**IMMUNOLOGY**

Tet2 coordinates with Foxo1 and Runx1 to balance T follicular helper cell and T helper 1 cell differentiation

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In response to various types of infection, naïve CD4+ T cells differentiate into diverse helper T cell subsets; however, the epigenetic programs that regulate differentiation in response to viral infection remain poorly understood. Demethylation of CpG dinucleotides by Tet methylcytosine dioxygenases is a key component of epigenetic programming that promotes specific gene expression, cellular differentiation, and function. We report that following viral infection, Tet2-deficient CD4+ T cells preferentially differentiate into highly functional germinal center T follicular helper (Tfh) cells that provide enhanced help for B cells. Using genome-wide DNA methylation and transcription factor binding analyses, we find that Tet2 coordinates with multiple transcription factors, including Foxo1 and Runx1, to mediate the demethylation and expression of target genes, including genes encoding repressors of Tfh differentiation. Our findings establish Tet2 as an important regulator of Tfh cell differentiation and reveal pathways that could be targeted to enhance immune responses against infectious disease.

**INTRODUCTION**

In response to viral infection, naïve antigen-specific CD4+ T cells proliferate and differentiate into T helper 1 (Th1) and T follicular helper (Tfh) subsets, each providing specialized functions that contribute to the broader adaptive immune response. Th1 cells mediate cellular immunity at sites of viral infection, while Tfh cells provide critical help for antibody responses. Tfh cells are required for the generation and maintenance of germinal centers (GCs), and within this specialized environment, Tfh cells provide help for B cells through the production of costimulatory molecules and cytokines, including CD40L, interleukin-21 (IL-21), and IL-4 (1, 2). These signals allow antigen-specific B cells to survive, proliferate, undergo affinity maturation, and, ultimately, differentiate into memory B cells and long-lived plasma cells. As Tfh cells develop in response to infection, they up-regulate the expression of the transcriptional repressor Bcl6 and chemokine receptor CXCR5. Bcl6 enforces Tfh polarization and prevents differentiation to other Th lineages, while CXCR5 allows Tfh cells to migrate to the B cell follicle (1–3). GC Tfh cells are a subset of Tfh cells that have the highest expression of CXCR5 and Bcl6. Unlike Thf cells, which reside at the periphery of B cell follicles, GC Tfh cells localize to the GC to provide enhanced help for B cells undergoing affinity maturation and are essential for generating long-lasting antibody-mediated immunity (2, 4).

CD4+ T cells that differentiate in response to viral infection must integrate and interpret multiple environmental signals to commit to either the Th1 or Tfh lineage. This process occurs with epigenetic changes that act to enforce transcriptional programing so that lineage-specific effector genes can be expressed or maintained in a poised state (5). Methylation of cytosine residues in CpG dinucleotides serves as a repressive epigenetic mark, and methylated CpG regions in gene enhancers and promoters are associated with transcriptional silencing, whereas demethylation of CpGs in these regions is associated with active or permissive gene expression (6). Ten-eleven translocation 2 (Tet2) is one of three Tet methylcytosine dioxygenases that catalyze the conversion of methylcytosine to hydroxymethylcytosine and other intermediates leading to unmethylated cytosine (6). Because of its ability to demethylate CpG regions, there has been increasing interest in the role of Tet2-mediated epigenetic programing in the development and function of immune cells. Recent studies have demonstrated an important role for Tet2 in the expression of key effector genes in Th1, Th17, and regulatory T cells (Tregs) (7–9). However, the exact role of Tet2 in CD4+ T cell differentiation and function in response to viral infection remains unclear.

In this study, we examined the role of Tet2 in antigen-specific CD4+ T cell differentiation and function in response to viral infection using a mouse adoptive transfer model with Tet2-deficient CD4+ T cells. We found that Tet2 is required to balance the differentiation of CD4+ T cells toward Th1 and Tfh lineages. In the absence of Tet2, CD4+ T cell differentiation is skewed toward the generation of highly functional GC Tfh cells. To identify the genes and biological pathways that are regulated by Tet2, we used genome-wide expression and methylation analyses that revealed that Tet2-mediated demethylation contributes to downstream Foxo1 and Runx1 signaling pathways and is required for the full expression of Foxo1 and Runx1 target genes. On the basis of these findings, we propose a model in which Tet2 coordinates with Foxo1 and Runx1, as well as other transcription factors to restrict GC Tfh differentiation in CD4+ T cells responding to viral infection.

**RESULTS**

Tet2 deficiency reduces Th1 differentiation and promotes enhanced formation of GC Tfh cells

To examine the role of Tet2 in CD4+ T cell differentiation and function in response to viral infection, we used an acute lymphocytic choriomeningitis virus (LCMV) infection model. Tet2 knockout (KO) mice were first crossed to SMARTA T cell receptor (TCR)
transgenic mice in which CD4\(^+\) T cells are specific for the LCMV GP\(_{66-77}\) epitope (10). Congenically marked wild-type (WT) (CD45.1\(^+\)) and Tet2 KO (CD45.1\(^+\)CD45.2\(^+\)) naïve CD4\(^+\) SMARTA cells were adoptively cotransferred at a 1:1 ratio (49% WT:51% Tet2 KO) into C57Bl/6 (B6) recipient mice (CD45.2\(^+\)) followed by infection with LCMV. Seven days post-infection (DPI), we observed that the frequency and number of WT and Tet2 KO SMARTA CD4\(^+\) T cells were equivalent in the spleens of recipient mice (Fig. 1, A and B), suggesting that loss of Tet2 does not affect the ability of naïve CD4\(^+\) T cells to survive or proliferate during clonal expansion. However, there was a significant decrease in both the frequency and the number of Tet2 KO cells in the livers of recipient mice compared to WT (Fig. 1, A and C), suggesting impaired differentiation into the T\(_{FH}\) cell subset, as T\(_{FH}\) effector cells migrate into peripheral tissues in response to viral infection (11). In agreement with this idea, we observed a substantial reduction in the frequency and number of Tet2 KO T\(_{FH}\) cells (Tbet\(^{High}\) CXCR5\(^+\)) in the spleen (Fig. 1, D to F). In addition, we observed a slight but significant reduction in the expression of Tbet within the T\(_{FH}\) subset of Tet2 KO CD4\(^+\) T cells (Fig. 1G). Tet2-deficient cells also expressed less interferon \(\gamma\) (IFN\(\gamma\)) and tumor necrosis factor \(\alpha\) (TNF\(\alpha\)) compared to WT counterparts, although there was no difference in production of IL-2 (Fig. 1A to F).

While we observed a decrease in the overall number of Tet2 KO T\(_{FH}\) cells, there was also an increase in the frequency of Tet2 KO T\(_{FH}\) cells, which are identified by low expression of Tbet and high expression of CXCR5 (Fig. 1D). Both the frequency and number of Bcl6\(^{High}\) GC T\(_{FH}\) cells significantly increased in Tet2 KO cells, increasing approximately fivefold compared to WT SMARTA cells (Fig. 1, H to J). In addition, Tet2 KO T\(_{FH}\) cells also expressed higher levels of Bcl6 compared to their WT counterparts (Fig. 1K). These data suggest that Tet2 plays an important role in balancing CD4\(^+\) T cell differentiation in response to viral infection and that, in the absence of Tet2, the balance shifts away from development of T\(_{FH}\) cells and instead skews toward enhanced GC T\(_{FH}\) cell differentiation.

To determine whether Tet2 acts early after cell activation to skew CD4\(^+\) T cell differentiation, we analyzed SMARTA cells 2 DPI. Early T\(_{FH}\) precursors were identified as Tim3\(^{High}\)Tbet\(^{High}\), while early T\(_{FH}\) precursors were identified as Tim3\(^{Low}\)Tbet\(^{Low}\) (12), as Tim3\(^{Low}\) cells do not express CXCR5 or Bcl6, while Tim3\(^{High}\) cells can express both (fig. S1G). Consistent with the observations at later time points, we observed a shift away from Tim3\(^{Low}\) precursors and a significant increase in the frequency of T\(_{FH}\) precursor cells within Tet2 KO CD4\(^+\) T cells (Fig. 1, I and M). Collectively, these results demonstrate that, in the absence of Tet2, the balance of CD4\(^+\) T cell differentiation is highly skewed toward the development of GC T\(_{FH}\) cells, and that Tet2-mediated programming is initiated early, within 2 days, in activated CD4\(^+\) T cells, and that this programming acts to promote T\(_{FH}\) differentiation and/or restrict T\(_{FH}\) differentiation.

**Tet2 KO CD4\(^+\) T cells have a cell-intrinsic programming that favors GC T\(_{FH}\) differentiation**

We next examined whether the strong shift in differentiation toward GC T\(_{FH}\) cells in the absence of Tet2 was CD4\(^+\) T cell intrinsic and occurs in polyclonal CD4\(^+\) T cell responses to virus infection. We generated mixed bone marrow chimeric mice using Tet2 floxed mice (13) crossed with CD4cre [Tet2 conditional KOs (Tet2cKO)] donor mice. Congenically marked recipient mice (CD45.1\(^+\)) were irradiated and reconstituted with bone marrow cells from Tet2cKO (CD45.2\(^+\)) and WT (CD45.1\(^+\)CD45.2\(^+\)) mice at a ratio of 1:2. Likewise, control bone marrow chimeras were generated by reconstituting irradiated recipients (CD45.1\(^+\)) with bone marrow cells from CD4cre control (CD45.2\(^+\)) and WT control (CD45.1\(^+\)CD45.2\(^+\)) mice (Fig. 2A). At 8 weeks after reconstitution, we observed no significant difference in the frequency of circulating CXCR5\(^+\) T\(_{FH}\) cells among donor cells in the blood between WT and CD4cre CD4\(^+\) T cells in control chimeras or WT versus Tet2\(^{lox}\)×CD4cre cells in the experimental chimeras, suggesting that loss of Tet2 does not bias differentiation before infection in naïve mice (Fig. 2B). We then infected these chimeric mice with LCMV and assessed the virus-specific CD4\(^+\) T cell response at 7 DPI using I-A\(^\beta\):GP\(_{66-77}\) tetramer (fig. S2A) and found no difference in the total number of tetramer-positive cells (Fig. 2C). While there was no significant difference in the frequency of WT versus CD4cre transgenic GC T\(_{FH}\) cells in the control chimeras, there was a significant increase in the frequency of Tet2\(^{lox}\)×CD4cre GC T\(_{FH}\) cells compared to WT in the experimental group (Fig. 2D). Furthermore, Tet2\(^{lox}\)×CD4cre T\(_{FH}\) cells expressed higher levels of Bcl6 compared to their WT counterparts (Fig. 2E). These results mirror those of the Tet2 KO SMARTA transfer (Fig. 1), reinforcing the concept that the impact of Tet2 on CD4\(^+\) T cell differentiation is cell intrinsic and occurs in polyclonal T cell responses to viral infection.

We next examined whether the enhanced T\(_{FH}\) phenotype occurs in the absence of B cells. Interaction with cognate B cells is necessary for full T\(_{FH}\) lineage commitment and maintenance as well as further differentiation into the GC T\(_{FH}\) subset (1, 2). We reasoned that if Tet2 acts intrinsically on CD4\(^+\) T cell differentiation, we would still observe an increase in Tet2 KO T\(_{FH}\)-like cells even in the absence of B cells. To test this hypothesis, we cotransferred WT SMARTA and Tet2 KO SMARTA cells into either B6 or B cell–deficient muM\(^{-}\) recipient mice, followed by infection with LCMV and analysis at 7 DPI. As expected, we observed a decrease in the frequency of WT GC T\(_{FH}\)-like cells (CXCR5\(^+\)Bcl6\(^+\)) within the muM\(^{-}\) recipients when compared to B6 (Fig. 2F). This was also true for Tet2 KO GC T\(_{FH}\)-like cells, which were reduced in frequency in the muM\(^{-}\) mice (Fig. 2F). However, Tet2 KO cells still generated a greater number of GC T\(_{FH}\)-like cells in muM\(^{-}\} recipients when compared to their WT counterparts in the same mice (Fig. 2G) and displayed enhanced expression of Bcl6 (Fig. 2H). These data indicate that loss of Tet2 alters CD4\(^+\) T cell–intrinsic programming such that even in the absence of signaling from cognate B cells, Tet2-deficient CD4\(^+\) T cells are still able to generate enhanced T\(_{FH}\)-like response compared to their WT counterparts.

**Tet2-deficient T\(_{FH}\) cells have an enhanced ability to support the GC B cell response**

Given the considerable increase in Bcl6 expression and GC T\(_{FH}\) cell formation in virus-specific CD4\(^+\) T cells, we reasoned that Tet2-deficient CD4\(^+\) T cells would be able to provide increased help for the B cell response and support a greater number of GC B cells. To test this idea, we transferred naïve WT SMARTA or Tet2 KO SMARTA CD4\(^+\) T cells into B6 or B cell–deficient muMt\(^{-}\} recipient mice, followed by infection with LCMV and analysis at 7 DPI. As expected, we observed a decrease in the number of WT GC T\(_{FH}\)-like cells (CXCR5\(^+\)Bcl6\(^+\)) within the muMt\(^{-}\} recipients when compared to B6 (Fig. 2F). This was also true for Tet2 KO GC T\(_{FH}\)-like cells, which were reduced in frequency in the muMt\(^{-}\) mice (Fig. 2F). However, Tet2 KO cells still generated a greater number of GC T\(_{FH}\)-like cells in muMt\(^{-}\} recipients when compared to their WT counterparts in the same mice (Fig. 2G) and displayed enhanced expression of Bcl6 (Fig. 2H). These data indicate that loss of Tet2 alters CD4\(^+\) T cell–intrinsic programming such that even in the absence of signaling from cognate B cells, Tet2-deficient CD4\(^+\) T cells are still able to generate enhanced T\(_{FH}\)-like response compared to their WT counterparts.
Fig. 1. Tet2 deficiency impairs T<sub>H</sub>1 differentiation and promotes enhanced formation of GC T<sub>FH</sub> cells. Congenically marked WT (CD45.1<sup>+</sup>) and Tet2 KO (CD45.1<sup>+</sup>CD45.2<sup>+</sup>) naïve SMARTA cells were adoptively cotransferred into CD45.2<sup>−</sup> naïve B6 recipients followed by LCMV Armstrong infection. In (A) to (K), recipient mice were given 1000 Tet2 KO and 1000 WT SMARTA cells, infected intraperitoneally with 2 × 10<sup>5</sup> PFU LCMV Armstrong, and analyzed at 7 DPI. In (L) and (M), recipient mice were given either 2 × 10<sup>6</sup> Tet2 KO or WT SMARTA cells, infected intravenously with 2 × 10<sup>6</sup> PFU LCMV Armstrong, and analyzed at 2 DPI. (A) Representative FACS plots gated on CD4<sup>+</sup>CD45.1+ donor SMARTA cells show the frequency of WT versus Tet2 KO cells in the spleen and liver at 7 DPI. (B) Frequency and number of SMARTA cells in the spleen. (C) Frequency and number of SMARTA cells in the liver. (D) Representative CXCR5 and Tbet analysis in the spleen. (E) Frequency of SMARTA cells that are CXCR5<sup>−</sup> Tbet<sup>High</sup> TH1 cells. (F) Number of SMARTA cells that are CXCR5<sup>−</sup> Tbet<sup>High</sup> TH1 cells. (G) Tbet mean fluorescence intensity (MFI) of CXCR5<sup>−</sup> SMARTA cells. (H) Representative CXCR5 and Bcl6 analysis in the spleen. (I) Frequency of CXCR5<sup>+</sup> Bcl6<sup>High</sup> GC T<sub>FH</sub> SMARTA cells. (J) Number of CXCR5<sup>+</sup> Bcl6<sup>High</sup> GC T<sub>FH</sub> SMARTA cells. (K) Bcl6 MFI of CXCR5<sup>+</sup> SMARTA cells. (L) Representative Tbet and Tim3 analysis in the spleen at 2 DPI. (M) Frequency of SMARTA cells that are Tbet<sup>Low</sup>Tim3<sup>−</sup> T<sub>FH-like</sub> cells at 2 DPI. For (A) to (K), n = 7 WT and n = 7 Tet2 KO. For (L) and (M), n = 6 WT and n = 7 Tet2 KO. Data were pooled from two independent experiments. Significant P values of <0.05 are indicated and were determined using a paired Student’s t test.
previous observations (see Fig. 1), we observed a greater frequency and number of Tet2 KO GC Tfh cells compared to WT SMARTA cells (Fig. 3C). As expected, NT control mice failed to generate a Fas<sup>+</sup>PNA<sup>+</sup> GC B cell response (Fig. 3, D and E). We observed a significant increase in both the frequency and number of GC B cells in the mice that received Tet2 KO cells compared to those that received WT SMARTA cells (Fig. 3, D and E) and a trend toward increased glycoprotein (GP)–specific antibody titers at day 60 (Fig. S3A). These
results demonstrate that loss of Tet2 within CD4^+ T cells increases the generation of GC TFH cells that are functional and able to support an enhanced B cell response in the context of viral infection.

**Loss of Tet2 disrupts gene expression pathways regulated by multiple transcription factors including Foxo1 and Runx1**

To determine the role of Tet2 in the transcriptional programing of virus-specific effector T cell differentiation, we performed RNA sequencing (RNA-seq) on donor WT and Tet2 KO T_H1 and T_FH cell subsets sorted from adoptive recipients at 7 DPI, as well as on WT and Tet2 KO naive CD4^+ T cells. Principal components analysis demonstrated that Tet2 KO and WT samples from each of the three sorted CD4^+ T cell subsets (naive, T_H1, and T_FH) clustered together within each subset (fig. S4A), suggesting that there may be relatively few key genes with differential expression between WT and Tet2 KO cells that contribute to the observed phenotype. Accordingly, when we assessed differential expression within CD4^+ T cell subsets, we obtained a relatively small list of genes that were significantly differentially expressed between WT T_H1 versus Tet2 KO T_H1 and WT T_FH versus Tet2 KO T_FH (273 in T_H1 and 314 in T_FH) (fig. S4B), again suggesting that the loss of Tet2 affects CD4^+ T cell programing and differentiation through a limited set of genes. There was no difference in Tet2 expression between WT T_H1 and WT T_FH cells (fig. S4C). In addition, Tet1 and Tet3 expression were similar between WT and KO cells, indicating that the other members of the Tet family are not up-regulated to compensate for loss of Tet2 (fig. S4D).

Heatmaps of all differentially expressed genes between WT T_H1 versus Tet2 KO T_H1 cells (fig. S4E) and WT T_FH versus Tet2 KO T_FH cells (Fig. 4A) demonstrated a clear pattern in which clusters of genes that were induced in WT T_H1 and T_FH cells remained under-expressed in their Tet2 KO T_H1 and T_FH counterparts (Fig. 4A and fig. S4E). These could be genes that are directly demethylated by Tet2 or are downstream of Tet2-mediated programing. In addition, we observed relatively little difference in expression between WT and Tet2 KO naive samples, reinforcing the concept that the effects of Tet2 programing on CD4^+ T cell differentiation are cell
intrinsic and occur after cell activation in response to infection. We also observed clusters of genes with higher expression in Tet2 KO TH1 and TFH cells compared to WT. These overexpressed genes could be downstream of repressors that are dependent on Tet2-mediated demethylation for expression. Among these were several genes that are known to play an important role in TFH cells, including Bcl6 (Fig. 4B) and Il21 (Fig. 4C), which correlates with the increased Bcl6 protein expression and GC TFH phenotype observed by flow cytometry (Fig. 1, H to K), and is consistent with our finding that Tet2-deficient cells provide enhanced help for GC B cells (Fig. 3).

Tet2 lacks a DNA binding domain and must be recruited to target sites by transcription factors to demethylate CpG regions (6).
Thus, in the absence of Tet2, the expression of downstream targets could be impaired even if the activating transcription factor itself remains highly expressed. To identify transcriptional pathways that are disrupted in the absence of Tet2, we used enrichment analysis of previously published chromatin immunoprecipitation sequencing (ChIP-seq) datasets to identify transcription factors with reported binding near the underexpressed genes in Tet2 KO T_{H1} and T_{FH} cells (16–18). Foxo1 was the top-ranked transcription factor pathway associated with Tet2 deficiency in both T_{H1} and T_{FH} cells (Fig. 4, D and E). Foxo1 restricts T_{FH} cell differentiation, and deletion of Foxo1 promotes the formation of T_{FH} cells (19). Runx1 was also highly ranked and significantly associated with the underexpressed genes (Fig. 4, D and E). A previous study demonstrated that Runx1 can bind to and recruit Tet2 (20). Neither Foxo1 nor Runx1 was differentially expressed between WT and Tet2 KO T_{H1} and T_{FH} cells, and both were highly expressed in naïve cells (Fig. 4, F and G), which was also confirmed for Foxo1 in naïve CD4^{+} T cells by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (fig. S4F). These results indicate that Tet2 is not directly required for proper Foxo1 and Runx1 gene expression, and instead suggest that Foxo1 and Runx1 factors could recruit Tet2 to target loci so that downstream expression of genes can be properly induced.

**Tet2 demethylates genes involved in pathways that regulate cell activation and T_{FH} lineage differentiation in both T_{H1} and T_{FH} cells**

To identify targets of Tet2-mediated demethylation, we performed whole-genome enzymatic methylation sequencing on sorted WT and Tet2 KO T_{H1} and T_{FH} cells 7 days after LCMV infection, as well as on naïve CD4^{+} T cells. Our analysis uncovered 1515 differentially methylated regions (DMRs) between WT and Tet2 KO T_{H1} cells and 853 DMRs between WT and Tet2 KO T_{FH} cells (fig. S5A). As expected, nearly all DMRs were hypermethylated in the Tet2 KO samples compared to WT. Only one gene (Myl12b) contained a hypomethylated region in Tet2 KO T_{H1} cells, and only three genes (Myl12b, Tubg1, and Gm26760) had regions of hypomethylation in Tet2 KO T_{FH} cells. In addition, there were no significant DMRs between WT and Tet2 KO naïve cells, again suggesting that loss of Tet2 does not significantly affect CD4^{+} T cells after antigen encounter and activation in our experimental system. Nearly 50% of DMRs in both T_{H1} and T_{FH} cells occurred in intronic regions, and most sites were located 50 to 500 kb downstream of transcription start sites (fig. S5, A and B). This finding was consistent with previous studies that showed that Tet2 predominantly binds to and mediates demethylation in gene bodies and distal regulatory regions rather than in promoter regions (21, 22).

To identify genes where Tet2-mediated demethylation contributes to gene expression, we compared the list of underexpressed genes with the list of genes containing hypermethylated DMRs in Tet2 KO T_{H1} and T_{FH} cells compared to WT T_{H1} and T_{FH} cells. We reasoned that underexpressed genes with hypermethylated DMRs were likely to be directly dependent on Tet2-mediated demethylation for proper expression. A significant number of genes were both hypermethylated and underexpressed in Tet2 KO T_{H1} and T_{FH} cells. Approximately 30% of underexpressed genes contained a hypermethylated DMR in Tet2 KO T_{H1} cells, and approximately 23% of underexpressed genes contained a hypermethylated DMR in Tet2 KO T_{FH} cells (fig. SSC). Underexpressed genes that did not contain hypermethylated DMRs may be downstream of Tet2-mediated programming.

We next examined the top 50 DMRs in WT versus Tet2 KO T_{H1} cells (Fig. 5A) and WT versus Tet2 KO T_{FH} cells (Fig. 5B) ranked by mean difference. We observed a clear pattern of demethylation that occurred as WT naïve CD4^{+} T cells differentiated into T_{H1} and T_{FH} effector cells; however, these same loci remained highly methylated in Tet2 KO effector cells (Fig. 5, A and B). Most regions that were hypermethylated in Tet2 KO T_{H1} cells were also hypermethylated in Tet2 KO T_{FH} cells, indicating that Tet2 acts to demethylate shared targets in both T_{H1} and T_{FH} subsets. Of note, several genes related to alternate effector CD4^{+} T cell lineages, including Ifng, Il10, and Batf, all contained hypermethylated regions in both T_{H1} and T_{FH} Tet2KO cells compared to WT cells (Fig. 5A). One of the few lineage-specific hypermethylated regions occurred in Prdm1, which encodes the transcription factor Blimp1 (Fig. 5, A and C). Blimp1 acts to directly repress Bcl6 and is a major negative regulator of T_{FH} differentiation (23). The Prdm1 locus contained several regions that were unmethylated only in WT T_{H1}; however, there was not a significant difference in Prdm1 expression between WT and Tet2 KO T_{H1} cells at 7 DPI (fig. SSD), although gene expression may have differed before day 7. This could reflect Tet2-mediated programing that is set earlier after T cell activation in response to virus. We found a region within the Tet2 gene that became demethylated between the naïve and effector stage in WT cells, which could indicate that Tet2 is targeted to the Tet2 locus to regulate this demethylation (Fig. 5A).

To better understand the biological processes and transcriptional pathways that were disrupted in the absence of Tet2, we used enrichment analysis with the lists of hypermethylated DMRs in Tet2 KO T_{H1} and T_{FH} cells. In both T_{H1} and T_{FH} cells, several of the top associated biological processes were leukocyte activation, cell activation, and lymphocyte activation, suggesting that Tet2-mediated demethylation plays a role in the transition of naïve cells to effector subsets (Fig. 5D). Several of the top-ranked DMRs occurred in genes that are known to play a role in TCR signaling and T cell activation, including Fyn and Ubash3b (Fig. 5, A and B) (24–27). TCR signal strength has been shown to influence Ifng versus Ifng differentiation (12, 28–30), which raises the possibility that Tet2 could contribute to balanced lineage commitment of CD4^{+} T cells by enabling the expression of genes that act to modulate TCR signal strength in response to viral infection.

Next, to identify transcription factors associated with the hypermethylated DMRs, we used enrichment analysis to search through published ChIP-seq data and identify transcription factors that have been shown to bind at sites within the hypermethylated genes (16–18). This revealed significant enrichment for binding of several key transcription factors within the hypermethylated DMRs of Tet2 KO T_{H1} compared to WT T_{H1} and Tet2 KO T_{FH} compared to WT T_{FH} cells (Fig. 5E). Again, both Foxo1 and Runx1 were highly ranked/enriched in T_{H1} and T_{FH} cells. Neither Foxo1 nor Runx1 contained any hypermethylated regions in their promoters or gene bodies (the nearest DMR to the Foxo1 locus is approximately 131 kb downstream). Combined with the fact that Foxo1 and Runx1 are not differentially expressed between WT and Tet2 KO cells, these data suggest that Foxo1 and Runx1 recruit Tet2 to specific loci to coordinate the demethylation of target genes directly regulated by Foxo1 and Runx1 transcription factors.

**Foxo1 colocalizes with genomic regions of Tet2-dependent demethylation and restricts GC T_{FH} formation**

Our data showing enrichment of Foxo1 ChIP-seq binding associated with underexpressed and hypermethylated genes in Tet2 KO effector
CD4+ T cells (Figs. 4, D and E, and 5E) suggest that Foxo1 may coordinate with Tet2 to restrict GC TFH cell differentiation. Given that Foxo1 is not differentially expressed in Tet2 KO cells (Fig. 4F), we hypothesized that Foxo1 recruits Tet2 to enable demethylation and expression of its target genes, which may then act to limit GC TFH differentiation. If this hypothesis is correct, we reasoned that regions of Tet2-dependent demethylation would colocalize with Foxo1 binding that is acquired in effector CD4+ T cells compared to naïve cells.
naive cells. Therefore, we performed Foxo1 ChIP-seq on sorted naive CD4⁺ T cells and CD4⁺Icos High effector SMARTA CD4⁺ T cells 7 days after LCMV infection. We identified 9713 Foxo1 binding peaks in activated cells and only 1743 peaks in naive CD4⁺ T cells, indicating that Foxo1 binding increases in effector T cells by 7 DPI. In addition, most peaks found in naive cells were maintained with activation (fig. S6A). Next, we compared the genes that contained Foxo1 binding peaks to genes that contained hypermethylated DMRs in Tet2 KO Th1 and Th17 cells to determine whether Foxo1 targeted the same genes. Using a hypergeometric test, a significant number of genes with hypermethylated DMRs overlapped with genes containing Foxo1 ChIP peaks in Tet2 KO Th1 and Th17 cells (Fig. 6A). Enrichment analysis revealed that regions of Foxo1-bound genes in activated CD4⁺ T cells were associated with many of the same biological processes (fig. S6B) as enrichment analysis of hypermethylated genes in Tet2 KO Th1 and Th17 cells (Fig. 5D), indicating that Foxo1 acts to regulate similar biological processes as Tet2 in activated CD4⁺ T cells.

We then chose to examine Foxo1 binding at key genes with defined roles in suppressing Th1 formation, such as Runx2 and Runx3 (31). Gene expression of both Runx2 and Runx3 significantly decreased in Tet2 KO Th1 cells relative to WT Th1 cells, and Runx3 expression also decreased in Tet2 KO Th1 cells relative to WT Th1 cells (Fig. 6, B and C). We then examined whether these genes contained Foxo1 binding peaks that directly overlapped or were adjacent to regions of Th1-dependent demethylation. Runx2 and Runx3 each contained regions that were methylated in naive cells (both WT and Tet2 KO), transitioned to an unmethylated state in WT Th1 and Th17 cells, but remained highly methylated in Tet2 KO effector CD4⁺ T cells (Fig. 6, D and E). Runx2 contained six hypermethylated DMRs, two of which directly overlapped Foxo1 peaks (Fig. 6D). The Runx3 promoter contained a small Foxo1 peak in naive cells that increased in effector cells and was immediately adjacent to a region of Tet2-dependent demethylation in Th1 and Th17 cells (Fig. 6E). In addition, induced Foxo1 binding in a Runx3 intronic region directly overlapped with a Tet2-dependent DMR (Fig. 6E). Furthermore, many Foxo1 binding peaks or DMRs coincided with candidate cis-regulatory elements (cCREs) with a distal enhancer-like signature reported by ENCODE (32) (Fig. 6, D and E). Runx2 and Runx3 have been shown to restrict Tfh cell formation by repressing the expression of key Tfh genes, including Icos and Cd200 (31). In addition to reduced Runx2 and Runx3 expression in Tet2 KO cells (Fig. 6, B and C), we found that both Icos and Cd200 had significantly higher expression in Tet2 KO effector CD4⁺ T cells compared to their WT counterparts (Fig. 6, F and G). Collectively, these results support a mechanism whereby Foxo1 and Tet2 coordinate at the Runx2 and Runx3 loci to promote their demethylation and gene expression, and Runx2 and Runx3 then repress Icos and Cd200 expression to limit Tfh differentiation.

We next examined the differentiation of Foxo1-deficient CD4 T cells to determine whether Foxo1 contributes to balancing CD4 T cell differentiation in response to viral infection. Foxo1 has been shown to modulate Tfh and GC Tfh differentiation in response to immunization and Listeria monocytogenes infection (19); however, the influence of Foxo1 on Tfh cell differentiation has not been clearly defined during acute viral challenge. We hypothesize that Foxo1-deficient CD4 T cells will skew toward enhanced GC Tfh differentiation due, in part, to reduced Runx2 and Runx3 expression. Naive WT SMARTA CD4⁺ T cells were cotransfected with Cas9 and guide RNAs targeting a nonsense sequence [control guide RNA (gRNA)] or Cas9 and gRNAs targeting Foxo1 (Foxo1 gRNA). Transfected SMARTA cells were then adoptively transferred into recipient B6 mice followed by infection with LCMV Armstrong. Using this CRISPR system, we were able to achieve deletion of Foxo1 in up to 56% of SMARTA CD4⁺ T cells at 7 DPI, as evidenced by a clear population of Foxo1-negative cells within the Foxo1 gRNA–transfected group (Fig. 6H and fig. S6C). Furthermore, there was a clear and significant decrease in the fluorescence intensity of Foxo1 in the Foxo1 gRNA population gated on Foxo1-negative cells (Fig. 6I). We next compared the Foxo1-negative and Foxo1-positive populations within the Foxo1 gRNA group to determine the impact of Foxo1 deletion on Th17 cell differentiation. There was a significant increase in the frequency of Cxcr5⁺ Bcl6 High GC Tfh cells within the Foxo1-deficient population (Fig. 6, J and K). In addition, the Foxo1-negative cells also had higher expression of Bcl6 compared to their Foxo1-positive counterparts (Fig. 6L). Furthermore, the frequency of Cxcr5⁺ Pd1 High GC Tfh cells was also significantly increased, further demonstrating that Foxo1 deficiency skewed Th17 cells toward a GC Tfh phenotype (fig. S6, D and E). Consistent with our prediction, we found that Foxo1-deficient Th1 and Th17 cells had significantly lower expression of Runx2 compared to their Foxo1-sufficient counterparts (Fig. 6, M and N), and this reduction correlated with a significantly higher frequency of CD200 High Th1 cells within the Foxo1-negative population (Fig. 6O and fig. S6P). We observed a significantly lower frequency of Cxcr5⁺ Icos High Th1 cells in the Foxo1-deficient population (fig. S6, G and H), a result that may be due to direct regulation of Icos by Foxo1, which has been described previously (19). Collectively, these results indicate that Tet2 and Foxo1 target shared pathways to regulate the balance of Th1/Th17 cell differentiation and are consistent with a model whereby Foxo1 recruits Tet2 to a set of target loci (including Runx2 and Runx3) to mediate their demethylation and expression to restrict Tfh differentiation.

Tet2-dependent demethylation colocalizes with Foxo1 and Runx1 binding at genes associated with TCR signaling and T cell activation

Given that Tet2 and Foxo1 were associated with similar biological processes involving lymphocyte activation and differentiation (Fig. 5D and fig. S6B), we next examined a select number of loci with reported roles in T cell activation and TCR signaling, including Ubash3b, Ahnak, Lgals3, and Fyn (24–27, 33, 34). All four genes had significantly lower expression in Tet2 KO effector cells when compared to their WT counterparts (Fig. 7, A to C, and fig. S7A). In addition, Ubash3b, Ahnak, and Fyn each contained hypermethylated regions in Tet2 KO Th1 and Th17 cells that directly overlapped or were adjacent to induced Foxo1 binding peaks (Fig. 7, D and E, and fig. S7B). These results suggest that Tet2 and Foxo1 may act together to mediate the expression of genes involved in T cell activation and TCR signaling.

Our enrichment analysis using published ChIP-seq datasets also pointed to an association between the transcription factor Runx1 and genes that were underexpressed and hypermethylated in Tet2 KO Th1 and Th17 cells (Figs. 4, D and E, and 5E). Runx1 has previously been shown to directly bind Tet2 and recruit it to mediate the demethylation of Runx1 target genes in Jurkat cells (20). Runx1 itself was not differentially expressed between WT and Tet2 KO cell types (Fig. 4G) and did not contain any hypermethylated DMRs, suggesting that Runx1 might instead interact with Tet2-mediated...
Fig. 6. Foxo1 colocalizes with genomic regions of Tet2-dependent demethylation and restricts GC T<sub>FH</sub> formation. (A) Overlap of genes containing Foxo1 ChIP-seq peaks in effector CD4<sup>+</sup> T cells and genes containing hypermethylated DMRs in Tet2 KO effector cells. Significance determined by hypergeometric test. (B and C) Expression of (B) Runx2 and (C) Runx3. (D and E) UCSC Genome Browser plots of (D) Runx2 (exons 4 to 7) and (E) Runx3. Tracks show ENCODE cCREs and Foxo1 ChIP-seq peaks in effector (black) and naive (gray) SMARTA CD4<sup>+</sup> T cells. Gray shading denotes DMRs. Columns within heatmaps depict methylation of individual CpGs. (F and G) Expression of (F) Icos and (G) Cd200. (H to O) Naive SMARTA cells were cotransfected with Cas9 and gRNAs targeting a nonsense sequence (control gRNA) or Foxo1 (Foxo1 gRNA) and transferred into recipient mice followed by infection and analysis at 7 DPI. (H) SMARTA cells were gated into Foxo1-negative and Foxo1-positive populations. Histograms show frequencies of Foxo1-negative versus Foxo1-positive SMARTA cells in control gRNA and Foxo1 gRNA groups. (I) Foxo1 MFI of control gRNA samples gated on Foxo1-negative cells and Foxo1 gRNA samples gated on Foxo1-positive and Foxo1-negative cells. (J) Representative FACS plots show the frequency of CXCR5<sup>+</sup>Bcl6<sup>High</sup> GC T<sub>FH</sub> cells between Foxo1-positive and Foxo1-negative cells within the Foxo1 gRNA group. (K) Frequency of CXCR5<sup>+</sup>Bcl6<sup>High</sup> GC T<sub>FH</sub> cells. (L) Bcl6 MFI of CXCR5<sup>+</sup> cells. (M and N) Runx2 MFI of (M) T<sub>FH</sub> and (N) T<sub>Ef</sub> cells. Significant P values of <0.05 were determined using an unpaired Student’s t test. For (I), dots represent individual mice, n = 8 control gRNA and n = 9 Foxo1 gRNA. Data are representative of three independent experiments. Significant P values of <0.05 were determined using an unpaired Student’s t test.
Fig. 7. Tet2-dependent demethylation colocalizes with Foxo1 and Runx1 binding at genes associated with TCR signaling and T cell activation. (A to C) Expression of (A) Ubash3b, (B) Ahnak, and (C) Lgals3 as determined by RNA-seq analysis. (D and E) UCSC Genome Browser plots of (D) Ubash3b and (E) Ahnak with tracks showing ENCODE cCREs, and Foxo1 ChIP-seq peaks in effector CD4^{high}CD4{T} cells (black) and naïve SMARTA CD4^{high}CD4{T} cells (gray). DMRs are marked with gray shading, and each column within the accompanying heatmaps depicts methylation of individual CpG sites within the selected DMRs for (D) Ubash3b and (E) Ahnak. (F) UCSC Genome Browser plot of Lgals3 with tracks for Encode cCREs and Runx1 ChIP-seq peaks from GSE67465 (35). DMRs are marked with gray shading, and each column within the accompanying heatmaps depicts methylation of individual CpG sites within the selected DMRs. For (A) to (C), gene expression is shown as normalized counts. Statistically significant P values of <0.05 are indicated and were determined by using an unpaired Student's t test.
DISCUSSION

Tet2-mediated demethylation of DNA at specific regulatory regions plays an important role in cellular differentiation and function. In CD4+ T cells, Tet2 is required for the proper expression of IFNγ and IL-17 in T H 1 and T H 17 cells, respectively, and Tet2 combined with Tet3 is necessary for the function of T reg and the stable expression of Foxp3 (7–9). In addition, reducing the availability of α-ketoglutarate (αKG), a critical cofactor for Tet2 enzymatic function, has been shown to enhance OTI1-specific T cell differentiation toward the T H 1 lineage (36). While recent studies have begun to address the role of Tet2 in CD4+ T cell programming, the mechanisms through which Tet2 affects T cell differentiation induced by viral infection and development remain unclear. In this study, using an acute viral infection model to study antigen-specific CD4+ T cell differentiation, combined with whole-genome enzymatic methyl sequencing and ChIP-seq, we show that loss of Tet2 alters the Foxo1- and Runx1-regulated transcriptional programs and leads to enhanced differentiation of GC T H 17 cells in response to viral challenge. Our findings indicate that cell-intrinsic Tet2-mediated programming acts to balance CD4+ T cell differentiation after viral challenge, exerting effects that are evident as early as 2 days after viral infection. In the absence of such programming, naïve CD4+ T cells preferentially differentiate toward the T H 1 lineage and highly functional GC T H 17 cells that provide enhanced help for GC B cells.

Numerous transcription factors contribute to regulating the differentiation of T H 17 and T H 1 cells in response to viral infection (31, 37). While the transcription factor Blimp1 can largely block T H 17 differentiation (23), many other transcription factors restrict or limit the degree of T H 17 and/or GC T H 17 cell differentiation that occurs in response to infection or vaccination, including factors such as Id2, Id3, Runx2, and Runx3 (15, 31). To understand why Tet2-deficient CD4 T cells have increased skewing toward GC T H 17 cells, we performed enrichment analyses to identify the transcription factors and pathways associated with the underexpressed and hypermethylated genes in Tet2 KO T H 17 and T H 17 cells. We found enrichment of multiple specific transcription factors including Foxo1, Runx1, and others. In the absence of Tet2, many Foxo1-regulated target genes fail to undergo demethylation and remain underexpressed, indicating that Tet2 contributes to proper transcriptional programming of Foxo1 target genes. Foxo1 gene expression was not disrupted, and the Foxo1 locus did not contain any regions of hypermethylation in the promoter or gene body in the absence of Tet2, indicating that Tet2-mediated demethylation does not contribute to Foxo1 expression. These combined observations are consistent with a model where Foxo1 recruits Tet2 to its target genes to enable their demethylation and expression, and this idea is supported by the direct overlap/immediate proximity of Foxo1 binding and Tet2-dependent demethylation at genes known to negatively regulate T H 17 cell differentiation. Specifically, our study identified that both Foxo1 binding and Tet2-mediated demethylation directly target Runx2 and Runx3, as these loci remained hypermethylated and exhibited decreased gene expression in Tet2 KO cells. A recent study identified Runx2 and Runx3 as key repressors of T H 17 differentiation by repressing CD200 and ICOS (31). On the basis of our combined findings, we propose a model whereby Tet2 and Foxo1 coordinate efforts to demethylate specific Foxo1 target genes, including Runx2 and Runx3, to reduce T H 17 cell differentiation. Stone et al. (19) previously demonstrated that Foxo1 restricts T H 17 differentiation, as demonstrated by the propensity of Foxo1 KO CD4+ T cells to differentiate toward the T H 17 lineage following immunization. Our experiments using the CRISPR-Cas9 system to delete Foxo1 demonstrated that, similar to Tet2 KO cells, Foxo1-deficient cells exhibit increased differentiation toward Bcl6 High GC T H 17 cells following acute viral infection. Consistent with our model, Foxo1-deficient CD4 T cells had reduced expression of Runx2 and an increased frequency of CD200 High T H 17 cells, consistent with our observations in Tet2-deficient cells. In contrast, Foxo1-deficient cells exhibited significantly reduced ICOS expression, whereas ICOS was significantly elevated in Tet2 KO cells. Reduced ICOS expression in Foxo1 KO cells has previously been described by Stone et al. (19), where ChIP and expression data demonstrate that Foxo1 positively regulates ICOS expression. Because Foxo1 expression is unaltered in Tet2 KO cells (compared to WT cells), the elevated ICOS expression that we observed in Tet2 KO cells likely results from Foxo1-dependent Icos gene activation combined with the reduced repression by Runx2 and/or Runx3 (due to their decreased expression) in Tet2 KO cells. Thus, we propose that the absence of Tet2-dependent demethylation reduces Runx2 and Runx3 expression to contribute to the enhanced GC T H 17 cell phenotype of Tet2 KO CD4+ T cells following viral infection (Fig. 8).

The results of our genome-wide methylation analysis indicate that Tet2-mediated demethylation contributes to T cell activation pathways in response to viral infection. Several of the genes that were both underexpressed and that contained hypermethylated regions in Tet2 KO T H 17 and T H 17 cells (compared to WT) are associated with T cell activation and TCR signaling, including Fyn (an Src family tyrosine kinase reported to both positively mediate TCR signal transduction and activate negative regulators of TCR signaling) (24, 25), Ubash3b (a phosphatase that can act to dephosphorylate Zap70 to reduce TCR signal transduction) (26, 27), Ahnak (a scaffold protein involved in T cell calcium signaling) (33), and Lgals3 (which encodes galectin-3 and has been implicated in negative regulation of TCR activation) (34). Studies have reported that differences in TCR signaling strength can bias CD4+ T cell differentiation toward or away from T H 17 differentiation (12, 28–30); however, given the various conclusions of these studies, the impact of increased TCR signal strength requires further investigation. Our results raise the
possibility that Tet2-mediated demethylation of genes involved in TCR signaling helps to tune the signal strength and contributes to balance the differentiation between T H 1 and T FH subsets. Many of these genes related to TCR strength of signal were found to be directly bound by either Foxo1 or Runx1. Runx1 has been shown to influence the differentiation of CD4+ T cell subsets (38, 39). Similar to Foxo1, in the absence of Tet2, Runx1 gene expression was not impaired and the Runx1 locus itself was not hypermethylated in Tet2 KO cells. Instead, Runx1 target genes (identified from published ChIP-seq studies) were underexpressed in Tet2 KO effector cells relative to WT effector cells and contained hypermethylated regions in Tet2 KO effector cells that were proximal to previously identified Runx1 binding peaks. Coimmunoprecipitation experiments have demonstrated that Runx1 and Tet2 can directly interact (20). Collectively, these results support a model in which Tet2 interacts with Foxo1, Runx1, and other transcription factors to mediate demethylation and proper expression of their specific target genes in CD4+ T cells. Besides the targeting of Tet2 to known repressors of T FH differentiation mentioned above (Runx2 and Runx3), the specific Tet2-dependent demethylation at genes that modulate TCR signaling may also contribute to the enhanced T FH phenotype in Tet2 KO cells. However, future studies will be needed to determine the precise impact of Tet2 on TCR signal strength and T FH cell differentiation.

Tet2 mutations contribute to altered immune cell development and generation of leukemias and lymphomas (40). Tet2 loss-of-function mutations are frequently found in angioimmunoblastic T cell lymphoma (AITL), a CD4+ peripheral T cell lymphoma that has a T FH -like phenotype and gene expression profile (41–43). In a recent study, Zang et al. (44) reported that Tet2 deficiency in CD4+ T cells, when combined with a mutation to the guanosine triphosphate (GTPase) RhoA, results in impaired Foxo1-mediated transcriptional programing and the development of a T FH -like cancer in a mouse model of AITL. The authors reported a slight decrease in Foxo1 expression within Tet2-deficient CD4+ T cells and a small increase to CpG methylation at the Foxo1 promoter (44). While our results also demonstrate that expression of Foxo1 target genes is disrupted in the absence of Tet2, we did not observe a defect in Foxo1 expression itself. In addition, our genome-wide methylation analysis showed no significant changes at the Foxo1 promoter and only slight hypermethylation downstream of Foxo1 in Tet2-deficient CD4+ T cells. These differing results between our study and the report by Zang et al. (44) may reflect the different model systems used (viral antigenic challenge versus malignant transformation). Our current study of Tet2 for programing virus-specific CD4+ T cells suggests that loss of Tet2 contributes to a skewing of cells toward a more polarized GC T FH phenotype, and these findings may have implications for understanding the origins and pathogenicity of CD4+ AITL lymphomas.

T FH cells are critical for generating long-lived antibody responses, which in turn are required for most successful vaccination strategies (45). Hence, there is considerable interest in better understanding the mechanisms that govern T FH cell differentiation and function. Our study provides novel insights into the epigenetic programs that affect CD4+ T cell lineage commitment and reveals that Tet2 acts as a key regulator of T FH cell differentiation in the context of viral infection. By modulating Tet2 activity, or the pathways that rely on Tet2-mediated demethylation, it may be possible to increase the generation of T FH cells to promote enhanced GC responses and improve antibody-mediated protection against pathogens.

MATERIALS AND METHODS

Mice and adoptive transfers

C57BL/6 mice were purchased from The Jackson Laboratory. Tet2 KO mice (46) were crossed to SMARTA TCR transgenic mice (10) to generate Tet2 KO×SM mice. Tet2 ΔGTPase mice (13) were crossed to CD4cre transgenic mice (47) to generate Tet2cKO. For adoptive transfer experiments, congenically marked naïve CD4+ T cells were isolated from the spleens of WT SM (CD45.1+) and Tet2 KO SM mice (CD45.1+ CD45.2+), combined at a 1:1 ratio, and intravenously cotransferred into naïve C57BL/6 mice (CD45.2) or B cell-deficient muMT mice. Approximately 24 to 72 hours after transfer, recipients were infected intraperitoneally with LCMV Armstrong.
For day 2 infection experiments, CD4+ T cell splenocytes were isolated from either WT SM or Tet2 KO SM mice and enriched before transfer using a magnetic CD4+ T cell negative selection kit (STEMCELL Technologies). For day 10 infection experiments, congenically marked CD45.1+ WT or Tet2 KO naïve SMARTA cells were adoptively transferred into CD45.2+ Bcl6floxCD4cre (Tfh cell–deficient) recipient mice (14) followed by infection with LCMV Armstrong approximately 24 hours later. All donor mice were between 6 and 8 weeks of age. Animal experiments were conducted in accordance with University of Utah Institutional Animal Care and Use Committee–approved protocols.

Generation of mixed bone marrow chimeras Tet2cKO and Cre control bone marrow chimeras were generated by reconstituting lethally irradiated CD45.1+ C57BL/6 recipient mice with congenically marked Cre control (CD45.2+) and WT (CD45.1+CD45.2+) bone marrow cells or Tet2cKO (CD45.2+) and WT (CD45.1+CD45.2+) bone marrow cells at a 1:2 ratio of Cre:WT. Chimeric mice were infected with 2 x 10⁸ plaque-forming units (PFUs) LCMV Armstrong at 8 weeks after reconstitution.

FACS analysis and sorting Cells were stained in 1x phosphate-buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS) for 30 min on ice with fluorochrome-conjugated antibodies (purchased from BD Biosciences, eBioscience, BioLegend, and Invitrogen) for cell surface antigens. For Foxo1 and Runx2 staining, antibodies were purchased from Cell Signaling Technology (Foxo1: 58223s, Runx2: 98059s). For tetramer staining, cells were first stained for 2 hours at 37°C with LCMV tetramer (provided by the National Institutes of Health (NIH) Tetramer Core) followed by staining for surface antigens. For CXCR5 staining, a three-step protocol was followed as described in (23) using purified rat anti-mouse CXCR5 (BD Biosciences), a secondary Biotin–SP–conjugated AffiniPure F(ab)ʹ2 goat anti-rat immunoglobulin G (IgG) (Jackson ImmunoResearch), and a streptavidin–APC or streptavidin–phycoerythin–cyanine 7 (Invitrogen). To label transcription factors, cells were first stained for surface antigens followed by permeabilization, fixation, and staining using the Foxp3 Permeabilization/Fixation kit (eBioscience). For intracellular cytokine staining, cells were first stimulated with GP61–80 peptide and brefeldin A (GolgiPlug, BD Biosciences) for 5 hours. Cells were then stained for surface antigens followed by permeabilization, fixation, and staining using the Cytofix/Cytoperm kit and protocol (BD Biosciences). Cell sorting was performed using FACSaria (BD Biosciences), and flow cytometry data were collected on FACSCanto (BD Biosciences). Fluorescence-activated cell sorting (FACS) data were analyzed using FlowJo software versions 9 and 10 (TreeStar).

RNA isolation and RNA-seq analysis RNA was isolated from sorted CD4+ T cells using an RNA extraction kit (Zymo). RNA-seq libraries were generated using the TruSeq Standard Total RNA kit with Ribo-Zero Gold (Illumina). Prepared libraries were sequenced using an Illumina NovaSeq 6000 system. Sequencing data were aligned to the mm10 reference genome using STAR in two-pass mode to output a BAM file sorted by coordinates. Mapped reads were assigned to annotated genes using featureCounts version 1.6.3, and differentially expressed genes were identified using DESeq2 version 1.30.1 with a 5% false discovery rate (48). Enrichment analysis of transcription factor association was performed using the Chea2016 module within Enrichr (16–18).

Quantitative RT-PCR RNA was isolated from sorted WT and Tet2 KO naïve CD4+ T cells using an RNA extraction kit (Zymo). Complementary DNA (cDNA) was synthesized using SuperScript IV Vilo Master Mix (Thermo Fisher Scientific), and qRT-PCR was carried out using PowerUP SYBR Green Master Mix (Thermo Fisher Scientific) with the following primers for Foxo1: GTGCGCTCTGTCTGAGAAATCC (forward) and CATTAGGCTGCTCAAGGCTGAA (reverse).

DNA library preparation and whole-genome enzymatic methyl sequencing Genomic DNA was isolated from sorted cells using a DNA Extraction Kit (Qiagen) and sonicated to generate fragments of approximately 350 to 400 base pairs using S220 Focused Ultrasonicator (Covaris). Unmethylated cytosines were converted to uracils, and sequencing libraries were created using the NEBnext Enzymatic Methyl Seq Kit (New England Biolabs) according to the manufacturer’s instructions. DNA libraries were sequenced using an Illumina NovaSeq 6000 system following the manufacturer’s protocols.

Genome-wide methylation analysis Sequencing data quality was assessed using FastQC v0.11.4. Adapters were trimmed from the sequencing reads using Trim Galore! v0.4.4 using options (trim_galore -s SOUTDIR --fastq --paired $FORWARD_READS $REVERSE_READS). Alignment to the mm10 reference genome was performed using Bismark (49) v0.19.0 with options (bismark --multicore 6 --bowtie2 -N 1 $MM10 -1 $FORWARD_READS -2 $REVERSE_READS). Deduplication was performed with deduplicate_bismark (49) (deduplicate_bismark -p -bam SBISMARK_ALIGNED_BAM). Library quality was assessed on the basis of the percentage of reads that aligned to the genome. Library quality was considered sufficient if greater than 50% of reads uniquely aligned to the genome. Enzymatic methyl conversion efficiency was assessed by evaluating the percent of methylation observed in the CHH genome context. Enzymatic methyl conversion was considered sufficient when this value was less than 3%. Genome coverage was assessed using the bedtools (50) genomewcov software v2.25.0. Library genome coverage was considered sufficient if 80% of the genome had a depth of at least 10 reads. For each library that met these quality metrics, methylation percentages at individual CpG positions in the reference genome were quantified using the Bismark Methylation Extractor (49) v0.19.0 program with options (bismark_methylation_extractor --p-comprehensive --bedgraph $BISMARK_DEDUPLICATED_BAM). DMRs among the datasets were detected using BSmooth DMR finder (51). DMRs have at least 10 CpGs and an absolute mean difference of >0.3. Visualization of CpG positions with at least 10× coverage on colored heatmaps (blue-white-red) reflects the percent methylated from 0% to 100%. Individual genomic loci were displayed using UCSC Genome Browser (52). Enrichment analysis to identify transcription factors associated with hypermethylated genes was performed using Chea2016 within Enrichr (16–18).

ChIP-seq analysis ChIP-seq was performed as described previously (53). Briefly, sorted cells were fixed with 16% formaldehyde to a final concentration of 1%, and cross-linking was stopped by the addition of glycine. Cells
were sonicated in radioimmunoprecipitation assay buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) and then incubated with Foxo1 antibody (Cell Signaling Technology, C29H4) at 4°C overnight. After washing, DNA was eluted with IP elution buffer (1% SDS/0.1 M NaHCO3). Samples were purified with QiaQuick spin columns (Qiagen). ChIP-seq libraries were constructed with the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs) according to the manufacturer’s instructions. Foxo1 ChIP-seq libraries were sequenced using Illumina NovaSeq. Between 58 million and 112 million paired-end Illumina sequencing reads were aligned to the mouse mm10 reference genome using Novocraft NovoAlign v4.0.2-02, allowing for one random alignment of multimapping reads and providing the adapter sequences for automatic trimming concordant with alignment. ChIP was analyzed using the MultiRepMacsChIPSeq pipeline v1.5.2 using options “-pe --optdist 10000 --dupfrac 0.05 --maq 10 --cutoff 2 --peaksize 300 --peakgap 100 --species mouse --chrskip ‘chrM|Phix’ --blacklist mm10.blacklist.bed.” ChIP-seq peaks were integrated with DNA methylation signal for downstream analysis. For analysis of Runx1 binding peaks, mm10 converted BED (q < 1 × 10^{-5}) and BigWig files for a published Runx1 ChIP-seq dataset GSE67465 (35) were downloaded from CHiP-Atlas (ID number: SRX974254) (54). Genes associated with Foxo1 and Runx1 peaks were determined by uploading the respective BED files into the genomic Regions Enrichment of Annotations Tool (GREAT) (55) and using the following settings: Background regions = Whole Genome, Basal plus extension, Proximal 5.0 kb upstream, 1.0 kb downstream, plus distal: up to 1000.0 kb. ChIP-seq peaks were displayed using the UCSC Genome Browser (52).

CRISPR-Cas9-mediated KO of Foxo1

The following CRISPR gRNAs were chosen to target the first exon of Foxo1 using the IDT predesigned CRISPR gRNA database (IDT): 5′-CCACCTGATGATCGGACAGg-3′ and 5′-CGGGTCGGTCCTC-CACGACCTggg-3′ (lowercase letters represent the PAM site). Nonspecific gRNA (computationally determined not to target any gene) was used as a negative control (IDT). Nonspecific or Foxo1-specific CRISPR-Cas9 ribonucleoproteins (RNPs) were generated from ATTO550-labeled tracrRNA (IDT), crRNA (IDT), and Cas9 nuclease (IDT) using commercial guidelines (IDT). Naïve SCID μT cells were isolated and cultured in complete medium (RPMI 1640 + 10% FBS + 4 mM L-glutamine) at 490 nm.

Supplementary materials

Supplementary material for this article is available at https://doi.org/10.1126/sciadv.abm4982

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