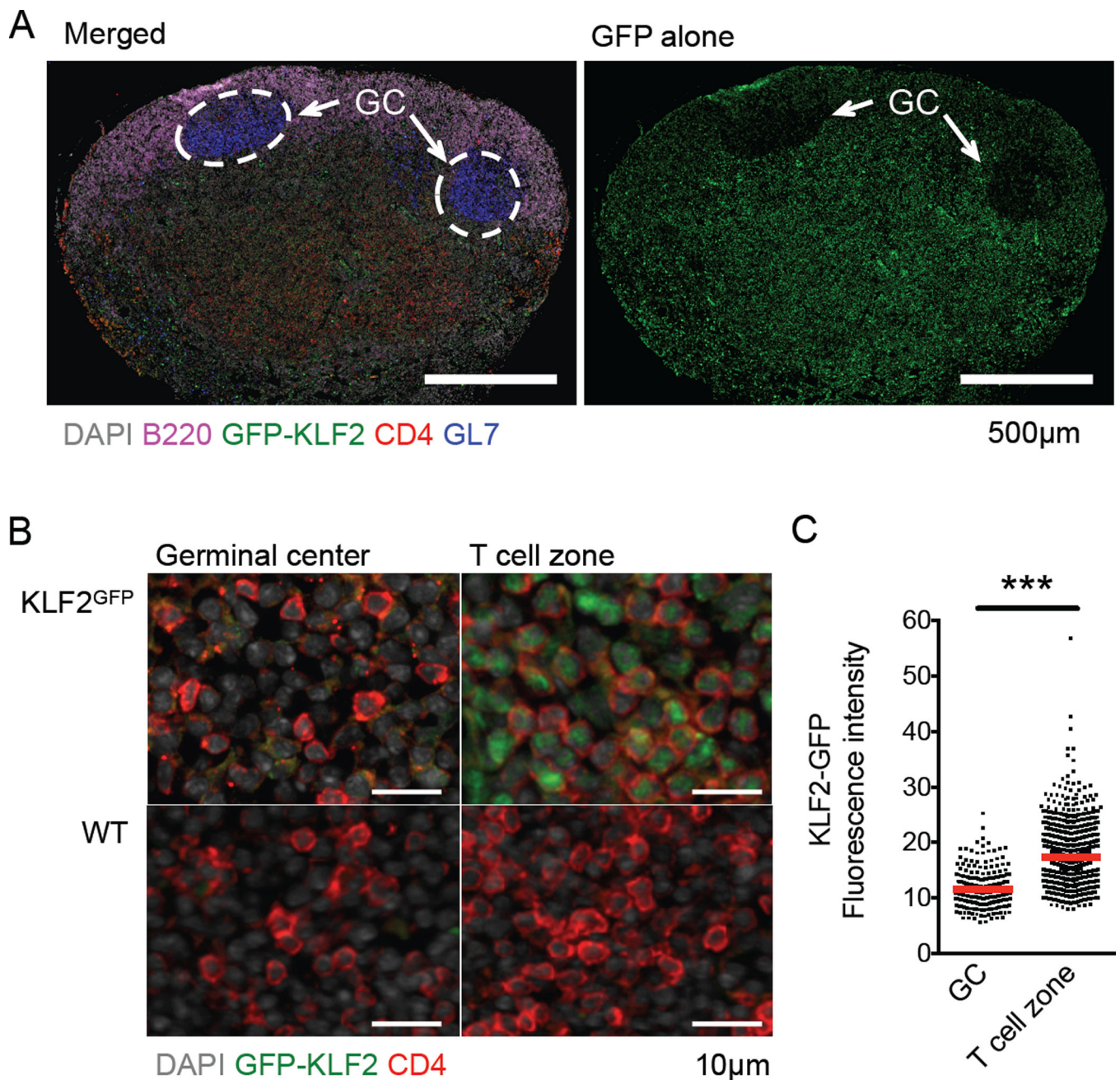
Figure 2. KLF2 expression by CD4⁺ T cells is reduced in the GC compared to T cell zone

Immunohistochemistry analysis of a draining lymph node (dLN) from KLF2-GFP reporter mice immunized subcutaneously with Phycoerythrin (PE) 14 days earlier. (A) In the left image, the indicated stains were used to identify B cell follicles (B220⁺) and T cell zone (B220⁻, CD4⁺), while germinal centers (GCs) were identified by GL7 staining (and confirmed by PE co-staining: data not shown). Two GCs are indicated by white arrows. The right image is the same section, but only the KLF2-GFP staining signal is shown. (B) The panels show the staining for KLF2-GFP (green) and CD4⁺ (red) for cells in the GC or T cell zone as indicated. The upper two panels are from immunized KLF2-GFP mice, while the

lower two panels are from immunized WT B6 mice. Gray, DAPI; Purple, B220; Green, KLF2-GFP; Red, CD4; Blue, GL7. The scale bars for the images are shown. (C) shows the KLF2-GFP fluorescence intensity of CD4$^+$ T cells in the GC or T cell zone (using the criteria defined in A, B). Each dot represents a single CD4$^+$ T cell and the red bar indicate average fluorescence intensity of each group. All experiments were repeated three times with similar results. Graphs show accumulated data from three independent experiments as mean ±s.e.m., two-tailed t-test. ***, P < 0.001.
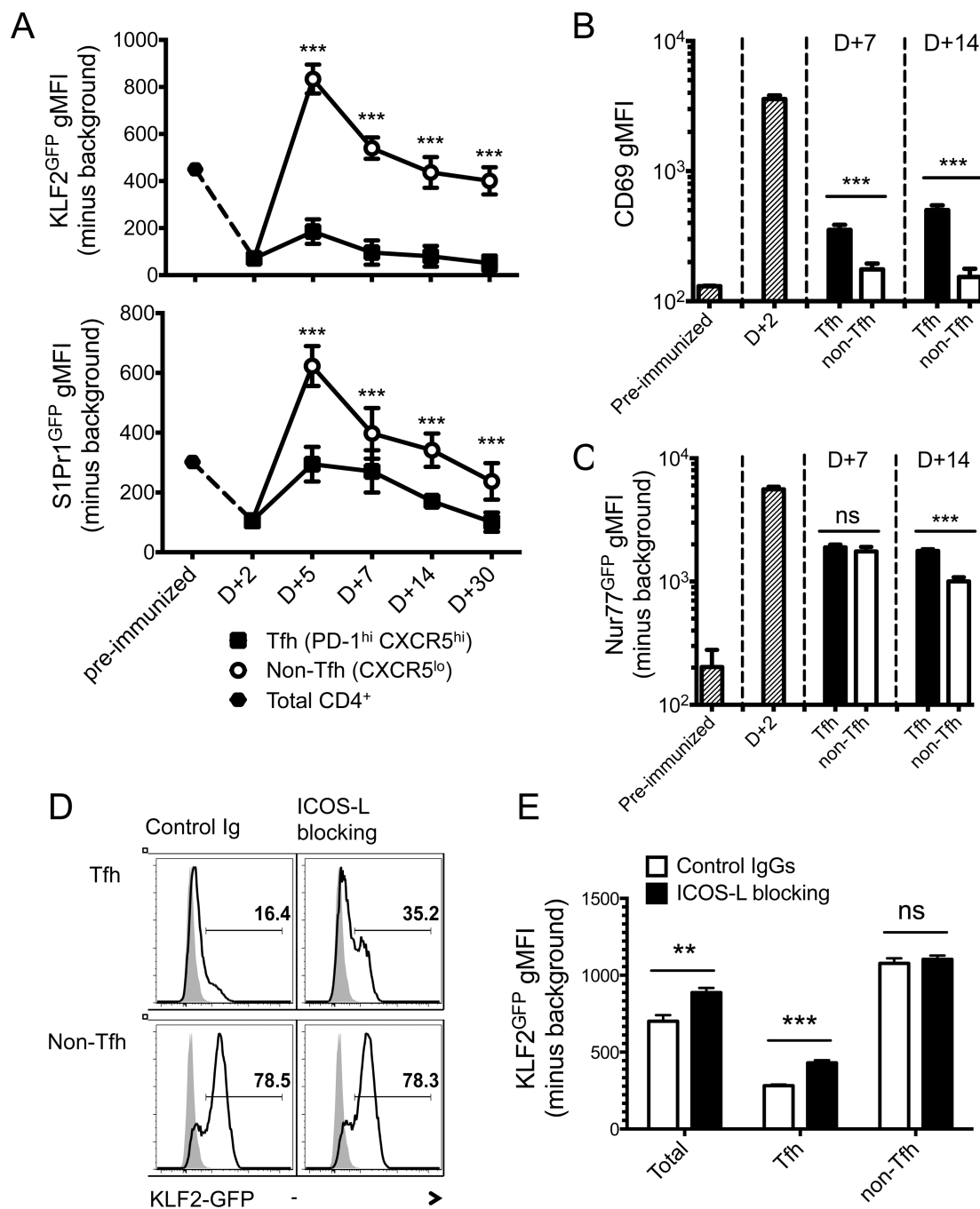
Figure 3. Downregulation of KLF2 and S1PR1 expression is maintained in Tfh cells

(A) Expression of KLF2-GFP (top) or S1PR1-GFP (bottom) by TEa CD4+ T cells at indicated time points following Eα-SA-DEL and CFA immunization. TEa cells were co-transferred with MD4 B cells into WT B6 recipients prior to priming. Data are presented as gMFI minus background gMFI of co-transferred non-transgenic TEa CD4+ T cells, after gating on the indicated phenotypic subsets (defined in Figure 1). Statistical significance was calculated relative to the non-Tfh population. (B) Cell surface expression of CD69 on Tfh and non-Tfh TEa CD4+ T cells at days 2, 7 and 14 post Eα-SA-DEL and CFA

immunization, presented as gMFI. (C) Expression of Nur77-GFP (a reporter for TCR signaling) by Tfh and non-Tfh TEa CD4$^+$ T cells (shown as gMFI, calculated as above). (D, E) KLF2-GFP TEa and MD4 B cell recipient mice were immunized with Eα-SA-DEL and CFA and treated with anti-ICOSL (ICOS-L blocking) or isotype control Ig at day 7 as schematically shown in Figure S2D. KLF2-GFP expression in Tfh or non-Tfh cells were analyzed 9 days p.i. (E) Data are presented as gMFI minus background gMFI of wild type CD4 T cells of the recipient mice. Data are from three independent experiments with a total of 9 recipient mice at each time points. Graphs show accumulated (A, B, C, E) or representative (D) data from the independent experiments as mean ±s.d. (A) or ±s.e.m. (B, C, E), two-tailed t-test. ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
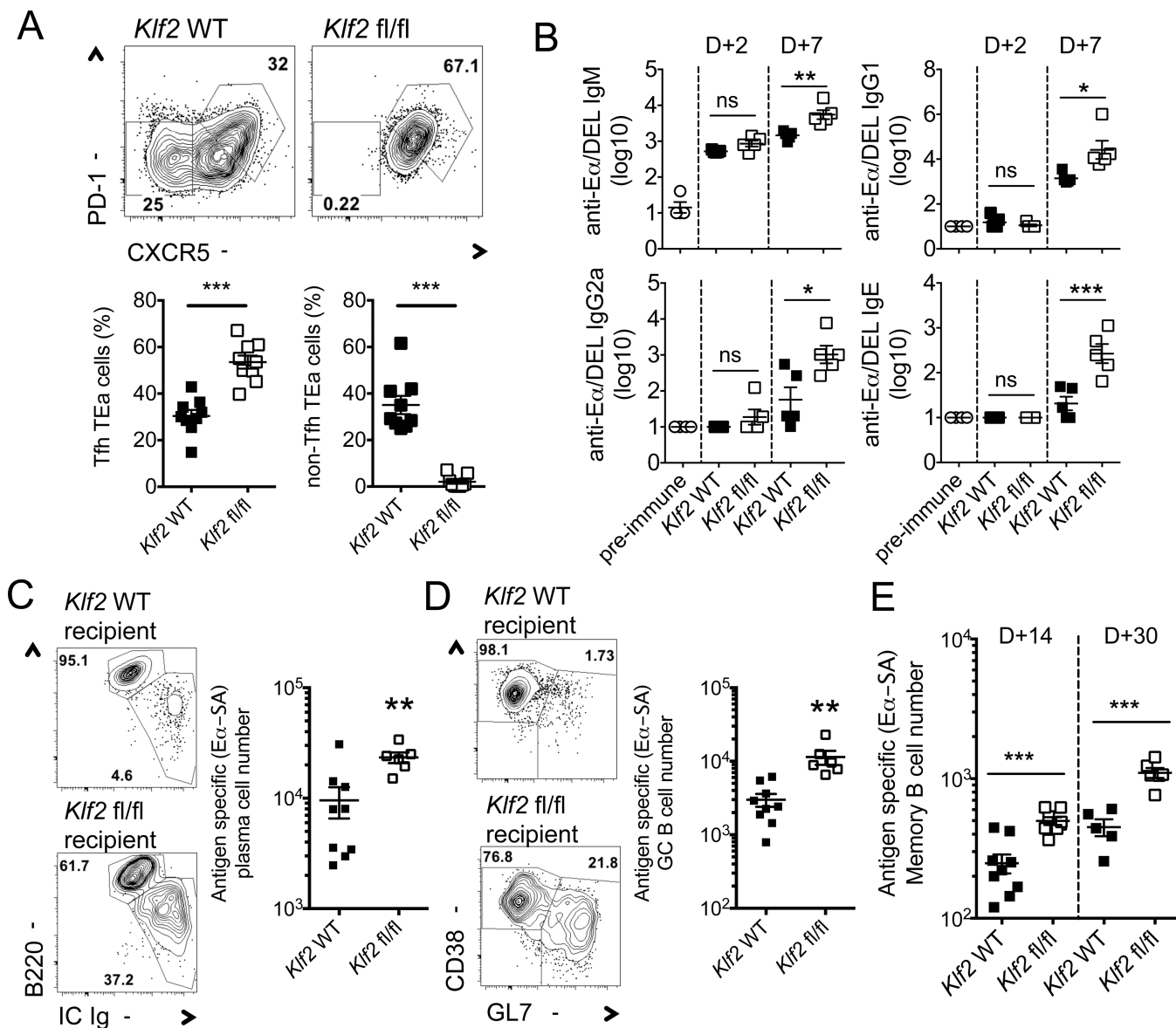
Figure 4. Inducible KLF2 deletion in CD4^{+} T cells enhances Tfh cell differentiation and the GC B cell response

(A). WT CD4^{+} TEa cells (Cre-ERT2 TEa) or KLF2 inducible knockout CD4^{+} TEa cells (*Klf2*^{fl/fl} Cre-ERT2 *Rosa26*-YFP TEa) were co-transferred with MD4 B cells into WT B6 recipients, and primed with Eα-SA-DEL and CFA subcutaneous immunization. Tamoxifen was administrated daily from day 2, and the cells analyzed at day 7. KLF2-deficient TEa CD4^{+} T cells were identified as YFP^{+}. (B, C, D, E) WT or KLF2-inducible knockout KLF2 TEa cells were transferred (without MD4 B cells) into *Tcra*–/– (B, C, E) or *Tcrb*–/– (E) recipients, which were immunized with Eα-SA-DEL in CFA and treated with tamoxifen from days 2 to 6. Animals were analyzed on day 7 (B, C, D) or day 14/30 (E) for phenotypic markers (defined in Supplementary Fig. 3. (B) Titers of Eα-SA-DEL specific antibodies in serum from mice receiving WT (n=5) or *Klf2*^{fl/fl} (n=5) TEa T cells. Serum samples were collected at the day of immunization (pre-immune), beginning of tamoxifen treatment (day 2

after immunization: "D+2") and 5 days of tamoxifen treatment (day 7 after immunization: "D+7"). (C, D) Quantification of endogenous Eα-SA-specific (C) plasma cells (intracellular immunoglobulin (Ig^{hi}, $B220^{low}$; left) and (D) GC B cells ($B220^{hi}$, $GL7^{hi}$; right) in *Tcra−/−* KO recipient mice at day 7 post immunization. (E) Quantification of endogenous Eα-SA specific isotype-switched memory B cell ($B220^{high}$, $CD38^{high}$, IgM^{neg}, IgD^{neg}) in TCRβ KO recipient mice at day 14 and 30 post immunization. Each symbol represents an individual mouse and small horizontal lines indicate the mean. Data are from three independent experiments with a total of 9 WT B6 recipient mice (A), or 9 (WT) or 6 (*Klf2*$^{fl/fl}$) *Tcra−/−* recipient mice (B, C, D), or 15 ($KLF2^{+/+}$) or 15 (*Klf2*$^{fl/fl}$) *Tcrb−/−* recipient mice (E). Graphs show accumulated data from the independent experiments as mean ±s.e.m., two-tailed t-test. ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.
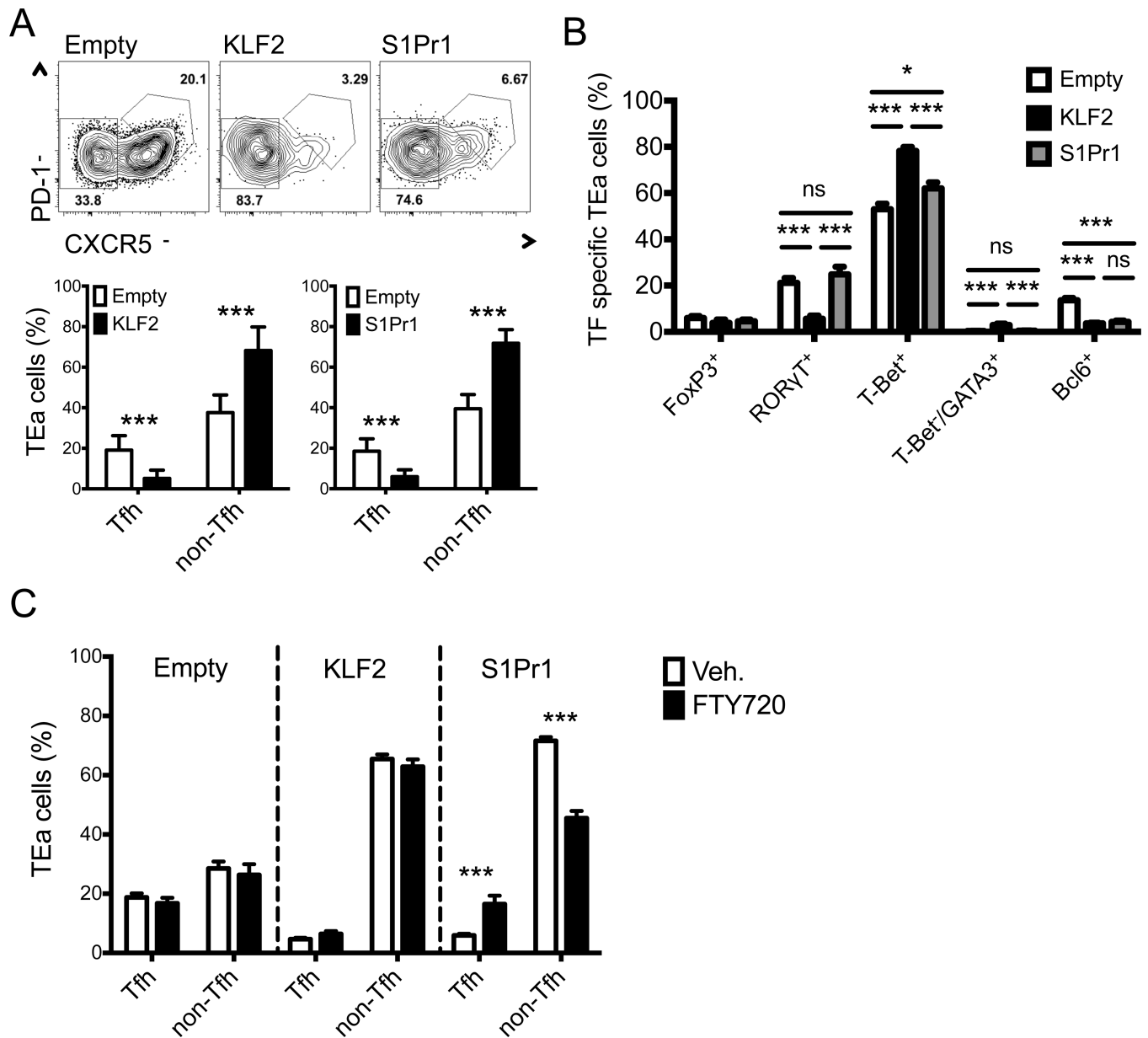
Figure 5. Forced expression of KLF2 or S1PR1 inhibits Tfh cell differentiation

(A) TEa CD4+ T cells were in vitro activated and transduced with MiT-based retroviruses encoding KLF2, S1Pr1 or no insert (empty) and adoptively transferred (with naïve MD4 B cells) in recipient mice that were immunized with Eα-SA-DEL in CFA. At day 7 of the response, transduced TEa cells (identified by the Thy-1.1 marker) were analyzed for phenotypic markers (defined in Figure 1). (B) Analysis of TEa CD4+ T cells for expression of lineage-defining transcription factors following transduction with indicated retroviruses at day 7 post immunization *in vivo*. (Gating strategy for each population is described in Supplementary Fig. 4B). (C) Effect of FTY720 treatment (S1PR1 blocking) on representation of Tfh and non-Tfh populations within the retrovirally transduced TEa T cells in vivo. FTY720 or vehicle control ("Veh.") was administrated by intra-peritoneal injection at day 2, 4, and 6 post immunization, and the phenotype of donor cells was analyzed at day 7

after the immunization. Data are from at least three independent experiments with a total of 11(A) or 9(B) recipient mice, and graphs show accumulated data from the independent experiments as mean ±s.e.m., two-tailed t-test. ***, P < 0.001.
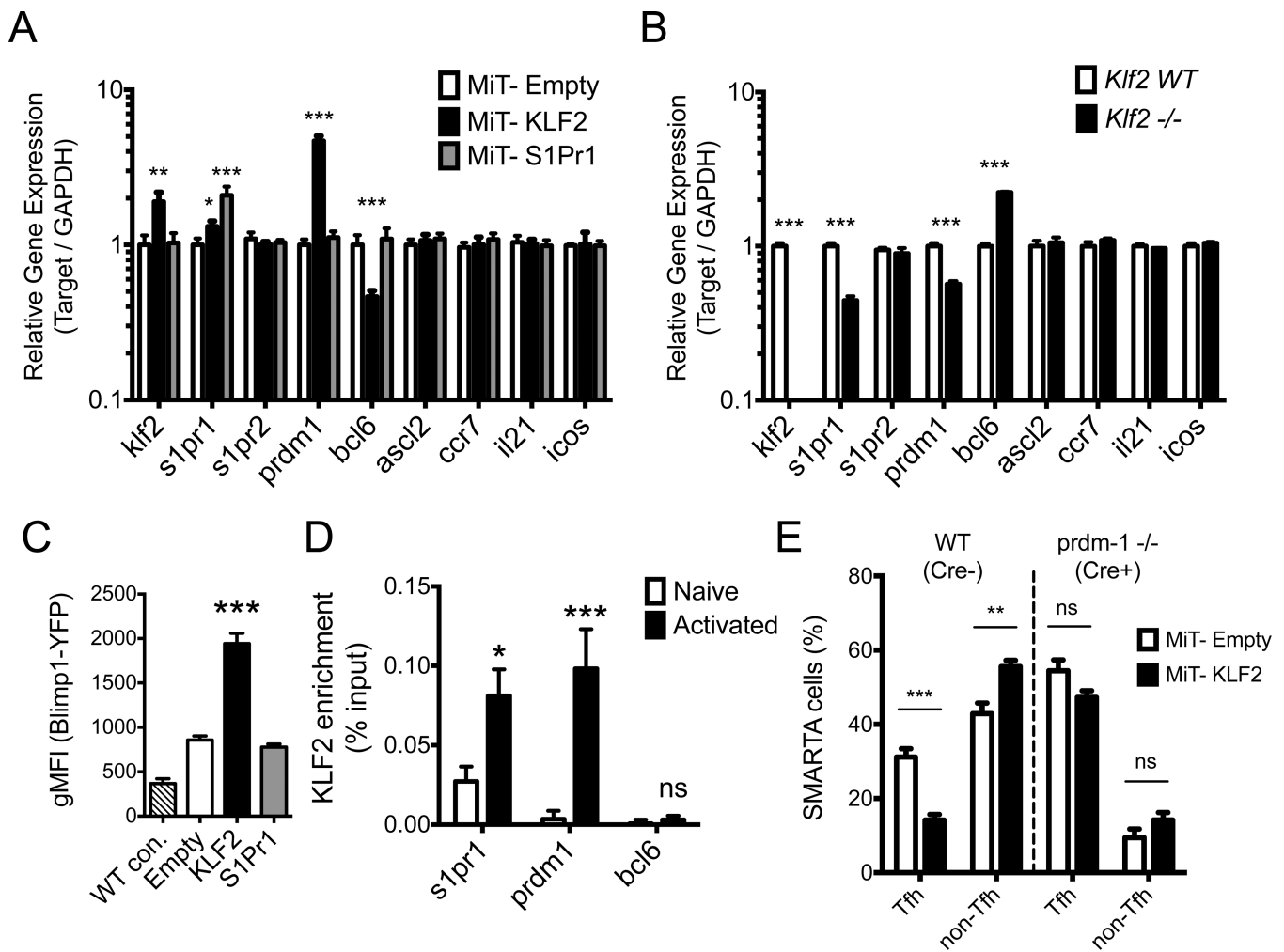
Figure 6. Transcriptional regulation of Blimp-1 by KLF2

(A, B) RT-PCR analysis of various genes (horizontal axis) for *in vitro* cultured retrovirus-transduced (A) or KLF2 KO (B) TEa CD4+ T cells after sorting based on expression of retroviral transduction marker (Thy1.1) or Cre-reporter signal (for *Klf2−/−* YFP+). (Detailed *in vitro* culture conditions are described in Supplementary Fig. 5A). (C) Induction of Blimp-1 in the retrovirus-infected Blimp-1-YFP reporter CD4+ T cells *in vitro* (identified by Thy1.1+ expression). (D) ChIP analysis of naïve or *in vitro* activated KLF2GFP-reporter TEa CD4+ T cells, followed by chromatin immunoprecipitation using rabbit IgG (control) or anti-GFP and quantitative PCR analysis of binding at the promotor regions of each gene (horizontal axis) (primers are listed in Materials and Methods). Results were normalized to those of a standardized aliquot of input chromatin, followed by subtraction of the signal obtained with IgG (nonspecific background). (E) prdm1fl/fl Bcl6+/− SMARTA CD4+ T cells, that were in vitro activated and co-transduced with MiT-based retroviruses encoding KLF2 (or no insert (Empty RV)) and Cre expressing retrovirus (MSCV-Cre-IRES-mAmetrine; Cre RV), were adoptively transferred in recipient mice and primed by LCMV infection. At day 7 of the response, transduced wild type (Cre−; mAmetrine negative) or *Prdm1−/−* (Cre+; mAmetrine positive) SMARTA cells (identified by the Thy-1.1 and mAmetrine expression)

were analyzed for phenotypic markers (defined in Supplementary Fig. 5C, 5D). All experiments repeated at least three times with similar results. Graphs show accumulated data from the independent experiments as mean ±s.e.m., two-tailed t-test. ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
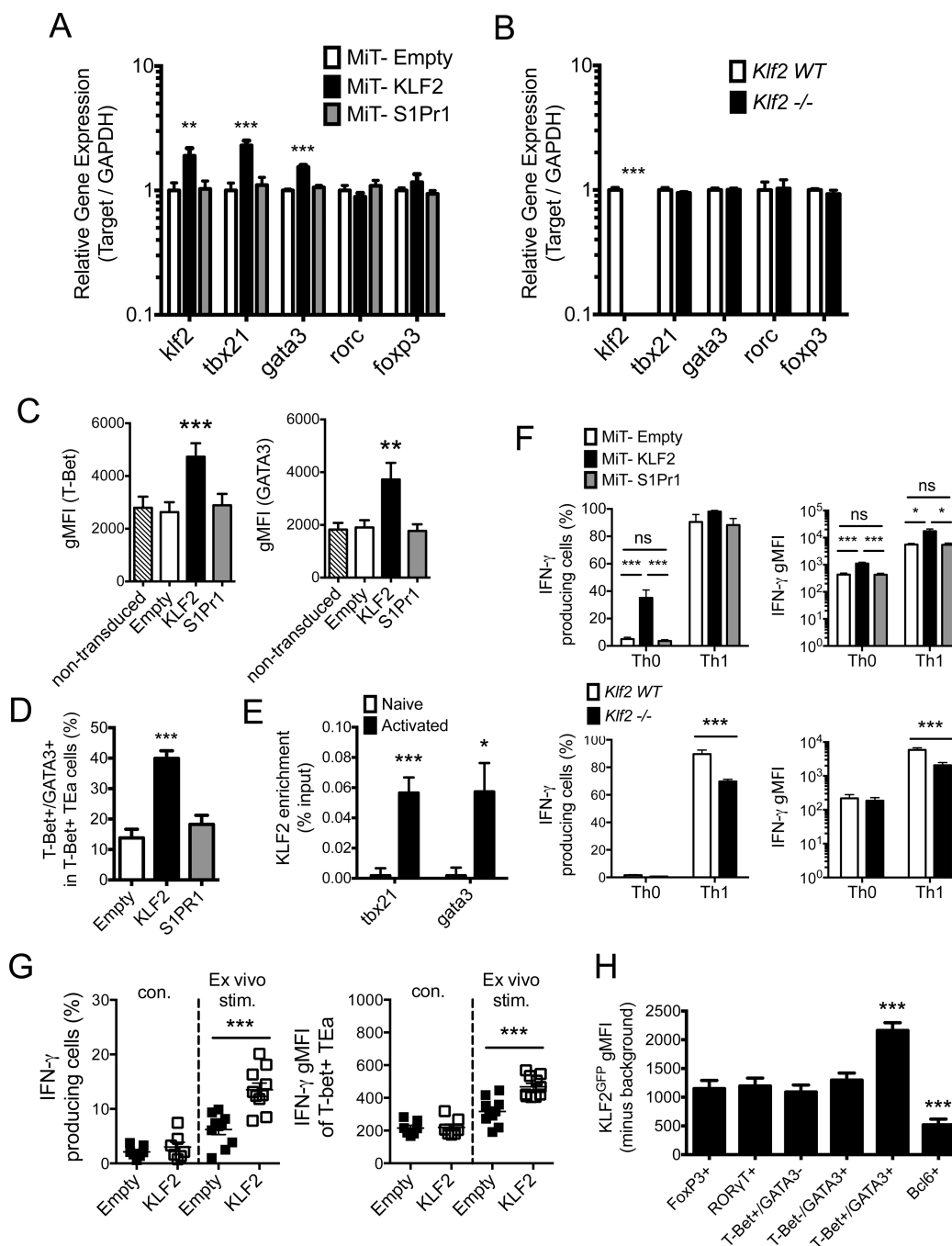
Figure 7. KLF2 induces T-bet and GATA3 expression, and enhances Th1 polarization during CD4+ T cell lineage commitment *in vivo*

(A, B) RT-PCR analysis of CD4+ T cell lineage specific transcription factor expression (horizontal axis) for *in vitro* cultured retrovirus-transduced (A) or KLF2 KO (B) TEa CD4+ T cells after sorting based on expression of retroviral transduction marker (Thy1.1) or Cre-reporter signal (for *Klf2–/–* YFP+). (Detailed *in vitro* culture conditions are described in Supplementary Fig. 5A). (C) FACS analysis of T-Bet and GATA3 expression in retrovirally-transduced TEa cells (identified by Thy1.1+ expression). (D) Frequency of T-

bet$^+$, GATA3$^+$ TEa CD4$^+$ T cells within the T-bet$^+$ population (Th1; T-bet$^+$FoxP3$^-$RORγT$^-$). (E) ChIP-PCR analysis of naïve or *in vitro* activated KLF2-GFP reporter TEa CD4$^+$ T cells, followed by chromatin immunoprecipitation using rabbit IgG (control) or anti-GFP and quantitative PCR analysis of binding at the promotor regions of *Tbx21* (T-bet) and *Gata3* (GATA3) (primers are listed in Supplementary experimental procedure). (F) Percentage of IFN-γ producing population (left) or level of IFN-γ production (right) upon PMA/Ionomycin stimulation for 3 hours in the retrovirus-infected (top) or KLF2 KO (bottom) TEa CD4$^+$ T cells *in vitro* (FACS plots in Figure S6A, S6B). (G) Percentage of IFN-γ producing population (left) or level of IFN-γ production in T-bet+ population (right) upon ex vivo PMA/Ionomycin stimulation for 3 hours in the retrovirus-infected TEa CD4$^+$ T cells *in vivo* (FACS plots in Figure S6C). (H) KLF2-GFP expression level (gMFI) within TEa CD4$^+$ T cells expressing the indicated transcription factors at day 7 post immunization. Data are from at least three independent experiments, and graphs show accumulated data from the independent experiments as mean ±s.e.m., two-tailed t-test. ns, not significant (P > 0.05); *, P < 0.05; and ***, P < 0.001.