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TCF1 Is Required for the T Follicular Helper Cell Response to Viral Infection

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**Cell Reports**

**TCF1 Is Required for the T Follicular Helper Cell Response to Viral Infection**

**Graphical Abstract**

- TCF1 and Blimp1 are reciprocally expressed in viral-specific TFH and Th1 cells
- TCF1 is intrinsically required for viral-specific TFH cell responses
- TCF1 forms negative feedback loops with IL-2 and Blimp1
- TCF1 maintains the transcriptional and metabolic signatures of TFH cells

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**In Brief**

TFH cells are critical for long-term humoral responses. Wu et al. examine the molecular mechanisms that govern the choice of activated CD4 T cells between TFH and Th1 fates, and they find that the transcription factor TCF1 promotes viral-specific TFH cell responses through a negative feedback loop with IL-2 and Blimp1.

**Accession Numbers**

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TCF1 Is Required for the T Follicular Helper Cell Response to Viral Infection

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SUMMARY

T follicular helper (TFH) and T helper 1 (Th1) cells generated after viral infections are critical for the control of infection and the development of immunological memory. However, the mechanisms that govern the differentiation and maintenance of these two distinct lineages during viral infection remain unclear. We found that viral-specific TFH and Th1 cells showed reciprocal expression of the transcription factors TCF1 and Blimp1 early after infection, even before the differential expression of the canonical TFH marker CXCR5. Furthermore, TCF1 was intrinsically required for the TFH cell response to viral infection; in the absence of TCF1, the TFH cell response was severely compromised, and the remaining TCF1-deficient TFH cells failed to maintain TFH-associated transcriptional and metabolic signatures, which were distinct from those in Th1 cells. Mechanistically, TCF1 functioned through forming negative feedback loops with IL-2 and Blimp1. Our findings demonstrate an essential role of TCF1 in TFH cell responses to viral infection.

INTRODUCTION

CD4 T cells constitute an essential force of the adaptive immune system and are critical for vaccination and immune responses against infections and tumors. CD4 T cells modulate the immune response through various mechanisms, including secretion of cytokines and direct cell-cell interaction. Depending on the antigen, microenvironment, and cytokine milieu, activated CD4 T cells can develop into distinct effector populations, each characterized by unique effector functions and differentiation programming (Crotty, 2011; Zhou et al., 2009). One major function of CD4 T cells is to help the humoral immune response, a function that is carried out by a CD4 subset known as follicular helper (TFH) cells (Cannons et al., 2013; Crotty, 2011). TFH cells express a set of surface markers, such as CXCR5, which enable them to migrate to the B cell follicle and distinguish them from other CD4 subsets. TFH cells provide crucial help for the initiation and maintenance of germinal centers (GCs), which are indispensable for antibody affinity maturation and the development of long-term humoral immunity conferred by long-lived plasma cells and memory B cells (Victora and Nussenzweig, 2012). TFH cells signal to antigen-presenting cognate B cells through the secretion of cytokines, such as IL-4 and IL-21, as well as the expression of CD40L and ICOS that engage their binding partners on B cells (Crotty, 2011).

TFH cells express high levels of Bcl6, a transcriptional repressor, which is essential for TFH cell differentiation (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009a). In contrast, Blimp1, an antagonist of Bcl6, is highly expressed by non-TFH effector cells and suppresses TFH cell differentiation (Johnston et al., 2009). Bcl6 expression is triggered in activated T cells early after antigen exposure through the interaction between dendritic cells (DCs) and T cells (Baumjohann et al., 2011; Choi et al., 2011). After priming by DCs, TFH cells upregulate CXCR5, downregulate CCR7, and move to the T-B zone border where they interact with cognate B cells (Allen et al., 2007; Baumjohann et al., 2011; Haynes et al., 2007). TFH and pre-GC B cells then migrate into the B cell follicle and initiate the GC reaction (Crotty, 2011). The interaction with cognate B cells is required for the maintenance and expansion of TFH cells (Baumjohann et al., 2011; Choi et al., 2011). In contrast, IL-2 signaling restricts the TFH cell response via STAT5- and Blimp1-mediated pathways (Ballesteros-Tato et al., 2012; Johnston et al., 2012). However, despite recent progress on the regulation of TFH cell differentiation, many molecular mechanisms involved in the initiation and maintenance of TFH cells remain to be elucidated.

T cell factor 1 (TCF1) is a key transcription factor of the Wnt signaling pathway, which activates Wnt target genes when bound by β-catenin (Verbeek et al., 1995). Multiple TCF1 isoforms are produced as a result of alternative splicing and dual promoter usage of the Tcf7 gene and can be grouped into long and short isoforms having or lacking the β-catenin-binding domain (Van de Wetering et al., 1996). TCF1 is induced by Notch signaling during T cell development and is highly expressed in thymocytes and mature naive T cells (Xue and Zhao, 2012). Various stages of T cell development, such as T cell lineage...
commitment of hematopoietic progenitor cells, β selection, and development from double-negative (DN) to double-positive (DP) thymocytes, are regulated by TCF1 (Germain et al., 2011; Okamura et al., 1998; Weber et al., 2011; Yu et al., 2012). During the CD8 T cell response, TCF1 is required for the development of central memory CD8 T cells and optimal recall response by memory cells (Zhou et al., 2010). In CD4 T cells, TCF1 promotes Th2 differentiation by inducing GATA3 expression and restricts IFNγ expression by T helper 1 (Th1) cells (Yu et al., 2009b). However, the role of TCF1 in the TFH cell differentiation is still unknown.

In this study, we found that, very early after viral infection, effector CD4 T cells differentiate into TCF1-high Blimp1-low TFH and TCF1-low Blimp1-high Th1 cells. Notably, Tcf7 deficiency led to a T-cell-intrinsic defect in viral-specific TFH cell responses, associated with decreased TFH cells and reduced GCs. Mechanistically, we found that TCF1 is required for the generation and maintenance of distinct transcriptional and metabolic signatures of TFH cells, including repression of Il2ra and Prdm1, the gene products of which limit TFH cell responses. Together, our data demonstrate that TCF1 is essential for the anti-viral TFH cell response.

RESULTS

Viral-Specific Effector CD4 T Cells Can Be Separated into TCF1-high Blimp1-low TFH and TCF1-low Blimp1-high Th1 Cells

During viral and intracellular bacterial infections, effector CD4 T cells can be divided into CXCR5-high Blimp1-high TFH and CXCR5-low Blimp1-high Th1 cells before the initiation of GCs (Choi et al., 2011, 2013; Pepper et al., 2011). However, it remains unclear how early these two lineages start to diverge. To address this, we used SMARTA CD4 T cells, which express a transgenic T cell receptor (TCR) that recognizes the GP66-77 epitope on lymphocytic choriomeningitis virus (LCMV), crossed to a Blimp1-YFP reporter to track Blimp1 expression during the response to LCMV (Fooksman et al., 2014; Oxenius et al., 1998). Strikingly, bimodal expression of Blimp1 was observed as early as day 1.5 post-infection (p.i.) (Figures 1A, S1A, and S1B), when SMARTA cells had only undergone the first few cell divisions (Figure 1B). Interestingly, at this early time point, CXCR5 was expressed by both Blimp1-high and Blimp1-low cells (Figure 1A). However, after day 2 p.i., Blimp1-high SMARTA cells started to express lower levels of CXCR5 than their Blimp1-low counterparts (Figures 1A and S1B). Accordingly, analysis of splenic sections from mice transferred with Blimp1-YFP reporter SMARTA cells revealed that both TFH (Blimp1-high) and Th1 (Blimp1-low) SMARTA cells could be found in splenic B cell follicles on day 2 p.i. (Figure S1D). In contrast, by day 3 p.i., most SMARTA cells in B cell follicles were Blimp1-low TFH cells, consistent with the lower expression of CXCR5 in Th1 cells (Figure S1E).

To further characterize the differentiation program of early TFH and Th1 cells, we looked for transcription factors that were differentially expressed between these cells. Based on our unpublished RNA sequencing studies and previously published data (Choi et al., 2013), TFH cells express much higher levels of Tcf7 transcripts than their Th1 counterparts. To evaluate this further, we analyzed the kinetics of expression of TCF1, the protein encoded by Tcf7, at a single-cell level early after LCMV infection by flow cytometry (Figures 1A, 1B, S1A, and S1C). SMARTA T cells could be readily separated into TCF1-high and TCF1-low populations starting from day 2 p.i., likely as a result of the gradual downregulation of TCF1 (which is expressed in naive cells) in a subset of SMARTA cells (Figures 1A and S1A). Notably, whereas TCF1-high SMARTA cells maintained high CXCR5, TCF1-low SMARTA cells expressed less CXCR5 after day 2 p.i. (Figures 1A and S1C). In addition, there was a clear inverse...
correlation between TCF1 and Blimp1, as well as a positive correlation between TCF1 and Bcl6 (Figures 1A and S1F), suggesting that TCF1 can be used to distinguish TFH cells from Th1 cells. Indeed, TCF1high SMARTA cells were almost exclusively found in the CXCR5highBlimp1low TFH subset, while TCF1low cells were primarily in the CXCR5lowBlimp1high Th subset (Figure S1G).

IL-2 signaling suppresses TFH development, and early Th1 cells express more CD25, the high-affinity IL-2 receptor, than early TFH cells (Ballesteros-Tato et al., 2012; Choi et al., 2011; Johnston et al., 2012; Pepper et al., 2011). We found that, while most SMARTA cells showed substantial CD25 expression on day 1.5 p.i., TCF1high TFH cells downregulated CD25 much faster than TCF1low Th1 cells (Figure S1F). We also found that expression of Tim3, a co-inhibitory receptor expressed by exhausted CD8 T cells (Jin et al., 2010; Sakushi et al., 2010), correlated well with Blimp1 (Figure S1F). We, therefore, used Tim3 as an early Th1 marker when the Blimp1-YFP reporter was not available.

To determine whether the dichotomy of Blimp1 and TCF1 expression between TFH and Th1 was maintained later during infection when TFH cell responses rely on antigen presentation by GC B cells (Baumjohann et al., 11; Choi et al., 2011), we examined their expression in SMARTA cells 1 week after LCMV infection. As expected, CXCR5high TFH cells mostly expressed low levels of Blimp1 (Figures 1C and S1H) and SLAM (Figure S1t; Johnston et al., 2009). Strikingly, TCF1 still negatively correlated with Blimp1 expression; co-staining of TCF1 and Blimp1 clearly separated SMARTA into two populations (Figures 1C and S1H). Furthermore, TCF1high cells were again primarily in the CXCR5highBlimp1low TFH population, while the CXCR5lowBlimp1high cells contained mostly TCF1low cells (Figure S1J) in which TCF staining was only slightly higher than that in B cells, which do not express TCF1 (Staal and Clevers, 2000; Figure 1C). Tcf7 encodes multiple isoforms as a result of alternative splicing and dual promoter usage (Van de Wetering et al., 1996). The flow cytometry antibody we used binds to the N-terminal β-catenin-binding domain and, therefore, only recognizes the long isoforms. To further assess TCF1 expression, we sorted TFH and Th1 SMARTA cells on day 3 p.i. and day 8 p.i., and we evaluated TCF1 protein levels by immunoblots using an antibody that recognizes all isoforms. While TFH cells from both time points expressed multiple isoforms with or without the β-catenin-binding domain, expression of all TCF1 isoforms was very low in Th1 cells, particularly on day 3 p.i. (Figure 1D). Thus, TCF1 protein levels are high in TFH but low in Th1 cells in both pre-GC phase and GC phase of the immune response to LCMV.

**Tcf7 Is Repressed by IL-2 and Blimp1**

The bifurcation in Tcf7 expression between TFH and Th1 cells prompted us to investigate which signals induce the loss of Tcf7 in Th1 cells. IL-2 signaling suppresses Tfh differentiation and Bcl6 expression in activated CD4 T cells (Ballesteros-Tato et al., 2012; Johnston et al., 2012; Oestreicher et al., 2012). The fact that TCF1lowTh1 cells expressed more CD25, the high-affinity IL-2 receptor, than TCF1high TFH cells (Figure S1F) suggested IL-2 might suppress Tcf7. To examine this possibility, we monitored the effect of IL-2 on in-vitro-activated CD4 T cells. We stimulated CD4 T cells with anti-CD3ε and CD28 for 3 days, then washed and cultured cells with or without IL-2 for 1 additional day. CD4 T cells receiving IL-2 expressed higher surface CD25, consistent with the role of IL-2 in promoting CD25 expression. Moreover, these cells expressed 50% less TCF1 than cells cultured without IL-2 (Figure 2A). Thus, differential IL-2 signals between TFH and Th1 cells may contribute to differences in TCF1 levels.

A previous study suggested that IL-2 suppresses Tfh differentiation via Blimp1 (Johnston et al., 2012). The inverse correlation between TCF1 and Blimp1 expression suggested that Blimp1 might also selectively repress the Tcf7 locus in Th1 cells. To examine this possibility, we transduced SMARTA cells with retroviral vectors expressing a scrambled small hairpin RNA (shRNA) or shRNA targeting Prdm1, which encodes Blimp1, and we analyzed the effect on TCF1 levels in Th1 cells. Knockdown of Blimp1 caused an increase in TCF1 expression in CXCR5lowCD25high Th1 SMARTA cells on day 3 p.i., compared to untransduced or control-vector-transduced Th1 SMARTA cells (Figure 2B). Interestingly, the region 30 kb upstream of the Tcf7 transcription start site (TSS) (Figure S2) contains evolutionarily conserved sequences with Blimp1-binding motifs (GAAAAG) (Kuo and Calame, 2004). This region is bound by TCF1 itself, as well as other transcription factors based on published chromatin immunoprecipitation sequencing (ChIP-seq) data from different cell types, and has been shown to regulate Tcf7 transcription (Germar et al., 2011; Steinke et al., 2014). Indeed, using ChIP assays, we found that Blimp1 physically interacted with this region in TFH and Th1 SMARTA cells (Figure 2C); however, the binding of Blimp1 was significantly stronger in Th1 cells than in TFH cells, consistent with the different abundance of Blimp1 in the two populations. We also observed more H3 K4 trimethylation (H3K4me3), which is associated with active transcription (Jenuwein and Allis, 2001), in this region in TFH cells than in Th1 cells, while more H3 K27 trimethylation (H3K27me3), which is linked to transcriptional repression (Cao et al., 2002), was found in Th1 cells than in TFH cells (Figure 2D). Furthermore, the ~30-kb region increased luciferase activity 2-fold relative to a control luciferase construct; however, overexpression of Blimp1 repressed the enhancer activity of this region (Figure 2E). Thus, Blimp1 may suppress Tcf7 expression in Th1 cells.

**Tcf7 Deficiency Causes a Severe Defect in the TFH Cell Response to LCMV**

The high expression of TCF1 in TFH cells suggested a potential role of TCF1 in TFH cell development. Tcf7 is a key player in multiple steps during T cell development (Verbeek et al., 1995; Weber et al., 2011). To study the immune response of mature T cells, we bred mice carrying a conditional Tcf7 allele to CD4-Cre mice, which initiate Cre activity in DP thymocytes (Steinke et al., 2014), to generate Tcf7 conditional (cKO) mice. Cre-mediated deletion caused more than a 10-fold reduction in TCF1 in CD4 T cells (Figure S3A). Although naive cKO mice had lower CD4 T cell frequencies than wild-type (WT) controls, frequencies of CD44high CD4 T cells were comparable (Figure S3B). We then infected cKO and WT mice with LCMV and analyzed LCMV-specific CD4 T cell responses using the GP66–77 IAb tetramer on day...
10 p.i. We used CXCR5, PD1, and Bcl6 to stain TFH cells and SLAM to stain Th1 cells. While the ratio of CXCR5^high^SLAM^low^ TFH to CXCR5^low^SLAM^high^ Th1 tetramer^+^ CD4 T cells was close to 1 in WT mice, the frequency of TFH cells among tetramer^+^ cells in cKO mice was markedly decreased (Figure 3A). We also observed a substantial loss of tetramer^+^ CXCR5^high^PD1^high^ and CXCR5^high^Bcl6^high^ GC-TFH populations in cKO mice. Accordingly, the number of GP66-77-specific TFH cells in the spleens of cKO mice was reduced more than 6-fold (Figure 3B). In contrast, the numbers of GP66-77-specific Th1 cells were not significantly different between cKO and WT mice. TCF1 levels in GP66-77-specific CD4 T cells were substantially decreased, even in the remaining CXCR5^+^ TFH cells, suggesting that the small fraction of TFH cells in cKO mice was not likely caused by incomplete recombination (Figure S3C). Moreover, defective TFH cell responses in cKO mice were not unique to one epitope, as the frequencies of TFH cells among total CD44^high^ CD4 T cells and total TFH counts in cKO spleens also were reduced (Figure 3C). Consistent with the role of TFH cells in supporting humoral responses and GC formation (Crotty, 2011), the numbers of GC (GL7^high^FAS^high^) B cells and activated (IgD^low^FAS^high^) B cells in the cKO mice were less than half of those in WT mice (Figure 3D).

To rule out that loss of TCF1 during thymocyte development compromised TFH differentiation in cKO mice, we crossed the conditional Tcf7 to ERT2-Cre mice, which activate Cre by tamoxifen treatment (Seibler et al., 2003). By treating the inducible Tcf7 knockout mice (iKO) with tamoxifen for several days immediately prior to LCMV infection, we ensured that iKO T cells had undergone thymic development similar to their ERT2-Cre^+^ counterparts (data not shown). Similar to cKO mice, TFH cell responses in tamoxifen-treated iKO mice on day 10 p.i. were severely compromised compared to vehicle-treated counterparts, confirming that Tcf7 is critical for TFH cell responses during LCMV infection (Figure 3E).

**TFH Cells Require TCF1 in a CD4 T-Cell-Autonomous Manner**

In the above experiments, TCF1 was deleted in both CD4 and CD8 T cells in cKO and iKO mice. To determine whether defective TFH cell responses in Tcf7 KO mice were intrinsic to CD4 T cells, we crossed Tcf7 cKO mice to SMARTA mice. Congenically marked naive CD4 T cells from WT and cKO SMARTA mice were isolated, mixed at 1:1 ratios, and adoptively transferred to WT CD45.1 mice, which were subsequently infected with LCMV (Figure 4A). By mixing WT and cKO SMARTA cells, we could compare the two within each recipient exposed to the same exact conditions and determine their phenotype with higher accuracy and sensitivity. TFH cell differentiation involves an early B-cell-independent phase and a subsequent B-cell-dependent phase (Baumjohann et al., 2011; Choi et al., 2011). To investigate the impact of Tcf7 deficiency on each phase of TFH differentiation, we monitored CD4 T cell responses on days 3 and 8 p.i. On day 3 p.i., loss of TCF1 preferentially reduced TFH cell responses as evidenced by the decreased frequencies of CXCR5^high^Tim3^low^ as well as CXCR5^high^Bcl6^high^ cells among cKO SMARTA cells (Figures 4B, 4C, and S4B). Tcf7 deficiency also caused a reduction in the number of Th1 (CXCR5^low^Tim3^high^) SMARTA cells and, to a lesser extent, the number of Th1 (CXCR5^low^Tim3^low^) SMARTA cells.
SMARTA cells (Figure S4C). We then monitored the differentiation of SMARTA cells at the peak of the immune response (day 8 p.i.) and observed an even more profound loss of TFH cells in cKO SMARTA cells compared to that on day 3 p.i. The frequencies of CXCR5 high SLAM low cells (Figure 4D) and CXCR5 high Bcl6 high cells (Figures 4E and S4E) were 4- to 5-fold lower in cKO cells than those in their WT counterparts. Similarly, there was close to a 10-fold reduction in the numbers of cKO T FH cells compared to those of WT, yet no significant difference between the numbers of cKO and WT Th1 cells (Figure S4F).

To delete TCF1 only in naive SMARTA cells, we generated Tcf7 iKO SMARTA mice and transferred mixed naive SMARTA CD4 T cells from iKO SMARTA mice and their ERT2-Cre counterparts (WT SMARTA) to WT recipients, which then were treated with tamoxifen followed by LCMV infection. Similar to cKO SMARTA cells, induced deletion of TCF1 preferentially affected T FH responses. Both the frequencies and numbers of CXCR5 high Tim3 low or CXCR5 high Bcl6 high iKO SMARTA cells on day 3 p.i. were lower than their WT counterparts (Figures S4H–S4J). Again, on day 8 p.i., we observed greater reductions in the frequencies and numbers of TFH (CXCR5 high SLAM low or CXCR5 high Bcl6 high) cells (Figures 4F, 4G, and S4L) within the iKO population, yet Th1 cell numbers were more similar to WT (Figure S4M). Together, our results demonstrate a cell-intrinsic requirement for TCF1 for viral-specific T FH cell responses.

Finally, to further evaluate the capacity of Tcf7-deficient CD4 T cells to support humoral responses, we adoptively transferred equal numbers of WT or iKO SMARTA CD4 T cells separately into SAP KO mice, which cannot generate GC responses because of defects in CD4 help (Crotty et al., 2003; Qi et al., 2008), treated the chimeras with tamoxifen, and then infected them with LCMV. On day 11 p.i., B cell responses were determined by the numbers of GC (GL7 high FAS high) and activated (IgD low FAS high) B cells. In SAP KO mice that received iKO SMARTA cells, the numbers of both GC and activated B cells were lower than in those that received WT cells (Figure 4H). Thus, T-cell-intrinsic defects caused by the loss of TCF1 led to a compromised humoral response.
**Tcf7** Deficiency Directly Compromises T<sub>FH</sub> Cell Expansion

To determine how Tcf7 deficiency affects T<sub>FH</sub> cells, we examined the proliferative capacity of cKO SMARTA cells, as determined by their incorporation of bromodeoxyuridine (BrdU). Interestingly, loss of TCF1 caused a reduction in BrdU incorporation in T<sub>FH</sub>, but not Th1, cKO SMARTA cells on day 5 p.i., at the middle of the clonal expansion phase (Figure S4N). Moreover, cKO T<sub>FH</sub> cells showed slightly stronger annexin V staining than their WT counterparts, suggesting that cKO T<sub>FH</sub> cells may be more prone to apoptosis (Figure S4O). To directly compare the ability of Tcf7-deficient and -sufficient T<sub>FH</sub> cells to expand, we sorted WT and iKO T<sub>FH</sub> (CXCR5<sup>high</sup>Tim3<sup>low</sup>) and Th1 (CXCR5<sup>low</sup>Tim3<sup>high</sup>) SMARTA cells on day 3 p.i., mixed equal numbers of WT and iKO T<sub>FH</sub> or WT and iKO Th1 SMARTA cells, and transferred the mixed T<sub>FH</sub> or Th1 cells into infection-matched recipients. Four days post-transfer (day 7 p.i.), 80% of the progeny from both WT and iKO donor T<sub>FH</sub> cells stayed as T<sub>FH</sub> cells (CXCR5<sup>high</sup>SLAM<sup>low</sup>), while a slightly higher percentage of progeny from iKO donor Th1 cells stayed as Th1 cells than those from WT donor Th1 cells (Figures S4P and S4Q). However, the numbers of progeny from iKO donor T<sub>FH</sub> cells were only half the numbers from WT donor T<sub>FH</sub> cells, suggesting that Tcf7 deficiency indeed compromised T<sub>FH</sub> cell expansion.

![Diagram of Tcf7 deficiency affecting T<sub>FH</sub> cell expansion](image-url)
the expansion of the transferred T\textsubscript{FH} cells, likely as a result of reduced proliferation and/or survival.

**Transcriptomic Analyses of Tcf7-Deficient T\textsubscript{FH} and Th1 Cells**

To gain further insights into the properties of Tcf7-deficient T\textsubscript{FH} and Th1 cells as well as the molecular pathways regulated by TCF1 in these cells, we set up adoptive transfers and infections as described in Figure 4A, and we performed microarray experiments to profile the transcriptomes of WT and cKO T\textsubscript{FH} and Th1 SMARTA cells sorted from the same mice on day 8 p.i. As the proportion of T\textsubscript{FH} cells was much lower among cKO SMARTA cells than their WT counterparts, we compared the transcriptomes between WT and cKO cells within the T\textsubscript{FH} or Th1 subset in order to filter out genes that were differentially expressed simply due to the reduced T\textsubscript{FH}:Th1 ratio caused by Tcf7 deficiency. We first generated T\textsubscript{FH} and Th1 signature gene sets by listing genes that were $\geq$ 2-fold (p < 0.05) more expressed in day 8 p.i. WT T\textsubscript{FH} cells than day 8 p.i. WT Th1 cells or vice versa (Table S1). Then, we used gene set enrichment analysis (GSEA) (Subramanian et al., 2005) to determine whether these signatures were enriched in cKO cells or WT cells. Strikingly, day 8 p.i. cKO SMARTA T\textsubscript{FH} cells exhibited reduced T\textsubscript{FH} gene expression signatures and increased Th1 signatures compared to WT SMARTA T\textsubscript{FH} cells (Figure 5A).

To better understand pathways affected by Tcf7 deficiency, we performed GSEA to compare the gene signatures of cKO and WT T\textsubscript{FH} cells using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and reactome-curated pathway databases. cKO T\textsubscript{FH} cells showed significantly reduced signatures related to gene expression (e.g., basal transcription factors, spliceosome, and aminoacyl-tRNA biosynthesis), DNA repair (e.g., nucleotide excision repair), and cell cycle and metabolism (e.g., valine, leucine, and isoleucine degradation; pentose phosphate pathway; fatty acid metabolism; and citrate cycle) (Tables S2 and S3). The reduced signatures related to branched-chain amino acid degradation, fatty acid metabolism, and citrate cycle in cKO T\textsubscript{FH} relative to WT T\textsubscript{FH} cells determined by GSEA using KEGG-curated pathway database is shown. (C–F) Experiments were set up as in Figure 4A. (C) Comparison of mitochondrial mass between day 8 WT T\textsubscript{FH} (CXCR5\textsuperscript{high}SLAM\textsuperscript{low}, solid line) and WT Th1 (CXCR5\textsuperscript{low}SLAM\textsuperscript{high}, shaded) SMARTA cells, as determined by MitoTracker Green FM, is shown. (D) Mitochondrial membrane potential of day 8 WT T\textsubscript{FH} (solid line) and WT Th1 (shaded) SMARTA cells, as determined by the MFI of DiOC6(3) staining, is shown. (E) Mitochondrial membrane potential of day 8 WT T\textsubscript{FH} and Th1 cKO (red) and WT (blue) SMARTA cells is shown. Data in (C)–(F) are from single experiments (n = 5) representative of two independent experiments. Statistical significance in (C)–(F) was determined by paired t tests. Each line represents data from a single mouse.

**Figure 5. Loss of TCF1 Changes the Transcriptomal and Metabolic Signatures of T\textsubscript{FH} Cells**

(A) Microarray analyses of day 8 T\textsubscript{FH} (CXCR5\textsuperscript{high} SLAM\textsuperscript{low}) and Th1 (CXCR5\textsuperscript{low}SLAM\textsuperscript{high}) cKO and WT SMARTA cells isolated by cell sorting from co-transfer experiments set up as in Figure 4A. Genes expressed $\geq$ 2-fold (p < 0.05) in WT T\textsubscript{FH} cells than in WT Th1 cells were listed as the T\textsubscript{FH} gene set; and those $\geq$ 2-fold (p < 0.05) expressed in WT Th1 cells were listed as the Th1 gene set (Table S1). Enrichment of gene sets in cKO relative to WT T\textsubscript{FH} cells were determined by GSEA. Positive enrichment scores (ESs) indicate enrichment in cKO T\textsubscript{FH} cells; negative ESs indicate enrichment in WT T\textsubscript{FH} cells.

(B) Enrichment of gene sets related to branched-chain amino acid degradation, fatty acid metabolism, and citrate cycle in cKO T\textsubscript{FH} relative to WT T\textsubscript{FH} cells determined by GSEA using KEGG-curated pathway database is shown.

(C)–(F) Experiments were set up as in Figure 4A. (C) Comparison of mitochondrial mass between day 8 WT T\textsubscript{FH} (CXCR5\textsuperscript{high}SLAM\textsuperscript{low}, solid line) and WT Th1 (CXCR5\textsuperscript{low}SLAM\textsuperscript{high}, shaded) SMARTA cells, as determined by MitoTracker Green FM, is shown. (D) Mitochondrial membrane potential of day 8 WT T\textsubscript{FH} (solid line) and WT Th1 (shaded) SMARTA cells, as determined by the MFI of DiOC6(3) staining, is shown. (E) Mitochondrial membrane potential of day 8 WT T\textsubscript{FH} and Th1 cKO (red) and WT (blue) SMARTA cells is shown. Data in (C)–(F) are from single experiments (n = 5) representative of two independent experiments. Statistical significance in (C)–(F) was determined by paired t tests. Each line represents data from a single mouse.
we also observed that mitochondrial mass was reduced in cKO SMARTA TFH cells and to a lesser extent in cKO SMARTA Th1 cells (Figure 5D). The intensity of DiOC6(3) staining in cells can be used to measure mitochondrial membrane potential, an indicator of mitochondrial function (Rottenberg and Wu, 1998). Again, we found that WT TFH cells showed stronger DiOC6(3) staining than WT Th1 cells (Figure 5E). However, both TFH and Th1 cKO SMARTA cells showed less DiOC6(3) staining than their WT counterparts (Figure 5F). Together, our data suggest that TFH cells exhibit metabolic differences from Th1 cells and, furthermore, that Tcf7-deficient TFH cells lose these characteristic properties of TFH cells. Thus, TCF1 appears to be required to generate and/or maintain TFH identity during viral infection.

**TCF1 Negatively Regulates the Expression of CD25 and Blimp1**

Among the genes upregulated in cKO TFH cells (Table S4) were Prdm1 and Il2ra, which encode Blimp1 and CD25, two proteins involved in pathways that suppress TFH cell differentiation. qRT-PCR results confirmed that Prdm1 and Il2ra transcripts in Tcf7-deficient TFH cells were higher than those in WT TFH cells on day 3 as well as day 8 p.i. (Figures 6A and 6B). The elevated Il2ra transcript and surface CD25 levels (Figure S5A) in Tcf7-deficient TFH cells suggested a potential increase in IL-2 responsiveness. To address this, we cultured WT and cKO SMARTA cells isolated on day 5 p.i. with or without IL-2 and measured IL-2 signaling by staining phosphorylated STAT5 (pSTAT5). The pSTAT5 levels were higher in TFH cKO SMARTA cells than in their WT counterparts (Figure 6C), indicating elevated IL-2 signaling in TFH cKO cells.

Published TCF1 ChIP-seq data from both thymocytes (Germar et al., 2011) and mature CD8 T cells (Steinke et al., 2014) indicate the presence of TCF1-binding peaks in the third intron of Prdm1 as well as the first intron of Il2ra and a region 23 kb upstream of Il2ra TSS (Figures S5B–S5D); the region in the third intron of Prdm1 has been shown to be important for Prdm1 expression in other cell types (Tunyaplin et al., 2004). To test whether TCF1 physically interacts with these regions in viral-specific CD4 T cells, we performed TCF1 ChIP experiments on sorted TFH and Th1 SMARTA cells. Strikingly, TCF1 bound strongly to all three regions in TFH cells, but not in Th1 cells (Figure 6D). Moreover, these TCF1-binding sites showed extensive H3K27me3 modification, associated with transcriptional
repression (Cao et al., 2002), in T_{FH} cells, but not in Th1 cells (Figure S5E). Thus, TCF1 can bind Prdm1 and Il2ra in T_{FH} cells, and loss of TCF1 leads to abnormal upregulation of these genes in T_{FH} cells.

Rescue of Tcf7-Deficient Phenotype by Enforced Expression of Bcl6

Our results show that TCF1 in T_{FH} cells suppressed the expression of Prdm1 (Figure 6). Given that Blimp1, encoded by Prdm1, can be antagonized by Bcl6 (Crotty, 2011), we reasoned that overexpression of Bcl6 might rescue the defective T_{FH} cell differentiation caused by loss of TCF1. To test this hypothesis, we overexpressed Bcl6 in WT or cKO SMARTA cells through retroviral transduction (Figure 7A). On day 8 p.i., enforced expression of Bcl6 rectified the defective T_{FH} cell differentiation in cKO SMARTA cells, greatly increasing the T_{FH} frequency of cKO SMARTA cells (Figure 7B). Thus, TCF1 acts upstream of the Bcl6-Blimp1 axis to regulate T_{FH} cell responses.

DISCUSSION

Upon viral or intracellular bacterial infections, effector CD4 T cells differentiate into Th1 cells, which protect the host through secreted cytokines such as IFNγ and TNFα and/or direct lysis of infected cells, and T_{FH} cells, which provide help for B cell responses (Choi et al., 2011; Crotty, 2011; Pepper et al., 2011; Swain et al., 2012). The differentiation of T_{FH} and Th1 cells occurs before GC initiation and depends on DC priming and ICOS-ICOSL interactions between activated CD4 T cells and DCs (Choi et al., 2011; Pepper et al., 2011). However, the molecular mechanisms that govern and maintain the balance of these two distinct subsets are still unclear. TCF1 is critical for thymic T cell development as well as Th2 polarization and the development of memory CD8 T cells during immune responses (Weber et al., 2011; Yu et al., 2009b; Zhou et al., 2010). Here we show that viral-specific T_{FH} cells maintain high levels of TCF1, while viral-specific Th1 cells downregulate TCF1 early after infection. We further demonstrate that both IL-2 and Blimp1 lead to TCF1 downregulation. Importantly, TCF1 was intrinsically required for robust antiviral T_{FH} cell responses as well as T cell help to B cells: in the absence of TCF1, we observed decreased numbers of T_{FH} cells and GC B cells, and the remaining Tcf7-deficient T_{FH} cells exhibited reduced T_{FH} cell transcriptional and metabolic signatures. We provide evidence that TCF1 also acts upstream of the Blimp1-Bcl6 axis and suppresses expression of both Prdm1 and Il2ra, the products of which suppress T_{FH} differentiation.

Previous studies have shown that induction of Bcl6 and CXCR5 is associated with early T_{FH} cells, while upregulation of Blimp1 and CD25 is associated with early Th1 cells (Baumjohann et al., 2011; Choi et al., 2011; Pepper et al., 2011). However, the precise timing and details of the mechanisms involved in the commitment of the two lineages remain obscure. In this study, we demonstrate that the pre-GC phase of effector CD4 T cell differentiation can be further divided into two stages. In the first stage, viral-specific CD4 T cells differentiate into either TCF1^{high}Blimp1^{low} T_{FH} or TCF1^{low}Blimp1^{high} Th1 cells. The two subsets expressed similar levels of CXCR5 and both could be found in B cell follicles at this stage. In the second stage, Th1 cells lost CXCR5 expression and were now excluded from B cell follicles. Interestingly, early T_{FH} cells downregulated CD25 rapidly and expressed high levels of Tcf7, closely resembling early memory precursor CD8 T cells (Arsenio et al., 2014; Locci et al., 2013). It is interesting to speculate that TCF1 may be a component in this shared signaling network, given its critical role in the development of memory CD8 T cells (Zhou et al., 2010).
Indeed, loss of TCF1 caused a profound defect in the TFH cell response to viral infection, as shown here and by two other recent studies (Choi et al., 2015; Xu et al., 2015). It is worth noting, however, that, in our hands, the loss of TFH cells in Tcf7−/− SMARTA cells on day 3 p.i. was less dramatic than that on day 8 p.i. It is possible that, while TCF1 is required for optimal TFH cell responses prior to GC formation, a redundant mechanism, such as the expression of the related transcription factor LEF1, may compensate for Tcf7 deficiency during TFH differentiation (Choi et al., 2015). However, we also found clear evidence for both reduced proliferation and reduced cell survival of TCF1−/− TFH cells between day 3 and day 7 p.i., which is a major part of the clonal expansion phase. Thus, TCF1 may be required for the full expansion of TFH cells, which may account for the differences in the extent of TFH defects between day 3 and day 8 p.i. It also should be noted that, although we have not found evidence of conversion from Tcf7−/− TFH cells to Th1 cells after tracking them for several days in infection-matched mice, we did see in Tfh expression gene and metabolic signatures in the absence of TCF1. TCF1 can also suppress expression of the Th1 cytokine IFNγ. Thus, TCF1 may be critical both for Tfh expansion/survival and for the generation and maintenance of TFH identity in the face of strong Th1-inducing signals, as seen in viral infections. In this respect, it is of interest that we have not seen a requirement for TCF1 for TFH responses during protein immunization with alun as an adjuvant, which is considered a Th2-inducing condition (T.W., unpublished data).

Previous studies have suggested that signaling mediated by CD25 and Blimp1, which are abundantly expressed in early Th1 cells, inhibit Tfh cell differentiation (Choi et al., 2011; Johnston et al., 2012; Pepper et al., 2011). Our ChIP and gene expression profiles of Tcf7−/− TFH cells suggest that TCF1 potentially represses expression of Pdlim1 and Il2ra. Indeed, CD25 expression was lost much faster in TFH cells than in Th1 cells early after infection. Given the high levels of TCF1 in TFH cells, TCF1 may contribute to their rapid loss of CD25. Furthermore, since IL-2 signaling increases CD25 expression, this could amplify IL-2 signaling in Th1 cells, generating a positive feedback loop that may contribute to the difference in CD25 expression between the two subsets. It is of note that two recent papers have found that loss of TCF1 or both TCF1 and LEF1 led to reduced Bcl6 as well as increased Blimp1 expression in Tfh cells (Choi et al., 2015; Xu et al., 2015). While we did not observe decreased Bcl6 expression, we did find that overexpression of Bcl6 rescues Tfh cell generation in the absence of TCF1, confirming that TCF1 acts upstream of the Blimp1/Bcl6 axes. Moreover, we found that TCF1 not only represses Blimp1 and CD25 expression, but also is repressed by Blimp1 and IL-2, suggesting that TCF1 is a critical component of negative feedback loops with IL-2 and Blimp1 that may regulate the differentiation and maintenance of TFH cells during viral infections.

Upon antigen encounter, T cells switch from oxidative phosphorylation to aerobic glycolysis, a pattern that is enforced in Th1 cells by IL-2 (Maclver et al., 2013; Oestreich et al., 2014). However, the metabolic profile of TFH cells remains largely unknown. We found that TFH cells have greater mitochondrial mass than Th1 cells. An enhanced mitochondrial mass also was observed in memory T cells, which is correlated with elevated fatty acid oxidation (van der Windt et al., 2012). Interestingly, our gene expression profiling data showed that Tcf7−/− TFH cells had reduced gene expression signatures related to branched amino acid degradation, fatty acid degradation, and the citrate cycle, suggesting reduced oxidative metabolism. Consistent with these findings, the loss of TCF1 led to reduced mitochondrial mass and function in TFH cells. Thus, TCF1 may contribute to the regulation of TFH cell metabolism and promote a more oxidative metabolic profile, although additional studies will be necessary to determine potential targets of TCF1 that contribute to these processes and whether they influence TFH cell differentiation.

In summary, our study has demonstrated that the distinct high levels of TCF1 expression distinguish TFH cells from Th1 cells and are critical for the development of viral-specific TFH cells. In addition, we have identified potential negative feedback loops linking TCF1 to IL-2 and Blimp1. Our findings unveil an essential role of TCF1 in contributing to the balance between immune responses mediated by two major CD4 subsets during viral infection and may help shed light on pathways important for the development of vaccines and immune therapies targeting viral infections.

**EXPERIMENTAL PROCEDURES**

**Mice and Infections**

Tcf7 conditional mice (Tcf7<sup>Fm/1aEUCCOMM/Hsd</sup>, Institut Clinique de la Souris) were crossed to Fip Deleter (T089, Taconic) and either CD4-Cre (<sup>Lee</sup>, et al., 2001) or ERT2-Cre (Seibler et al., 2003) (Taconic) to generate TCF1 recognition the LCMV GP66-77 epitope (Oxnium et al., 1990). Blimp1-YFP mice (Fooksman et al., 2014) were crossed to SMARTA mice. Other mouse strains, in vitro activation, retroviral transduction, flow cytometry, microscopy, RNA and protein analyses, ChIP, and luciferase assays are described in the Supplemental Experimental Procedures.

For adoptive transfers, 10<sup>6</sup> (for days 1.5, 2, and 3) or 10<sup>5</sup> (for other time points) SMARTA CD4 T cells were transferred to recipient mice, unless indicated. Mice were intravenously (i.v.) injected with 2 × 10<sup>5</sup> (for days 1.5, 2, and 3) or 2 × 10<sup>6</sup> (for other time points) plaque-forming units (PFUs) of LCMV Armstrong. For ERT2-Cre inducible KO mice, 2 mg tamoxifen in corn oil was injected intraperitoneally (i.p.) daily for 3–5 days before LCMV infection. Controls were either Cre<sup>−</sup> mice or mice transferred with ERT2-Cre SMARTA injected with tamoxifen or ERT2-Cre<sup>−</sup> animals injected with vehicle. All animal husbandry and experiments were approved by the NHGRI or NINDS Animal Use and Care Committees.

**Statistical Analysis**

Two-tailed paired or unpaired Student’s t test was performed with GraphPad Prism 6 to calculate p values (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

**ACCESSION NUMBERS**

The accession number for the microarray data reported in this paper is GEO: GSE65660.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.049.

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