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REVIEW

Tcf1 at the crossroads of CD4$^+$ and CD8$^+$ T cell identity

Jodi A. Gullicksrud[1,2], Qiang Shan (✉)[1], Hai-Hui Xue (✉)[1,2]

[1] *Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA*
[2] *Interdisciplinary Immunology Graduate Program, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA*

Abstract **Transcription factors and DNA/histone modification enzymes work in concert to establish and maintain cell identity. CD4$^+$ and CD8$^+$ T cells are key players in cellular immunity with distinct functions. Recent studies offer novel insights into how their identities are established in the thymus and maintained in the periphery during immune responses. During thymic maturation, Thpok, HDAC1 and HDAC2 guard CD4$^+$ T cells from activation of CD8$^+$ cytotoxic genes, and Tcf1 and Lef1 utilize their intrinsic HDAC activity to shut down CD4$^+$ lineage-associated genes in CD8$^+$ T cells. In activated CD4$^+$ T cells, Tcf1 and Lef1 act upstream of the Bcl6-Blimp1 axis to direct differentiation of follicular helper T (Tfh) cells, and prevent diversion of Tfh to IL-17-producing cells. In parallel, T-bet, together with Eomes or Blimp1, ensures proper induction of the cytotoxic program in CD8$^+$ effectors elicited by acute infection, and prevents generation of pathogenic, IL-17-producing CD8$^+$ effector T cells. Antigen persistence due to chronic viral infection leads to CD8$^+$ T cell exhaustion. A portion of exhausted CD8$^+$ T cells has the capacity to activate the Tfh program in a Tcf1-dependent manner. Those Tfh-like CD8$^+$ T cells exhibit enhanced proliferative capacity in response to PD-1 blockage therapy and are more effective in curtailing viral replication. Thus, dissecting the molecular aspects of T cell identity, during development and immune responses, may lead to new therapies for treating autoimmunity, tumors, and persistent infections.**

Keywords Tcf1, Lef1, HDAC, CD4$^+$ T cells, CD8$^+$ T cells, cell identity

CD4$^+$ and CD8$^+$ T cells are functionally distinct lymphocytes that play essential roles in immune defenses against foreign pathogens and transformed cells. They are generated in the thymus following step-wise maturation processes, with CD4$^+$CD8$^+$ double positive (DP) thymocytes as immediate precursors (Yui and Rothenberg, 2014). The mature CD4$^+$ or CD8$^+$ thymocytes exit the thymus and migrate to and populate secondary lymphoid tissues. When presented with cognate antigens by dendritic cells, CD4$^+$ and CD8$^+$ T cells are activated and undergo differentiation to exert effector functions. CD4$^+$ T cells provide versatile help, tailored to specific pathogen types, whereas CD8$^+$ T cells are cytotoxic cells that kill infected or transformed target cells (Williams and Bevan, 2007; Harty and Badovinac, 2008; Zhu et al., 2010). The distinct identities of CD4$^+$ and CD8$^+$ T cells are established after DP thymocytes make the lineage-choice decision in the thymus, and must be properly guarded after activation in the periphery to ensure their distinct functionality.

Cell identity is established by lineage-determining transcription factors, which initiate and sustain expression of cell type-specific genes while repressing those in alternative lineages (Smale, 2003; Natoli, 2010). The TFs then engage epigenetic means to ensure heritable transcriptomes and maintain cell identity. In the lymphoid lineage, Pax5 directs B cell development in the bone marrow and continues to protect B cell identity from dedifferentiation to progenitor-like cells which can give rise to T cells (Cobaleda et al., 2007), and Bcl11b protects thymocytes at all developmental stages from activating the natural killer cell program (Li et al., 2010). Within the T cell lineage, mature CD4$^+$ and CD8$^+$ T cells, either in the thymus or in the periphery, show remarkably similar transcriptomes in spite of their distinct functions (Mingueneau et al., 2013). Therefore, regulation of CD4$^+$ and CD8$^+$ T cell identity must not solely rely on lineage-restricted factors.

Tcf1 and Lef1 (encoded by the *Tcf7* and *Lef1* genes, respectively) are members of the high mobility group (HMG) family transcription factors. In T cells, both factors are expressed in multiple isoforms, all of which contain a highly

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Correspondence: [a]Qiang Shan; [b]Hai-Hui Xue
E-mail: [a]qiang-shan@uiowa.edu; [b]hai-hui-xue@uiowa.edu

conserved C-terminal, HMG DNA binding domain (Fig. 1). The long isoforms contain a unique N-terminal, β-catenin binding domain that interacts with the β-catenin coactivator stabilized by extracellular cues such as Wnt or prostaglandin stimulation. During early T cell development, Tcf1 and Lef1 have well-established roles in promoting T lineage specification, β-selection, and survival (Ioannidis et al., 2001; Germar et al., 2011; Weber et al., 2011; Yu et al., 2012), and these topics have been reviewed elsewhere (Staal and Sen, 2008; Xue and Zhao, 2012; Steinke and Xue, 2014). In this review, we focus on their newly discovered regulatory roles in thymocytes at late stages of maturation and in peripheral T cells responding to infections.

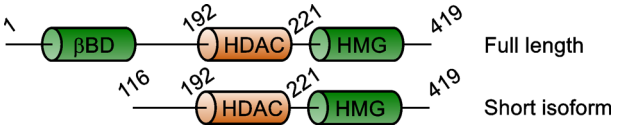

Figure 1 Diagram showing the functional structure of Tcf1 and Lef1 proteins. βBD, β-catenin binding domain; HDAC, histone deacetylase activity domain; HMG, high mobility group DNA binding domain. The numbers denote boundaries of highlighted functional domains based on the full length Tcf1 protein.

CD4$^+$ and CD8$^+$ T cell identity in the thymus

At very early stages of T cell development, thymus-seeding hematopoietic progenitors undergo T cell specification and commitment (Yui and Rothenberg, 2014; De Obaldia and Bhandoola, 2015). Once committed to becoming T cells, they begin to express CD4 and CD8 coreceptors and become DP thymocytes. To ensure proper responsiveness to cognate antigens, DP thymocytes undergo vigorous positive and negative selections. The post-selected DP thymocytes then face a lineage-choice decision of becoming either CD4$^+$ or CD8$^+$ T cells. This lineage-choice process is orchestrated by multiple pathways and transcriptional regulators (Taniuchi and Ellmeier, 2011). At the center are the Thpok transcription factor and Runx1/3-CBFβ complex that have mutually antagonistic roles. Thpok contains multiple copies of zinc-finger DNA binding domains in the C terminus and has a central role in instructing DP thymocytes to the CD4$^+$ T cell fate (Vacchio and Bosselut, 2016). Runx1 and Runx3 both have an N-terminal *Runt* DNA binding domain; and the non-DNA binding cofactor, CBFβ, binds to the Runx factors to enhance DNA binding affinity and stability. The Runx1/3-CBFβ complex is essential in directing DP thymocytes to the CD8$^+$ T cell lineage (Collins et al., 2009). *Thpok* is one of the few genes that show restricted expression in naïve CD4$^+$ T cells. After directing CD4$^+$ lineage commitment, Thpok continues to exert the role of 'constant supervision' of CD4$^+$ T cell identity. Using a newly developed human CD2 promoter-driven Cre recombinase transgene (hCD2-Cre), Vacchio et al. specifically ablated Thpok in mature CD4$^+$ T cells, bypassing the requirement for Thpok in the thymus (Vacchio et al., 2014). In the resting state, Thpok-deficient CD4$^+$ T cells showed variegated expression of both CD4 and CD8 coreceptors, with a portion expressing diminished levels of CD4 and another portion showing CD8 upregulation. After activation *in vitro* or *in vivo*, Thpok-deficient CD4$^+$ T cells showed more pronounced CD8 induction and abundant upregulation of cytotoxic CD8$^+$ lineage-associated molecules, such as granzyme B and Eomes. Significantly, ablating CBFβ prevented aberrant activation of the cytotoxic CD8$^+$ T cell program in Thpok-deficient CD4$^+$ T cells, in both resting and activated states. Thus, Thpok-mediated repression of Runx3/CBFβ remains active beyond the DP thymocyte stage and appears to be a recurring regulatory axis that maintains CD4$^+$ T cells in the helper lineage. Intestinal intraepithelial lymphocytes (IELs) have long-term exposure to luminal antigens, and at least a portion of IEL CD4$^+$ T cells downregulates Thpok, upregulates Runx3, and hence acquires features of cytotoxic CD8$^+$ T cells (Mucida et al., 2013; Reis et al., 2013). This naturally occurring conversion of CD4$^+$ to CD8$^+$ T cells may have beneficial effects, such as avoiding generation of pathogenic IL-17-producing helper T (Th17) cells that cause tissue damage and inflammation in the gut.

Histone modification enzymes, in particular histone deacetylases (HDACs), are critical for regulating T cell identity. Unlike Thpok expression, which is restricted to naïve CD4$^+$ T cells, several HDACs are expressed in both CD4$^+$ and CD8$^+$ T cells. Classical HDACs are divided into three classes: HDAC 1, 2, 3 and 8 comprise class I; HDAC 4-7, 9 and 10 comprise class II; and HDAC11 is the sole member of class VI (Yang and Seto, 2008). Targeting HDAC1 and HDAC2 in thymocytes using CD4-Cre has an impact on CD4$^+$ T cell identity that is remarkably similar to the Thpok ablation discussed above (Boucheron et al., 2014). HDAC1/2-deficient CD4$^+$ T cells showed variegated upregulation of CD8 coreceptor in a resting state, and strong induction of the cytotoxic CD8$^+$ lineage-associated genes including granzyme B, perforin, and Eomes after activation under both T helper neutral (Th0) and T helper 1 (Th1) conditions in vitro. In HDAC1/2-deficient CD4$^+$ T cells, aberrant activation of the cytotoxic CD8$^+$ T cell program also depended on upregulation of Runx3, which was not accompanied by loss of Thpok expression. Although the *Runx3* gene in HDAC1/2-deficient CD4$^+$ T cells was marked with moderately increased lysine 9 acetylation of H3 histone (H3K9Ac), it remains to be determined if HDAC1 and HDAC2 modulate Runx3 expression through H3K27 or H4 histone, or through non-histone substrate(s). *In vitro* co-immunoprecipitation assay showed that Thpok could interact with several HDAC proteins but not HDAC1 or HDAC2 (Rui et al., 2012). These observations suggest that HDAC1/2-mediated Runx3 repression is independent of Thpok and that Thpok and HDAC1/2 may act in parallel to guard CD4$^+$ T cell identity.

We have recently demonstrated that Tcf1 and Lef1 promote

$CD4^+$ T cell lineage choice by acting upstream of Thpok (Steinke et al., 2014), where we used CD4-Cre to conditionally target both factors (CD4-Cre^+*Tcf7*$^{fl/fl}$*Lef1*$^{fl/fl}$, called *Tcf7*$^{-/-}$*Lef1*$^{-/-}$) in thymocytes. We further crossed this line onto a $\beta2m^{-/-}$ background, where MHC class I expression is defective. As expected, in Tcf1/Lef1-sufficient $\beta2m^{-/-}$ mice, very few $CD8^+$ T cells were generated in either immature ($CD24^+CD69^+$) or mature ($CD24^-CD69^-$) $TCR\beta^+$ post-selected thymocytes (Fig. 2A). In contrast, mature $CD8^+$ thymocytes did appear in the absence of Lef1, constituted approximately 50% of all mature thymocyte pool in the absence of Tcf1, and reached > 90% when both factors were ablated (Fig. 2A). These data indicate that, without Tcf1 and/or Lef1, MHC-II-selected $TCR\beta^+$ thymocytes are redirected from $CD4^+$ to the $CD8^+$ lineage. A portion of *Tcf7*$^{-/-}$ or *Tcf7*$^{-/-}$*Lef1*$^{-/-}$ mature $CD8^+$ T cells showed aberrant upregulation of the CD4 coreceptor, owing to a role of Tcf1 and Lef1 in *Cd4* gene silencing in cooperation with Runx3 (Steinke et al., 2014). In *Tcf7*$^{-/-}$ $TCR\beta^+$ post-selected DP thymocytes on a wild-type background, we found Thpok expression is reduced by half (Steinke et al., 2014). Thpok is induced by positively selecting TCR signals, and the induction is more potent by MHC-II- than MHC-I-derived signals (Muroi et al., 2008). In analyzing transcripts from sorted *Tcf7*$^{-/-}$*Lef1*$^{-/-}$$\beta2m^{-/-}$ and control $\beta2m^{-/-}$ post-select DP thymocytes, we found Tcf1 and Lef1 deficiency caused > 10-fold reduction of Thpok in MHC-II-selected DP thymocytes, with concomitant induction of Runx3 transcripts from the $CD8^+$-specific distal promoter (called *Runx3d*, Fig. 2B). These data indicate that Tcf1 and Lef1 are required for proper Thpok induction by TCR signals, and further strengthen our conclusion that Tcf1 and Lef1 act upstream of Thpok to promote the $CD4^+$ T cell-lineage choice.

The regulation of $CD8^+$ T cell identity was not addressed directly until recently. Despite that Tcf1 and Lef1 are not required for $CD8^+$ T cell fate decision, Tcf1/Lef1-deficient $CD8^+$ T cells exhibited variegated expression of not only the CD4 coreceptor but also other $CD4^+$ lineage-associated proteins, including CD40 ligand, Foxp3 and Rorγt; and these gene loci were associated with increased levels of H3K9Ac and H3K27Ac (Xing et al., 2016). Mechanistic studies largely excluded the possibility of direct regulation or recruitment of HDACs and/or histone acetyl transferases (HATs) by Tcf1 and Lef1 in $CD8^+$ T cells, and eventually led to a surprising finding: Tcf1 and Lef1 have intrinsic HDAC activities. For Tcf1, HDAC activity was mapped to a 30-amino acid region located between the β-catenin binding and the HMG DNA binding domains (Fig. 1), as measured with H3K9- and/or H3K27-acetylated synthetic peptides or protein substrates. Reintroducing full-length wild-type Tcf1 (the p45 kDa full-length isoform) into Tcf1/Lef1-deficient $CD8^+$ T cells partly diminished histone acetylation signals at the $CD4^+$ lineage-associated genes and repressed their expression; in contrast, reintroducing a Tcf1 mutant with compromised HDAC activity failed to do so. These data highlight an unexpected requirement for the Tcf1 HDAC activity in establishing the proper histone acetylation landscape and hence the identity of naïve $CD8^+$ T cells.

These new findings suggest that transcription factors, at least a portion of them, have an intrinsic capacity to modify histones. This capacity confers efficiency and enhances flexibility in gene regulation by directly marking target gene loci with proper epigenetic modifications upon DNA binding. Until now, HDAC activity was only found in a set of dedicated proteins, including HDAC1-11 (Yang and Seto, 2008). The sensitivity of Tcf1 HDAC activity to a pan-HDAC inhibitor, Vorinostat, and an HDAC6-seletive inhibitor, Tubacin, is consistent with the structural similarity between the HDAC domains of Tcf1/Lef1 and those of classical HDACs, as projected by homology modeling analysis (Xing et al., 2016). Resolving the crystal structure of Tcf1 HDAC domain will elucidate its distinct folding feature(s) and catalytic center(s) as new members of the HDAC family. It is particularly noteworthy that, although a number of HDACs are expressed in T lineage cells, they cannot compensate for the loss of Tcf1 and Lef1 in histone deacetylation in $CD8^+$ T cells. This consideration highlights the uniqueness of HDAC activity in Tcf1 and Lef1 and/or the requirements for proper positioning of the HDAC activity through direct binding of Tcf1/Lef1 to the target gene loci. In the study by Boucheron et al. (2014), HDAC1/2-deficient $CD8^+$ T cells derepressed Thpok but not CD4 itself; on the other hand, in Tcf1/Lef1-deficient $CD8^+$ T cells, Thpok was only minimally upregulated, unlike other $CD4^+$ lineage-associated genes (Xing et al., 2016). Thus, Tcf1/Lef1 and HDAC1/2 might share 'divided labor' in guarding $CD8^+$ T cell identity. It would be of interest to know if these factors act in synergy in establishing $CD4^+$ T cell identity in the thymus, and if they play a role of 'constant supervision' in maintaining identity of both $CD4^+$ and $CD8^+$ T cells in the periphery.

Th1 and Tfh bifurcation in $CD4^+$ T cell response to acute infection

Mature T cells are activated in secondary lymphoid organs by antigen presenting cells, which capture and process foreign antigens. $CD4^+$ T cells differentiate into distinct helper lineages that coordinately help clear pathogens (Zhu et al., 2010). In the context of acute viral or intracellular bacterial infections, activated $CD4^+$ T cells mount two major types of responses: Th1 responses and follicular helper (Tfh) T cell responses. Th1 cells secrete inflammatory cytokines and migrate from secondary lymphoid organs to inflamed tissues; and Th1 differentiation is directed by the T-bet transcription factor, probably with assistance of Blimp1 (Zhu et al., 2010). On the other hand, Tfh cells upregulate the CXCR5 chemokine receptor, which allows them to migrate into B cell follicles, where they undergo further maturation and help B cells to produce class-switched, high affinity antibodies. Tfh differentiation is directed by the Bcl6 transcription factor

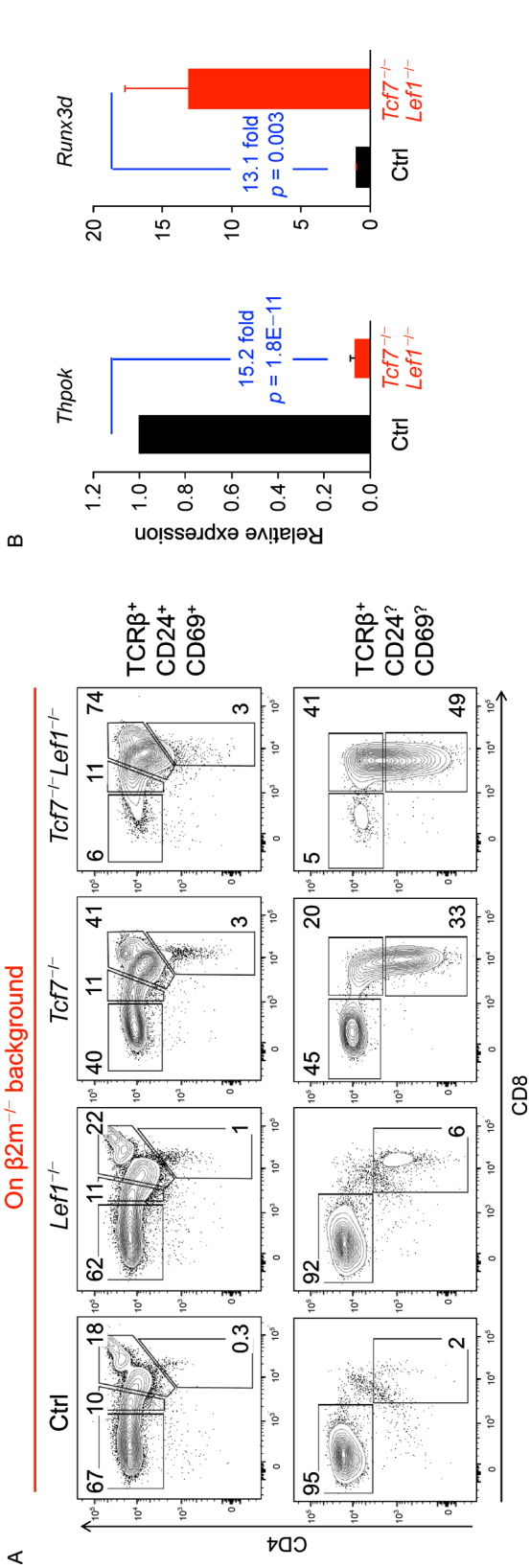

Figure 2 Tcf1 and Lef1 regulate CD4$^+$ lineage choice by acting upstream of Thpok. TCRβ$^+$ post-select thymocytes were analyzed in control, *Tcf7*$^{-/-}$, *Lef1*$^{-/-}$, and *Tcf7*$^{-/-}$*Lef1*$^{-/-}$ mice on β2m$^{-/-}$ background. (A) Immature (top) and mature (bottom) post-select thymocytes were analyzed for CD4 and CD8 expression. Immature cells contained DP, CD4$^+$CD8^{lo} intermediates, CD4$^+$ and CD8$^+$ subsets, and mature cells contain CD4$^+$, CD8$^+$ subsets, and an additional CD4$^+$CD8$^+$ subset in *Tcf7*$^{-/-}$ and *Tcf7*$^{-/-}$*Lef1*$^{-/-}$ mice. Percentage of each subset is marked in representative contour plots from≥3 experiments (n≥5). (B) The expression of *Thpok* and *Runx3* genes in TCRβ$^+$ post-select DP thymocytes sorted from β2m$^{-/-}$ and *Tcf7*$^{-/-}$*Lef1*$^{-/-}$β2m$^{-/-}$ mice, as determined by qRT-PCR. The expression of each gene in β2m$^{-/-}$ control cells is set as 1, and that in *Tcf7*$^{-/-}$*Lef1*$^{-/-}$β2m$^{-/-}$ cells was normalized accordingly. *Runx3d* denotes a *Runx3* transcript from CD8$^+$ lineage-specific distal promoter. Data are means±s.d. from 3 experiments (n = 3 for β2m$^{-/-}$, and n = 6 for *Tcf7*$^{-/-}$*Lef1*$^{-/-}$β2m$^{-/-}$). p values were determined by Student's t-test.

but is antagonized by Blimp1 (Crotty, 2014). Several recent studies give new insight into how Th1 and Tfh identity is established and maintained.

Tcf1 is highly expressed in naïve CD4$^+$ T cells, as marked by a Tcf1-EGFP reporter. In response to infections by lymphocytic choriomeningitis virus (LCMV), vaccinia virus, and *Listeria monocytogenes* bacteria, the Tcf1-EGFP reporter was greatly diminished in Th1 cells, but sustained in Tfh cells (Choi et al., 2015). This expression pattern was independently verified by intracellular staining for Tcf1 protein (Wu et al., 2015; Xu et al., 2015). We used the hCD2-Cre described above to ablate Tcf1 and/or Lef1, specifically in mature CD4$^+$ T cells, to bypass their requirements for thymic development. Whereas Lef1 deficiency showed a modest effect, loss of Tcf1 substantially diminished Tfh differentiation elicited by infection with vaccinia virus, and deleting both factors caused the strongest defects (Choi et al., 2015). Accordingly, without Tcf1 and Lef1, formation of germinal center (GC) B cells and plasma cells was severely compromised. The requirement for Tcf1 or Lef1 in Tfh differentiation in response to viral infection was also established, on a per-cell basis, using short-hairpin RNA-mediated knockdown, CD4-Cre-mediated deletion and estrogen receptor-Cre (ER-Cre)-mediated inducible deletion approaches (Choi et al., 2015; Wu et al., 2015; Xu et al., 2015). Although Tcf1/Lef1 deficiency does not abrogate Tfh differentiation completely, the residual remaining Tcf1-deficient Tfh cells were functionally impaired, and fail to provide efficient B cell help (Wu et al., 2015; Xu et al., 2015).

Further molecular dissection revealed that, in Tfh cells, Tcf1 and Lef1 act upstream of the Bcl6-Blimp1 axis, with Tcf1/Lef1-deficient Tfh cells showing diminished Bcl6 expression and aberrant Blimp1 upregulation (Choi et al., 2015). Tcf1 binds to Bcl6 promoter and a –2.8 kb upstream regulatory region as well as Blimp1 intron 3 and a –24 kb upstream region (Choi et al., 2015; Xu et al., 2015). Some of these regulatory elements were functionally tested for Bcl6 activation and Blimp1 repression, using an ex vivo dual reporter assay system previously developed in our laboratory (Zhou et al., 2010; Xu et al., 2015). It is notable that Tcf1 occupies these regulatory elements in naïve CD4$^+$ T cells, where both Bcl6 and Blimp1 are expressed at low, basal levels. These Tcf1 binding events were preserved in Tfh cells but lost in Th1 cells, suggesting that 1) Tcf1 binding and its continued expression facilitate differentiation of naïve CD4$^+$ T cells to the Tfh lineage, and 2) after CD4$^+$ T cell activation, Tcf1 must cooperate with incoming signals to activate Bcl6 and repress Blimp1. It will be interesting to find whether Tcf1 HDAC activity participates in this process. Recent studies demonstrated that the mTOR kinase critically regulates Tfh differentiation (Yang et al., 2016; Zeng et al., 2016); and mTORC2-deficient Tfh cells exhibited reduced expression of Tcf1. Forced expression of WT Tcf1, but not the Tcf1 HDAC mutant, partly rectified Tfh differentiation defects caused by loss of mTORC2 (Yang et al., 2016), suggesting a requirement for the Tcf1 HDAC activity. Elucidation of the 'yin-yang' regulatory function by Tcf1 awaits future in-depth analysis.

In addition to the Bcl6-Blimp1 axis, Tcf1, together with Lef1, acts on multiple other aspects of the Tfh program, which include promoting optimal expression of Tfh functional cytokines (IL-4 and IL-21), ICOS, IL-6 receptor α and gp130 chains, CXCR5, and the Ascl2 transcription factor (Choi et al., 2015; Xu et al., 2015). In fact, a GSK3β inhibitor, which stabilizes the β-catenin co-activator, was shown to induce CXCR5 and Ascl2 expression (Liu et al., 2014). On the other hand, loss of Tcf1 and Lef1 resulted in upregulation of *Rorc* and *Il17a* transcripts in Tfh cells, suggesting a key role of these factors in regulating Tfh identity and preventing activation of the Th17 program. Tcf1-mediated repression of Th17-associated genes is a recurrently used regulatory circuit, as it has been also observed in thymocytes, Tγδ17 cells, and NKp46$^+$ innate lymphoid cells (Ma et al., 2011; Malhotra et al., 2013; Mielke et al., 2013). In Th1 cells, Blimp1 repressed Tcf1 expression by binding to a –30 kb regulatory region in the *Tcf7* gene (Wu et al., 2015), revealing that Blimp1 and Tcf1 are mutually antagonistic in promoting Th1 *vs.* Tfh differentiation (Fig. 3).

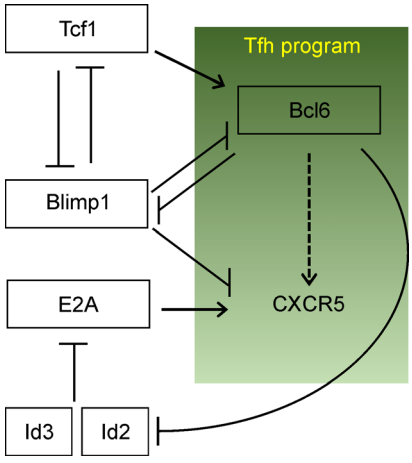

Figure 3 Conserved regulatory circuits upstream of the Tfh program. Regulatory roles of each transcription factor/regulator (in boxes) are observed in both CD4$^+$ Tfh cells (generated in response to acute infection) and Tfh-like CXCR5$^+$CD8$^+$ cells (generated in response to chronic viral infection). Arrows denote positive regulation, lines ending in bars denote negative regulation, and dotted line marks indirect regulation.

E proteins and their inhibitory cofactors, Id proteins, are another group of factors that regulate Th1 and Tfh differentiation. In E proteins (including E2A, HEB, and E2-2), a basic DNA binding region abuts the helix–loop–helix (HLH) domain that allows protein dimerization. The Id proteins (including Id1-Id4) all have an HLH domain but lack a basic region, and they heterodimerize with E proteins and inhibit their binding to DNA (Kee, 2009). Similar to Tcf1, Id3 is highly expressed in naïve CD4$^+$ T cells, sustained in Tfh

but downregulated in Th1 cells, whereas Id2 is strongly induced in Th1 cells but remains expressed at a low level in Tfh cells (Shaw et al., 2016). Loss of Id2 impaired Th1 differentiation; and residual Id2-deficient Th1 (or Thl-like) cells expressed some but not all Tfh-characteristic genes, suggesting Id2 has a role in fully establishing Th1 identity. Although Tfh cells exhibit an Id2^{lo}Id3^{hi} phenotype, deleting Id2 (or Id3) modestly enhanced Tfh differentiation; further increasing Id3 expression by retroviral transduction diminished Tfh cells (Liu et al., 2014; Shaw et al., 2016). These results suggest that balanced expression of Id proteins is critical for maintaining the size of the Tfh cell pool in response to infections. All three E proteins are expressed in Tfh cells, with E2A and HEB gene transcripts being more abundant than E2-2 (Choi et al., 2015). Each E protein may have distinct roles, and may bind Id proteins with different affinity. It is therefore possible that the relative abundance of the E-Id protein complex determines the outcome of Th1 and Tfh differentiation. Nonetheless, forced expression of E2A or Ascl2 induced CXCR5 expression in CD4$^+$ T cells; and co-expression of Id2 diminished CXCR5 induction by E2A (but not Ascl2) (Liu et al., 2014; Shaw et al., 2016). Forced expression of Bcl6 was not sufficient to induce CXCR5 expression, and no Bcl6 binding sites were found in proximity of the *CXCR5* gene locus (Hatzi et al., 2015; Liu et al., 2014). Bcl6 appears to suppress expression of Id2 as a direct target, and hence indirectly allows E2A to activate CXCR5 expression in Tfh cells (Shaw et al., 2016).

These studies suggest Tcf1/Lef1 and E proteins may control a cooperative regulatory circuit that promotes Tfh differentiation (Fig. 3). The relationship between Tcf1/Lef1 and E-Id protein complexes remains to be elucidated. Our previous RNA-Seq analysis shows that Tcf1/Lef1-deficient Tfh cells express fewer transcripts of *Tcf12* (encoding HEB), *Ascl2*, and *Id3* genes (Choi et al., 2015). In addition, loss of Id2 was associated with increased expression of Tcf1 (Shaw et al., 2016). These observations suggest a positive "feed-forward" regulation between Tcf1/Lef1 and E proteins that coordinately enforces a Tfh fate. The underlying molecular details merit further investigation.

Regulation of cytotoxic CD8$^+$ T cell identity during acute infection

After acute viral or bacterial infection, mature CD8$^+$ T cells are activated and undergo clonal expansion to generate a large quantity of antigen-specific effector CD8$^+$ T cells that are equipped with cytotoxic molecules, including perforin and granzymes. The CD8$^+$ effectors are responsible for eliminating target cells infected by intracellular pathogens as well as tumor cells (Williams and Bevan, 2007; Harty and Badovinac, 2008). Following the peak response, only a small portion of CD8$^+$ effectors survives the contraction phase and gives rise to memory CD8$^+$ T cells, which provide enhanced protection against the same pathogen. In this context, the CD8$^+$ effectors are dedicated cytotoxic cells and also called Tc1 cells. CD8$^+$ T cells are shown to have the capacity of producing IL-4 (Tc2), IL-9 (Tc9), and IL-17 (Tc17), either under *in vitro* polarization conditions, at specific anatomical locations, or under specific allergic/inflammatory conditions *in vivo*. These unique propensities were recently reviewed (Mittrucker et al., 2014).

Maintaining the identity of Tc1 CD8$^+$ effectors is essential for their cytotoxic capacity. In this regard, the best-known transcriptional regulators are the T-bet and Eomes transcription factors (Intlekofer et al., 2008). T-bet and Eomes are homologous proteins containing T-box DNA binding domains (Pearce et al., 2003). Whereas deletion of either factor had no pronounced effect, deletion of both severely impaired induction of IFN-γ, granzyme B and perforin, resulting in failure of viral clearance. Instead, T-bet/Eomes-deficient CD8$^+$ T cells aberrantly activated the Th17 program, including upregulating Rorγt, the Th17 master regulator, along with the IL-17A and IL-21 cytokines, and IL-23 receptor. These IL-17-producing, T-bet/Eomes-deficient CD8$^+$ T cells caused progressive inflammatory and wasting syndrome.

CD8$^+$ effectors are functionally heterogeneous, with the KLRG1^{hi}IL-7Rα$^-$ subset representing short-lived effector cells (SLECs), and the KLRG1^{lo}IL-7Rα$^+$ subset having increased potential to give rise to memory CD8$^+$ T cells, hence considered memory precursor effector cells (MPECs) (Joshi et al., 2007). Blimp1 is potently induced in CD8$^+$ effectors, in particular SLECs (Kallies et al., 2009; Rutishauser et al., 2009). Whereas loss of Blimp1 did not compromise CD8$^+$ effector clonal expansion or cytokine production, Blimp1-deficient CD8$^+$ effectors were defective in granzyme B and perforin expression and impaired in cytotoxicity. In addition, Blimp1-deficient CD8$^+$ effectors were skewed to the MPEC phenotype, an effect accompanied by increased expression of Tcf1 and Id3 (discussed below). Although T-bet and Blimp1 appear to be induced independently in CD8$^+$ effectors, a recent study demonstrated a cooperativity between the two factors in 1) activating the cytotoxic program and 2) repressing the Th17 program, a feature reminiscent of the functional redundancy of T-bet and Eomes (Xin et al., 2016). Similar to T-bet/Eomes-deficient animals, mice reconstituted with T-bet and Blimp1 double-deficient bone marrow (BM) cells succumbed to CD8$^+$ T cell-mediated immune pathology, whereas mice reconstituted with either T-bet or Blimp1 single-deficient BM cells were unaffected. Of note, CD8$^+$ effectors lacking T-bet alone or both T-bet and Blimp1 expressed Rorγt and IL-17A at similar levels. Thus, it is likely that, in addition to activating the Th17 program, T-bet and Blimp1 double deficiency caused additional undefined changes in CD8$^+$ effectors. It remains to be investigated how T-bet, Eomes, and Blimp1 act together to coordinate repression of Th17-associated genes in CD8$^+$ effectors.

In addition to repressing CD4$^+$ lineage-associated genes in

mature $CD8^+$ T cells, Tcf1 and Lef1 play another key role in $CD8^+$ T cells before antigen encounter: they restrain expression of cytotoxic effector molecules, including Blimp1, Fas ligand, and granzymes. Importantly, this function is also mediated by Tcf1 HDAC activity (Xing et al., 2016). Concomitant with induction of the cytotoxic program, Tcf1 expression is downregulated in acute infection-elicited $CD8^+$ effectors (Zhao et al., 2010). Tcf1 expression is also downregulated but partly preserved in MPECs. In fact, ablating both Tcf1 and Lef1 almost completely abrogated formation of MPECs (Zhou and Xue, 2012). As noted above, Blimp1-deficient $CD8^+$ effectors showed increased Tcf1 expression (Rutishauser et al., 2009; Xin et al., 2016), suggesting that the mutually antagonistic Tcf1-Blimp1 axis is utilized in naïve $CD8^+$ T cells as well as $CD8^+$ effectors. Similar to the expression pattern during naïve $CD4^+$ to Tfh differentiation, Id3 is highly expressed in naïve $CD8^+$ T cells and MPECs; and Id3 deficiency impairs generation of memory $CD8^+$ T cells (Ji et al., 2011; Yang et al., 2011). On the other hand, Id2 is robustly induced in $CD8^+$ effectors and critically regulates SLEC differentiation and survival (Cannarile et al., 2006; Yang et al., 2011). It is proposed that both Id2 and Blimp1 negatively regulate Id3 in SLECs but more studies are needed to elucidate the interplay among Tcf1/Lef1, Id protein, Blimp1, and T-bet/Eomes, to determine whether they control other aspects of $CD8^+$ effector identity in response to acute infection.

Activation of the Tfh program in $CD8^+$ T cells in chronic infection

During chronic infection, antigen-specific $CD8^+$ T cells initially acquire effector functions but gradually become less functional due to antigen persistence in the host (Wherry and Kurachi, 2015). The loss of functional capabilities, known as $CD8^+$ T cell exhaustion, occurs in a progressive and stepwise manner. Exhausted $CD8^+$ T cells lose proliferative potential and IL-2 production at an early stage, and then lose the ability to produce tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) as the exhaustion progresses. Exhausted $CD8^+$ T cells expresses multiple inhibitory receptors including PD-1, LAG3, TIM3 and 2B4 (CD244), in addition to high levels of CD43 and CD69 but low levels of CD62L and IL-7Rα. $CD8^+$ T cell exhaustion is likely one of the major reasons for ineffective viral control in experimental models and human chronic infections by human immunodeficiency virus (HIV), hepatitis B and C viruses (Khaitan and Unutmaz, 2011; Ye et al., 2015).

Tfh-like $CXCR5^+CD8^+$ T cells have been described in human peripheral blood lymphocytes and tonsils (Quigley et al., 2007) but their physiologic functions were not fully elucidated until recently. Several recent studies identified and extensively characterized $CXCR5^+CD8^+$ T cells in chronic viral infection models and HIV-infected human patients (He et al., 2016; Im et al., 2016; Leong et al., 2016). Whereas this population was not evident in effector or memory $CD8^+$ T cells in the context of acute viral infections (Im et al., 2016; Leong et al., 2016), the $CXCR5^+CD8^+$ T cells were readily detectable in protein immunization and B cell lymphoma-bearing models, where antigens persist for prolonged time (Leong et al., 2016). In addition to the Tfh-characteristic CXCR5 molecule, $CXCR5^+CD8^+$ T cells exhibited other Tfh-like features, including higher expression of Bcl6, Tcf1 and ICOS. In addition, the Tfh-like $CXCR5^+CD8^+$ T cells expressed lower levels of inhibitory receptors including PD-1, LAG-3, 2B4 and CD160, suggesting they were less exhausted than their $CXCR5^-CD8^+$ counterparts. In fact, sorted $CXCR5^+CD8^+$ T cells gave rise to both $CXCR5^+CD8^+$ and $CXCR5^-CD8^+$ T cells upon adoptive transfer into infection-matched hosts, whereas $CXCR5^-CD8^+$ T cells appeared to be terminally exhausted effector $CD8^+$ T cells. This result hence elucidates the lineage relationship, identifying the Tfh-like $CXCR5^+CD8^+$ T cells as long sought-after self-renewing precursors that maintain the pool size of exhausted $CD8^+$ T cells.

Exhausted $CD8^+$ T cells remain critical in curtailing viral replication, in spite of their diminished functionality (Wherry and Kurachi, 2015). $CXCR5^+CD8^+$ T cells are more effective in viral control than $CXCR5^-CD8^+$ T cells (He et al., 2016; Im et al., 2016; Leong et al., 2016). This enhanced capacity is not necessarily associated higher expression of cytokines and/or cytotoxic molecules but may lie in two other aspects. First, $CXCR5^+CD8^+$ T cells showed more proliferative capacity than $CXCR5^-CD8^+$ T cells when transferred into either infection-matched or naïve recipients (He et al., 2016; Im et al., 2016). Blocking signals from inhibitory receptors is an effective approach to at least partly reinvigorate exhausted $CD8^+$ T cells. Indeed, $CXCR5^+CD8^+$ T cells responded more robustly than $CXCR5^-CD8^+$ T cells to PD-1 blockage treatment (He et al., 2016; Im et al., 2016). The second aspect concerns the anatomical localization of $CXCR5^+CD8^+$ T cells; and two of these studies show distribution of the $CXCR5^+CD8^+$ T cells is enriched in B cell follicles in addition to the T cell zone in secondary lymphoid organs, in both murine models and HIV-infected patients (He et al., 2016; Leong et al., 2016). Recent studies have demonstrated that $CD4^+$ Tfh cells are the major $CD4^+$ T cell compartment that harbors replication-competent and infectious HIV (Fukazawa et al., 2015; Banga et al., 2016). The ability of $CXCR5^+CD8^+$ T cells to enter B cell follicles thus provides a window of opportunity for eliminating the infected cells in the B cell follicle sanctuary.

These studies also identified several transcription factors that are responsible for the generation of $CXCR5^+CD8^+$ T cells in response to chronic infection. Tcf1 appears to be a foremost critical factor in the process. As noted above, Tcf1 has complex roles at the early and late stages of T cell development. Im et al. (2016) used hCD2-Cre to specifically ablate Tcf1 in mature $CD8^+$ T cells and demonstrated an

intrinsic requirement for Tcf1 in generating CXCR5$^+$CD8$^+$ T cells and maintaining the pool size of the antigen-specific exhausted CD8$^+$ T cells in multiple organs. Another study identified a Tcf1^{hi} CD8$^+$ T cell subset in response to chronic viral infection, which depends on Tcf1 for persistence, reinvigorated expansion by PD-1 blockage, and recall response by a secondary challenge (Utzschneider et al., 2016). These Tcf1^{hi} CD8$^+$ T cells expressed higher levels of CXCR5 and many other Tfh genes than the Tcf1^{lo} counterparts (Werner Held, personal communication). These data highlight the essential role of Tcf1 in activating the Tfh program in CD8$^+$ T cells activated by chronic infections.

A second set of TFs involves the Bcl6-Blimp1 axis. Similar to CD4$^+$ Tfh cells, forced expression of Bcl6 or ablation of Blimp1 increased the abundance of CXCR5$^+$CD8$^+$ T cells, whereas ablation of Bcl6 diminished, but did not completely abrogate, the generation of CXCR5$^+$CD8$^+$ T cells (Leong et al., 2016). A previous study used granzyme B-Cre to specifically ablate Blimp1 in CD8$^+$ T cells after activation by chronic viral infection, and found that loss of Blimp1 was associated with improved production of multiple cytokines and diminished expression of several inhibitory receptors in exhausted CD8$^+$ T cells (Shin et al., 2009). Furthermore, deleting one Blimp1 allele improved granzyme B expression, degranulation and cytotoxicity of exhausted CD8$^+$ T cells. These beneficial effects are likely ascribed to increased population of CXCR5$^+$CD8$^+$ T cells, and it is of interest to revisit the role of Blimp1 haploinsufficiency during CD8$^+$ T cell exhaustion.

E-Id proteins also contribute to generation of CXCR5$^+$ CD8$^+$ T cells. Whereas CXCR5$^-$CD8$^+$ T cells exhibit an Id2^{hi}Id3^{lo} phenotype, CXCR5$^+$CD8$^+$ T cells are Id2^{lo}Id3^{hi}. In spite of different expression levels of the Id proteins, ablating either Id2 or Id3 increased the abundance of CXCR5$^+$CD8$^+$ T cells, and forced expression of E2A showed similar effect (He et al., 2016; Leong et al., 2016). On the other hand, forced expression of Id3 diminished CXCR5$^+$CD8$^+$ T cell frequency, and Id2 overexpression antagonized E2A-induced expansion of CXCR5$^+$CD8$^+$ T cells. These studies further showed that Blimp1 and E2A co-occupied a + 10.7 kb region in the sole intron of *Cxcr5;* and the Blimp1 and E2A sites were shown to function as negative and positive regulatory elements, respectively, using the *ex vivo* dual reporter assay (Yu et al., 2012). Both Blimp1 and E2A had additional binding sites upstream of the *Cxcr5* gene. Similar to the situation in Tfh cells where Bcl6 may not directly induce CXCR5, it is proposed that Bcl6 indirectly upregulates *Cxcr5* expression through antagonizing Blimp1-mediated repression in CXCR5$^+$CD8$^+$ T cells. Although not directly tested, it is possible that Bcl6 may also repress Id2 and thus allow E2A to activate CXCR5 in exhausted CD8$^+$ T cells (Fig. 3).

The role of T-bet and Eomes was not directly addressed in these studies, although loss of either severely diminished antigen-specific CD8$^+$ T cell responses and resulted in failure to control viral replication in multiple organs during chronic infection (Paley et al., 2012). Therefore, CXCR5$^+$CD8$^+$ T cells likely depend on T-bet and Eomes for long-term maintenance; and the interplay between these T-box factors with Tcf1 is of interest. We previously demonstrated a positive effect of forced expression of Tcf1 and/or constitutively active β-catenin in expanding the memory CD8$^+$ T cell pool in acute infection (Zhao et al., 2010). Additionally, although the relative importance of Id2 and Id3 proteins were not addressed, they may prove to be useful therapeutic targets by lifting their inhibitory effect and hence favoring generation of CXCR5$^+$CD8$^+$ T cells in chronic infections. Elucidating the regulatory circuits in CXCR5$^+$CD8$^+$ T cell differentiation thus afford an intervention opportunity to direct cytotoxic CD8$^+$ T cells to the 'hideout' sites of persistently replicating viruses.

In this review, we highlighted recent progresses in understanding the regulation of CD4$^+$ and CD8$^+$ T cell identity, both during development and differentiation after activation. For each factor, versatile and conserved mechanisms are utilized to meet functional needs. During CD8$^+$ T cell development, Tcf1 uses its intrinsic HDAC activity to repress CD4$^+$ lineage-associated genes to guard CD8$^+$ T cell identity. Meanwhile, Tcf1-mediated activation of Bcl6 and repression of Blimp1 appear to be a shared mechanism in promoting CD4$^+$ Tfh differentiation in acute infection and CXCR5$^+$CD8$^+$ T cell differentiation in chronic infection. This progress not only provides answers but also brings new questions. For example, since Tcf1 and Lef1 are highly expressed in both CD4$^+$ and CD8$^+$ T cells, 1) how do Tcf1 and Lef1 (in particular via their HDAC activities) affect expression of CD4$^+$ lineage-associated genes in CD4$^+$ T cells per se? and 2) what are shared and distinct Tcf1 occupancy sites in CD4$^+$ and CD8$^+$ T cells and what is their connection with biological output? It remains enigmatic as to how Tcf1 and Lef1 exert positive or negative regulatory effects in the context of different genes in the same cell, in particular, how Tcf1/Lef1 HDAC activity is opposed/masked so as to activate downstream target genes. Certainly, a viable possibility is that there is differential recruitment of co-activators or co-repressors. Whereas the controversy over the requirements for the β-catenin coactivator in T cells remains to be fully resolved (Steinke and Xue, 2014), an unusual biological effect of β-catenin interaction with Tcf/Lef factors has been reported in embryonic stem cells (ESCs). Tcf3 (encoded by *Tcf7l1*) negatively regulates ESC self-renewal (Yi et al., 2011), and interaction of β-catenin with Tcf3 does not convert Tcf3 to a transcriptional activator but rather removes Tcf3 from DNA and causes its degradation (Shy et al., 2013). Might Tcf1 and Lef1 use a similar regulatory mechanism in T cells? It is also noteworthy that Lef1 bound to target locations can bend DNA by 130 degrees (Giese et al., 1992). Tcf1 and Lef1 may therefore contribute to chromatin folding/looping and influence three-dimensional chromatin conformation in CD4$^+$ and CD8$^+$ T cells. This structural effect would likely affect accessibility of genes by other transcription factors or

the epigenetic machinery. To develop therapeutics for treating autoimmunity, cancer, and persistent infections, an in-depth dissection of the regulatory circuits should provide useful tools to prevent unnecessary identity diversion and at the same time confer the plasticity desired.

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Compliance with ethics guidelines

Jodi A. Gullicksrud, Qiang Shan, and Hai-Hui Xue declare that they have no conclict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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