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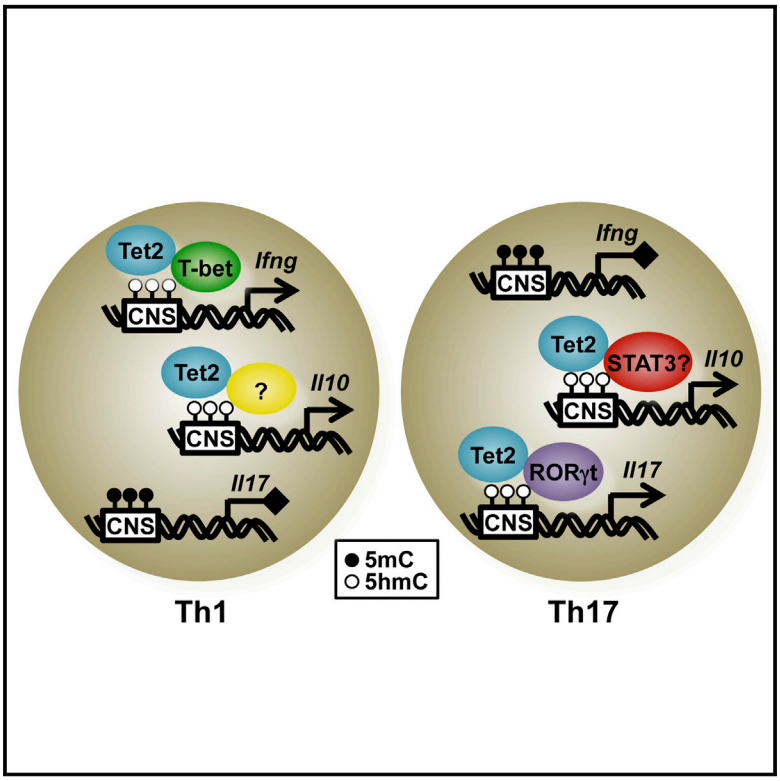
Immunity

The Methylcytosine Dioxygenase Tet2 Promotes DNA Demethylation and Activation of Cytokine Gene Expression in T Cells

Graphical Abstract



Authors

Kenji Ichiyama, Tingting Chen, ..., Lai Wei, Chen Dong

Correspondence

weil9@mail.sysu.edu.cn (L.W.), chendong@tsinghua.edu.cn (C.D.)

In Brief

Tet proteins are methylcytosine dioxygenases that convert methyalated DNA (5mC) to hydroxymethylated DNA (5hmC). Dong and colleagues generate a genome-wide 5hmC map in T helper (Th) cells and demonstrate that Tet2-mediated DNA demethylation plays a crucial role in control of signature cytokine expression in Th cells.

Highlights

- 5hmC marks transcriptional regulatory regions of lineage-specific signature genes
- Tet2 and key transcription factors cooperatively regulate signature gene expression
- Tet2 promotes the signature cytokine expression in Th1 and Th17 cells in vitro
- Tet2 controls cytokine production by T cells in autoimmune disease

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Article

The Methylcytosine Dioxygenase Tet2 Promotes DNA Demethylation and Activation of Cytokine Gene Expression in T Cells

Kenji Ichiyama,[1] Tingting Chen,[2] Xiaohu Wang,[3] Xiaowei Yan,[4] Byung-Seok Kim,[5] Shinya Tanaka,[6] Delphine Ndiaye-Lobry,[7] Yuhua Deng,[2] Yanli Zou,[2] Pan Zheng,[1] Qiang Tian,[4] Iannis Aifantis,[7] Lai Wei,[2,*] and Chen Dong[3,*]
[1]Center for Cancer and Immunology Research, Children's National Medical Center, Washington, DC 20010, USA
[2]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China
[3]Institute for Immunology, Tsinghua University, Beijing 100084, China
[4]Institute for Systems Biology, Seattle, WA 98109, USA
[5]Department of Immunology, MD Anderson Cancer Center, Houston, TX 77054, USA
[6]Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan
[7]Howard Hughes Medical Institute and Department of Pathology, New York University School of Medicine, New York, NY 10016, USA
*Correspondence: weil9@mail.sysu.edu.cn (L.W.), chendong@tsinghua.edu.cn (C.D.)
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SUMMARY

Epigenetic regulation of lineage-specific genes is important for the differentiation and function of T cells. Ten-eleven translocation (Tet) proteins catalyze 5-methylcytosine (5mC) conversion to 5-hydroxymethylcytosine (5hmC) to mediate DNA demethylation. However, the roles of Tet proteins in the immune response are unknown. Here, we characterized the genome-wide distribution of 5hmC in $CD4^+$ T cells and found that 5hmC marks putative regulatory elements in signature genes associated with effector cell differentiation. Moreover, Tet2 protein was recruited to 5hmC-containing regions, dependent on lineage-specific transcription factors. Deletion of *Tet2* in T cells decreased their cytokine expression, associated with reduced p300 recruitment. In vivo, Tet2 plays a critical role in the control of cytokine gene expression in autoimmune disease. Collectively, our findings suggest that Tet2 promotes DNA demethylation and activation of cytokine gene expression in T cells.

INTRODUCTION

Upon activation by antigens, naive $CD4^+$ T cells differentiate into one of several lineages of helper T (Th) cells, including Th1, Th2, Th17, and iTreg cells, defined by their patterns of cytokine production and immune function (Zhu et al., 2010). Th cell differentiation depends on the complex network of cytokine signaling imposed on lineage-specific transcription factors (Kanno et al., 2012). In addition to transcription factors, growing evidence shows that epigenetic mechanisms are crucial for controlling Th cell differentiation (Kanno et al., 2012). Wei et al. (2009) reported that histone modifications on the lineage-specific genes correlate with gene expression in Th cell differentiation. Moreover, we previously reported the recruitment of histone acetyltransferase (p300) and H3K27 demethylase (JMJD3) in the *Il17a* and *Il17f* loci in a Th17-cell-specific manner (Wang et al., 2012). Together, these findings suggest that histone modification is an important mechanism for Th cell differentiation.

DNA methylation at the 5-position of cytosine (5-methylcytosine [5mC]) is one of the key epigenetic mechanisms in development and gene regulation (Bird, 2002), and the alterations in DNA methylation patterns have been implicated in various diseases (Robertson, 2005). The 5-hydroxymethylcytosine (5hmC) was first identified in the T-even bacteriophage and was later found in several tissues (Shen and Zhang, 2013). 5hmC exists in mouse, bovine, and rabbit zygotes as well as mouse embryonic stem cells and accumulates specifically in the paternal pronucleus coinciding with a reduction in 5mC (Shen and Zhang, 2013), implying a potential biological function of 5hmC and a role of DNA demethylation in early development. Furthermore, Tsagaratou et al. reported that the pattern of 5hmC modification dynamically changes in Th1 and Th2 differentiation, as well as thymic T cell development (Tsagaratou et al., 2014). Recently, several studies identified the Ten-Eleven-Translocation (TET) proteins TET1, TET2, and TET3 as a new family of a-ketoglutarate and Fe^{2+}-dependent enzymes that alter the methylation status of DNA by converting 5mC into 5hmC (Pastor et al., 2013). Functional analyses using Tet-deficient cells have demonstrated their crucial roles in diverse biological processes (Pastor et al., 2013). Although it is becoming increasingly clear that Tet-mediated 5mC oxidation at functional genomic elements is physiologically an important epigenetic process in mammals, the roles of 5hmC and Tet proteins in the immune system remain to be understood.

Here, we generated genome-wide maps of 5hmC in various Th cells and found that 5hmC exists at putative regulatory elements of lineage-specific genes in appropriate Th cells. Tet2 was associated with 5hmC-containing regions, and deletion of Tet2 inhibited cytokine expression by Th1 and Th17 cells, resulting in reduction of 5hmC and key transcription factors binding. Finally, we confirmed Tet2 function in regulating the cytokines expression in vivo. Collectively, our findings prove that Tet-mediated active DNA demethylation is an essential epigenetic mechanism for regulation of Th cell function.

RESULTS

Genome-wide Distribution of 5hmC in CD4$^+$ T Cells

To study the role of DNA demethylation and particularly 5hmC during CD4$^+$ T-cell-mediated immune responses, we conducted global mapping of 5hmC via DNA immunoprecipitation coupled with high-throughput sequencing (DIP-seq) (Ku et al., 2011). As a control, 5mC was analyzed in the same manner. We assessed these modifications in five types of CD4$^+$ T cells: freshly isolated CD4$^+$CD25$^-$CD44^{lo}CD62L^{hi} (naive) T cells and naive CD4$^+$ T cells cultured under Th1, Th2, Th17, and TGF-β-induced Treg (iTreg) cell differentiation conditions. The appropriate polarization of each Th cell subset was confirmed by intracellular staining as well as qPCR. We found enrichment of lineage signature genes in polarized Th cells (Figure S1A). Furthermore, we observed approximately 90% of cells expressing lineage-specific transcription factors in polarized Th cells, although the percentages of signature cytokine-producing cells varied from 7% (Th2) to 50% (Th1 and Th17) under various conditions (Figure S1B). Immunoprecipitated samples were amplified and subjected to Illumina sequencing. A total of 172 million short reads from all samples were aligned onto the mouse genome (mm9 Build 37 assembled by NCBI). In all Th cell subsets, we identified 372,892 5hmC-occupied peaks and 260,315 5mC-occupied peaks (Table S1). To determine whether our sequencing depth can cover the size of the 5mC and 5hmC libraries, we performed a scaling analysis. Randomly sampled fractions of reads from each sample were subjected to peak identification via the SICER program (Zang et al., 2009). We found that about 15 or 6 million reads were sufficient to identify most of peaks for 5mC or 5hmC samples, respectively (Figure S1C). Therefore, our sequencing depth was able to cover the size of all libraries. We then analyzed the distribution of these peaks in the mouse genome in four kinds of regions: promoter (1 kb upstream and downstream of transcription start site), exon, intron, and intergenic, according to the annotation of "known genes" from the UCSC Genome Browser (Kent et al., 2002). As shown in Figure 1A and Table S1, each Th cell subset displayed similar patterns of 5hmC and 5mC distribution. The majority of 5hmC- and 5mC-associated regions were found in introns and intergenic regions, ranging from 39% to 57%, suggesting that they might potentially function in enhancers. In contrast, only around 4% of 5hmC- and 5mC-occupied peaks were localized to promoters and less than 7% in exons (Figure 1A and Table S1), slightly more than the presence of these two regions in the whole mouse genome (Table S1).

It is unclear whether 5hmC and 5mC colocalize in the same genomic regions during Th cell differentiation or function independently. We searched for regions with binary marks of both 5hmC and 5mC. 17%–23% of 5hmC-occupied regions in all Th cell subsets share both marks (Figure 1B and Table S2), indicating that a potentially active DNA demethylation process occurred in these regions. However, the majority of conversion from 5mC to 5hmC has been maintained in Th cell subsets.

We next analyzed the distribution of 5hmC and 5mC within gene bodies and 5 kb regions 5′ and 3′ of them. Although similar amounts and chromosomal distribution of 5mC were found in all Th cell subsets, the highest amounts of 5hmC in extended genic regions were found in naive T cells (Figure 1C). Moreover, this analysis also revealed the depletion of 5hmC and 5mC islands from regions proximal to the transcription start sites (TSSs) (Figure 1C), consistent with the observation that only a minority of the 5hmC and 5mC islands were located in promoter regions (Figure 1A).

In mammals, the majority of CpG dinucleotides are methylated, whereas unmethylated CpGs are found primarily in those regions of DNA with relatively high density of CpG, so-called CpG islands (CpGIs) (Bird, 1986). We therefore analyzed the distribution of 5hmC and 5mC among CpGIs. Our results showed that 5hmC was depleted from the centers of CpGIs in all Th cell subsets, whereas 5mC was depleted only at the regions upstream and downstream of the centers of CpGIs (Figure 1D). Similarly, naive T cells have the highest amounts of 5hmC in CpGIs, suggesting that the change in 5hmC around TSSs and CpGIs might play a critical role during Th cell differentiation.

5hmC Associates with Lineage-Specific Signature Genes

We next examined whether 5hmC and 5mC presented in lineage-specific genes in each Th cell subset. First, we assessed 5hmC and 5mC patterns in *Ifng*, *Il4*, and *Il17* cytokine genes, which serve as the defining lineage markers for Th1, Th2, and Th17 cells, respectively. As shown in Figure 2A, 5hmC was strongly associated with *Ifng*, *Il4*, and *Il17*, particularly in some of the evolutionarily conserved non-coding sequences (CNSs) and some promoter regions. Furthermore, we confirmed the distribution of 5hmC and 5mC in naive, Th1, and Th17 cells by qPCR after immunoprecipitation of 5hmC or 5mC. Consistent with sequencing analysis, the CNS(−6) at *Ifng*, known as an enhancer (Hatton et al., 2006), was highly hydroxymethylated in Th1 cells but hypermethylated in other Th cells (Figure S2A). Similarly, the CNS2 *Il17a* and *Il17f* promoters of the *Il17* locus were strongly hydroxymethylated in Th17 cells but were hypermethylated in other Th cells (Figure S2B). In addition to lineage-specific cytokines, we also analyzed *Il10*, which is expressed by virtually every Th cell subset (Ouyang et al., 2011). As expected, 5hmC was closely marked with some CNSs of *Il10* in Th1, Th2, and Th17 cells and naive T cells showed strong 5mC peaks in these regions (Figures 2A and S2C). On the other hand, we could not detect substantial IL-10 production or augmented 5hmC signals in iTreg cells (Figure 2A and data not shown). It was also obvious that many 5hmC peaks were shared by several lineages, while some lineage-specific peaks were associated with the promoter and CNS regions of lineage-specific genes such as *Ifng*, *Il17a*, and *Il17f* (Table S3). As we mentioned above, cells cultured with in vitro polarized conditions are a heterogeneous population regarding cytokine production. To assess whether the existence of non-cytokine-producing cells affect the results of 5hmC mapping, we used cytokine gene reporter mice (*Ifng*^{fyp}, *Il4*^{gfp}, and *Il17f*^{Cre}*Rosa26*^{yfp}) to purify fully polarized Th cells. DIP-PCR analysis revealed that DNA hydroxymethylation on signature genes was very similar before and after cell sorting (Figure S3).

Lineage-specific transcription factors such as T-bet, GATA3, RORγt, and Foxp3 are well known as master regulators essential for development and function of Th1, Th2, Th17, and Treg cells, respectively. Therefore, we next examined the distribution of 5hmC and 5mC in genes encoding these key transcription

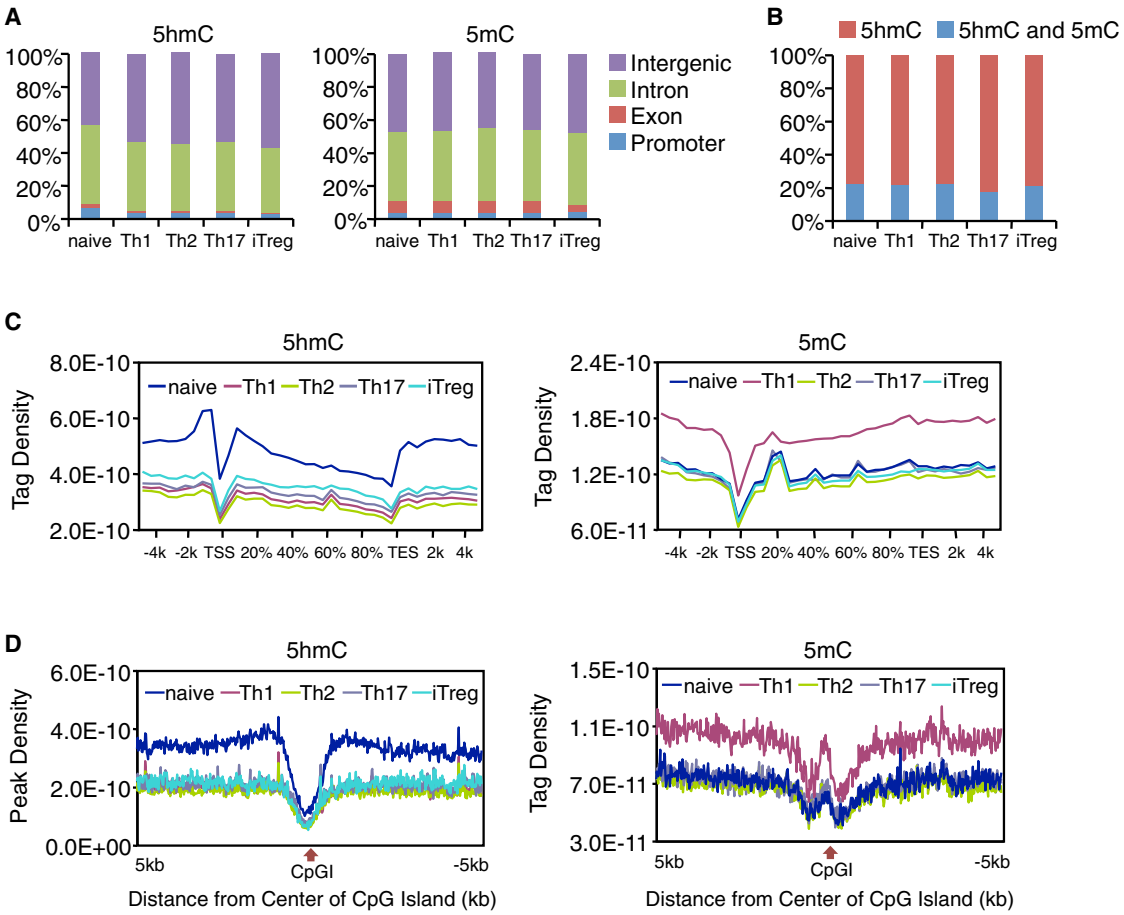


Figure 1. Comparison of Genome-wide 5hmC and 5mC Distribution in Each T Cell Subset

(A) The distribution of 5hmC and 5mC modifications was analyzed on the basis of location: promoter (within 1 kb upstream from the transcription start site), exon, intron, and intergenic regions.

(B) The bar graph showing the percentage of peaks uniquely associated with just 5hmC or both 5hmC and 5mC.

(C) The normalized tag density profiles for 5hmC (left) and 5mC (right) across gene body ± 5 kb flanking regions with 200 bp resolution are shown.

(D) The normalized tag density profiles for 5hmC (left) and 5mC (right) around ± 5 kb regions flanking CpGI centers with 100 bp resolution are shown.

See also Figure S1 and Tables S1 and S2.

factors. *Tbx21* and *Foxp3* were indeed associated with high 5hmC in Th1 and iTreg cells, respectively, implying regulation of these genes by active DNA demethylation (Figure 2B). Especially, CNS2 in *Foxp3* was intensely demethylated in iTreg cells. This is consistent with a report on hypomethylation of CNS2 in Foxp3 expression (Ohkura et al., 2012). However, prominent 5hmC peaks were located in *Rorc* and *Gata3* in even non-expressing cell lineages (Figure 2B). Thus, these observations suggest that the expression of *Gata3* and *Rorc* might not be regulated by active DNA demethylation.

Collectively, our data prove that 5mC and 5hmC at lineage-specific signature cytokine genes seem to correlate with their expression in Th cell subsets and suggest that active DNA demethylation might be one important mechanism for gene regulation during Th cell development.

5hmC Marks Transcriptional Regulatory Regions

Epigenetic control of gene expression involves dynamic regulation of DNA methylation and histone modifications. Although several studies assessed the regulation of DNA methylation by histone modification patterns (Cedar and Bergman, 2009), the relationship between active DNA demethylation and histone modification remain unclear. To correlate DNA demethylation and histone modifications, we used published chromatin data from Th cell subsets available in databases (Wei et al., 2009). In silico analysis revealed that 5hmC peaks in Th-cell-specific signature genes partly colocalized with H3K4me3 modification, a histone marker associated with transcriptionally active state (Figure S4A). These findings support previous reports that 5hmC is enriched in euchromatin in cerebellar cell types (Mellén et al., 2012) and that enhanced H3K4me3 appeared to be correlated with CpG hypomethylation within the Treg-cell-associated genes (Ohkura et al., 2012). Yet the overall picture of H3K4me3 distribution pattern seemed not to match that of the 5hmC distribution pattern in each Th cell subset (Figure S4B). As previously reported, the majority of H3K4me3 is enriched near TSSs (Wei et al., 2009), whereas the majority of 5hmC is depleted from TSSs (Figures 1 and S4B). In addition, we sought to calculate

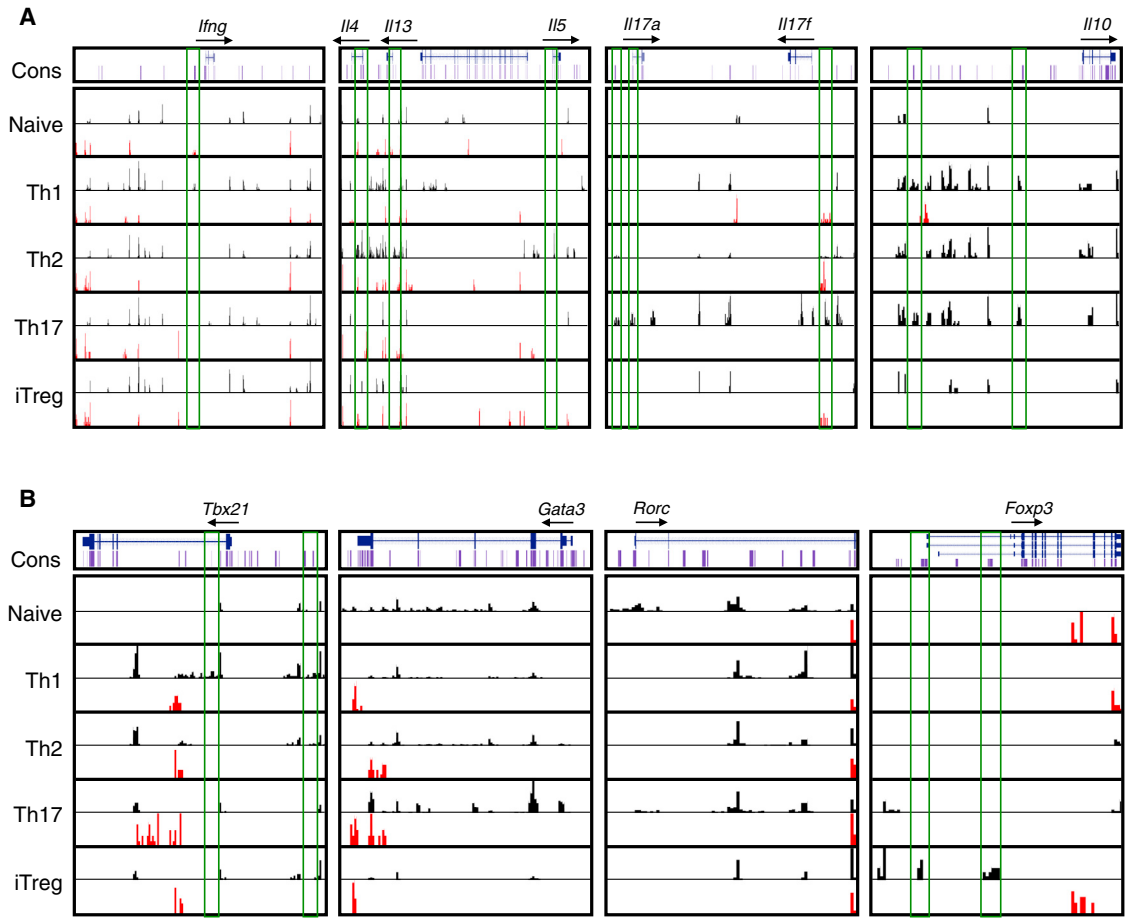


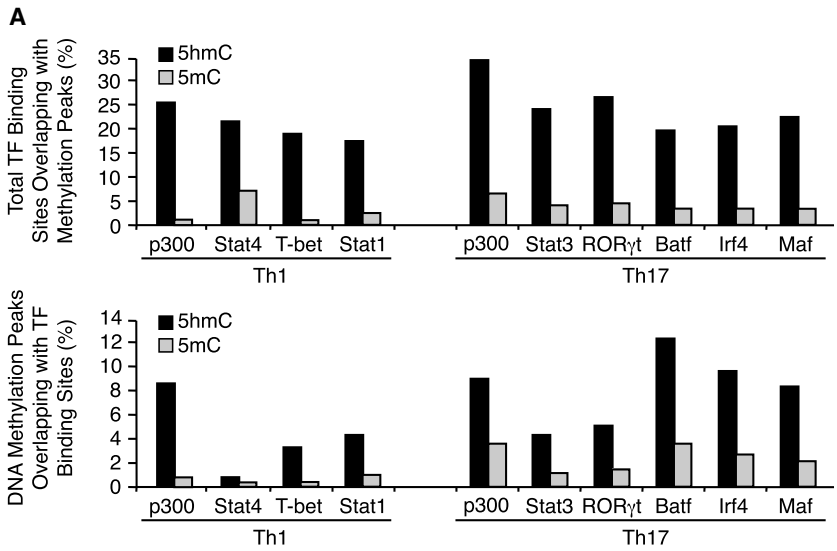
Figure 2. 5hmC and 5mC Modifications of Signature Cytokine Genes and Transcription Factor Genes in Th Cells

Distribution of 5hmC and 5mC in the *Ifng* (chr10: 117,810,000–117,940,000), *Il4*, *Il13*, *Il5* (chr11: 53,420,500–53,553,500), *Il17a*, *Il17f* (chr1: 20,713,500–20,787,300), *Il10* (chr1: 132,884,100–132,923,100), (A) *Tbx21* (chr11: 96,958,500–96,987,500), *Gata3* (chr2: 9,777,000–9,802,000), *Rorc* (chr3: 94,175,000–94,191,200), and *Foxp3* (chrX: 7,153,000–7,170,500) (B) genomic regions in each T cell subset is shown. All figures with views of 5hmC and 5mC distribution are labeled such that the arrow represents the direction of gene transcription. Gene structure is downloaded from the UCSC Genome Browser, and only tags on islands are shown. The islands labeled in black represent 5hmC. The islands labeled in red represent 5mC. Scales are kept constant among cell types. Unique peaks are highlighted by green squares. See also Figures S2 and S3 and Table S3.

the total number of peaks that are overlapped between modifications. Expectedly, H3K4me3 preferentially overlapped with 5hmC rather than 5mC. However, only around 10% to 20% of H3K4me3 sites were shared with 5hmC islands in each Th cell subset (Figure S4C). Thus, generally speaking, a minority of these two modifications was overlapped in each Th cell subset. Collectively, although active DNA demethylation is correlated with the enhancement of H3K4me3, this relationship seems to be limited to certain regions of linage-specific signature gene loci.

To further characterize whether 5hmC contributes to the transcriptional regulation during Th cell differentiation, we then analyzed whether 5hmC was colocalized with binding sites for active enhancer-defining factor p300. The genome-wide p300 binding sites have been previously defined by Vahedi et al. (2012) in Th1 cells as well as by Ciofani et al. (2012) in Th17 cells. As shown in Figure 3A, 26%–34% of p300 binding sites colocalized with 9%–10% of 5hmC peaks in Th1 and Th17 cells, whereas only 2%–8% of p300 binding sites colocalized with 1%–4% of 5mC peaks in these cells. Taken together, our data demonstrate a genome-wide enrichment of 5hmC modification on the active enhancers in effector T cells.

Although our data indicated that 5hmC was depleted from TSSs and proximal promoters as well as the centers of CpGIs, we found that the binding sites of key transcription factors as well as evolutionarily conserved enhancers marked by p300 co-localized with 5hmC but not 5mC on lineage-specific signature gene loci in Th1 and Th17 cells (Figure 3B). We therefore explored the colocalization between 5hmC and binding sites of key transcription factors governing Th1 and Th17 cell development, particularly Stat4, T-bet, and Stat1 in Th1 cells (Vahedi et al., 2012), as well as Stat3, RORγt, Batf, Irf4, and Maf (Ciofani et al., 2012). As expected, 17%–26% of these transcription factor binding sites were localized at 5hmC-occupied genomic regions, whereas only 1%–7% of them co-localized with 5mC peaks (Figure 3A). Moreover, enrichment of 5hmC modification was found on the binding sites of all tested transcription factors (Figure 3A).



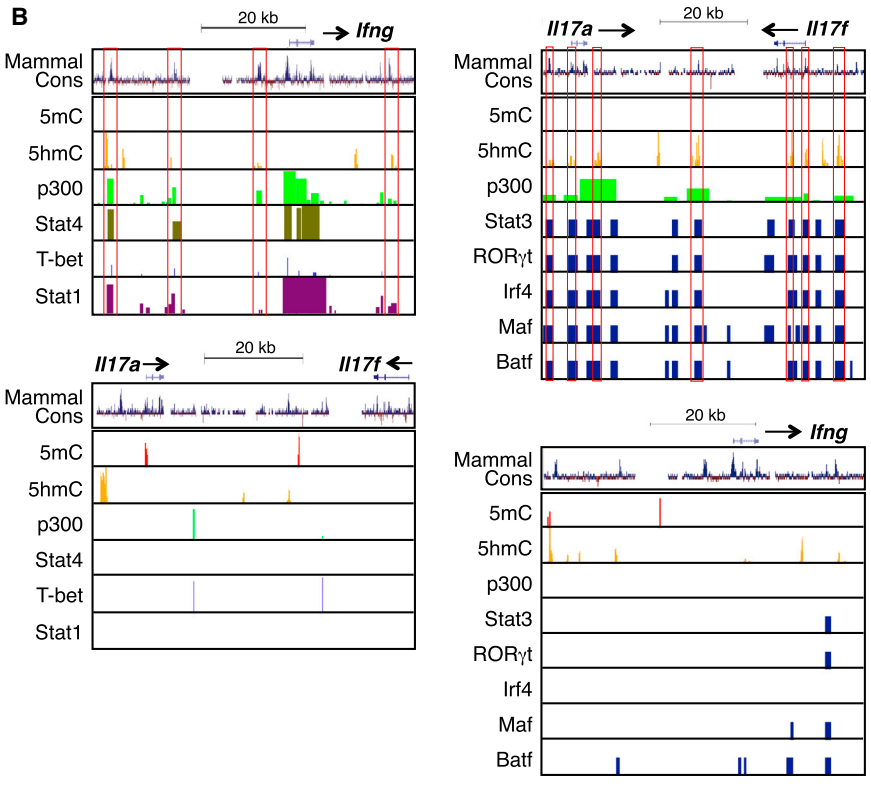


Figure 3. Colocalization of 5hmC with Active Enhancers Bound by p300 and Key Transcription Factors in Th1 and Th17 Cells

(A) Top: the percentages of total p300 and transcription factor binding sites colocalized with 5mC (gray) and 5hmC (black) peaks was shown in Th1 and Th17 cells. Bottom: the percentages of 5mC (gray) and 5hmC (black) peaks that were co-localized with p300, Stat4, T-bet, and Stat1 binding sites in Th1 cells (left), as well as with p300, Stat3, Rorc, Batf, Irf4, and Maf binding sites in Th17 cells (right) is shown.

(B) Distribution of 5hmC, 5mC, p300, and transcription factors binding sites along *Ifng*, *Il17a*, and *Il17f* genomic region in Th1 (Stat4, T-bet, Stat1) (left) and Th17 (Stat3, RORγt, Irf4, Maf, Batf) (right) cells are shown. The regions of overlapping peaks are highlighted by red squares.

See also Figure S4.

Tet2 Recruitment to the Cytokine Genes Is Dependent on Lineage-Specific Key Transcription Factors

Recently, it has been reported that proteins of Tet family (TET1, TET2, and TET3) can regulate gene transcription by converting 5mC to 5hmC. As a first step to verify the role of Tet proteins in Th cell development, we examined the mRNA expression of *Tet* in each Th cell subset. Consistent with a previous report (Ko et al., 2010), naive $CD4^+$ T cells expressed all *Tet* family members in high amounts (Figure 4A). Although the expression of all *Tet* members was downregulated after TCR-mediated activation, *Tet2* was highly expressed in each Th cell subset compared to other *Tet*. This result led us to a hypothesis that Tet2 might play a crucial role in Th cell differentiation.

Because we have identified 5hmC-associated regions, we analyzed whether Tet2 is recruited to these loci in various Th cell lineages by chromatin immunoprecipitation (ChIP) assay. As expected, in Th1 cells, Tet2 was found recruited to CNS(−6) and promoter regions of the *Ifng* locus that contain 5hmC, but not to promoter region of *H19* imprinted gene (Figure 4B). Tet2 needs co-factor(s) for binding to DNA, because it does not have a CXXC DNA binding domain (Pastor et al., 2013). Previously, one group reported that T-bet was bound to CNS(−6) and promoter regions of the *Ifng* locus in Th1 cells (Hatton et al., 2006). Therefore, we decided to assess the role of T-bet in Tet2 recruitment. According to hMeDIP and ChIP analysis with *Tbx21*-deficient T cells, 5hmC and Tet2 recruitment in *Ifng* were reduced in *Tbx21*-deficient Th1 cells (Figure 4B). Thus, these results suggest that Tet2 induces DNA demethylation at the *Ifng* locus in a T-bet-dependent manner in Th1 cells.

Similarly, we examined the recruitment of Tet2 to the *Il17a* and *Il17f* loci in Th17 cells. We previously reported that the CNS2 region of the *Il17a* and *Il17f* loci is essential for the function of ROR factors in *Il17a* expression (Wang et al., 2012). Therefore, we examined the association of Tet2 in CNS2 and *Il17a* promoter region. As shown in Figure 4C, Tet2 associated with CNS2 and *Il17a* promoter regions, which correlated with existence of 5hmC at these sites. Furthermore, 5hmC and Tet2 recruitment were diminished in the absence of CNS2 region or RORγt (Figure 4C), indicating that Tet2 induces DNA demethylation at the *Il17* locus in a RORγt-dependent manner in Th17 cells. In

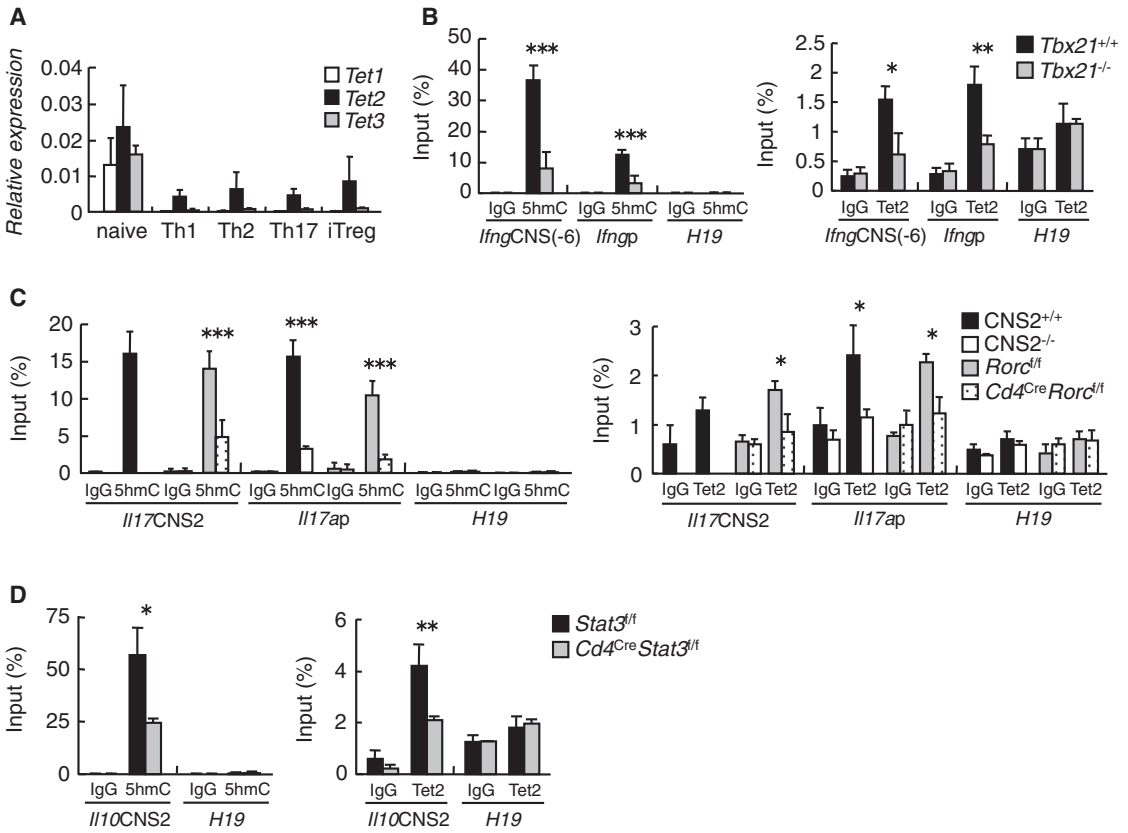


Figure 4. The Recruitment of Tet2 to the Signature Cytokine Gene Loci Is Dependent on Lineage-Specific Transcription Factors

(A) Naive T cells were cultured under differentiation conditions for each Th cell subset. Tet family mRNA expression was analyzed by real-time RT-PCR.

(B) Naive T cells from WT and *Tbx21*$^{-/-}$ mice were cultured under Th1 cell conditions for 4 days. 5hmC (left) and Tet2 accessibility (right) in *Ifng* were analyzed by hMeDIP- and ChIP-PCR, respectively.

(C) Naive T cells from littermate control and *CNS2*$^{-/-}$ or *CD4*^{Cre}*Rorc*$^{f/f}$ mice were cultured under Th17 cell conditions for 4 days. 5hmC (left) and Tet2 accessibility (right) in *Il17* were analyzed by hMeDIP- and ChIP-PCR, respectively.

(D) Naive T cells from *CD4*^{Cre}*STAT3*$^{f/f}$ and littermate control mice were cultured under Th17 cell conditions for 4 days. 5hmC (left) and Tet2 accessibility (right) in *Il10* were analyzed by hMeDIP- and ChIP-PCR, respectively.

The data represent the average of at least three independent experiments and shown as mean ± SD. See also Figure S5.

contrast, although 5hmC peaks of lineage signature genes were observed in Th2 and iTreg cells, we could not detect the strong recruitment of Tet2 to these regions (Figures 2, S5A, and S5B).

A previous study indicated that Th17 cells produce IL-10 in a STAT3-dependent manner (Stumhofer et al., 2007). Therefore, we also analyzed the *Il10* locus under the Th17 cell condition by use of STAT3-deficient T cells or their controls. Tet2 was found recruited to the CNS2 region of the *Il10* locus, which contained 5hmC, in a manner dependent on STAT3 (Figure 4D). Collectively, these results suggest that Tet2 induces active DNA demethylation of signature cytokine genes in a particular Th cell subset and that its action is dependent on lineage-specific transcription factors.

Tet2 Regulates Th1 and Th17 Cell Differentiation

The above analysis has suggested 5hmC and Tet2 in Th cell differentiation. To further assess the role of Tet2 in Th cells, we analyzed the differentiation of each Th cell subset by using *Tet2*-deficient (*Tet2*$^{-/-}$) CD4$^+$ T cells derived from *Cd2*^{Cre}*Tet2*$^{f/f}$ mice (Moran-Crusio et al., 2011) in vitro. In accordance with previous reports (Ko et al., 2011), *Cd2*^{Cre}*Tet2*$^{f/f}$ mice had normal development of CD4$^+$ and CD8$^+$ T cells and B cells (data not shown). In support of Figures S5A and S5B, both intracellular staining and qPCR analysis revealed that the differentiation of Th2 and iTreg cells were not affected in *Tet2*$^{-/-}$ T cells (Figures S5C–S5E, S5G, and S5H). Additionally, 5hmC marks in signature genes were similar between *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ T cells (Figures S5F and S5I). In contrast, deletion of Tet2 led to 80%–83% reduction in genome-wide 5hmC peaks in Th1 and Th17 cells (Figure S6A). Most of the 20% residual 5hmC peaks in *Tet2*$^{-/-}$ cells existed in wild-type Th1 and Th17 cells, suggesting that in addition to Tet2, other Tet family members might also be responsible for maintaining the DNA demethylation process in Th1 and Th17 cells. We also analyzed the general difference in tag density of 5hmC around gene promoters, CpGIs, as well as all 5hmC sites that appeared in wild-type Th cells. As shown in Figure S6A, the depletion of 5hmC around TSSs and CpGIs was only slightly reduced in Tet2-deficient Th1 cells. However, the depletion of 5hmC in TSSs and CpGIs in WT Th17 cells was totally reversed in Tet2-deficient Th17 cells. Moreover, the reduction of 5hmC

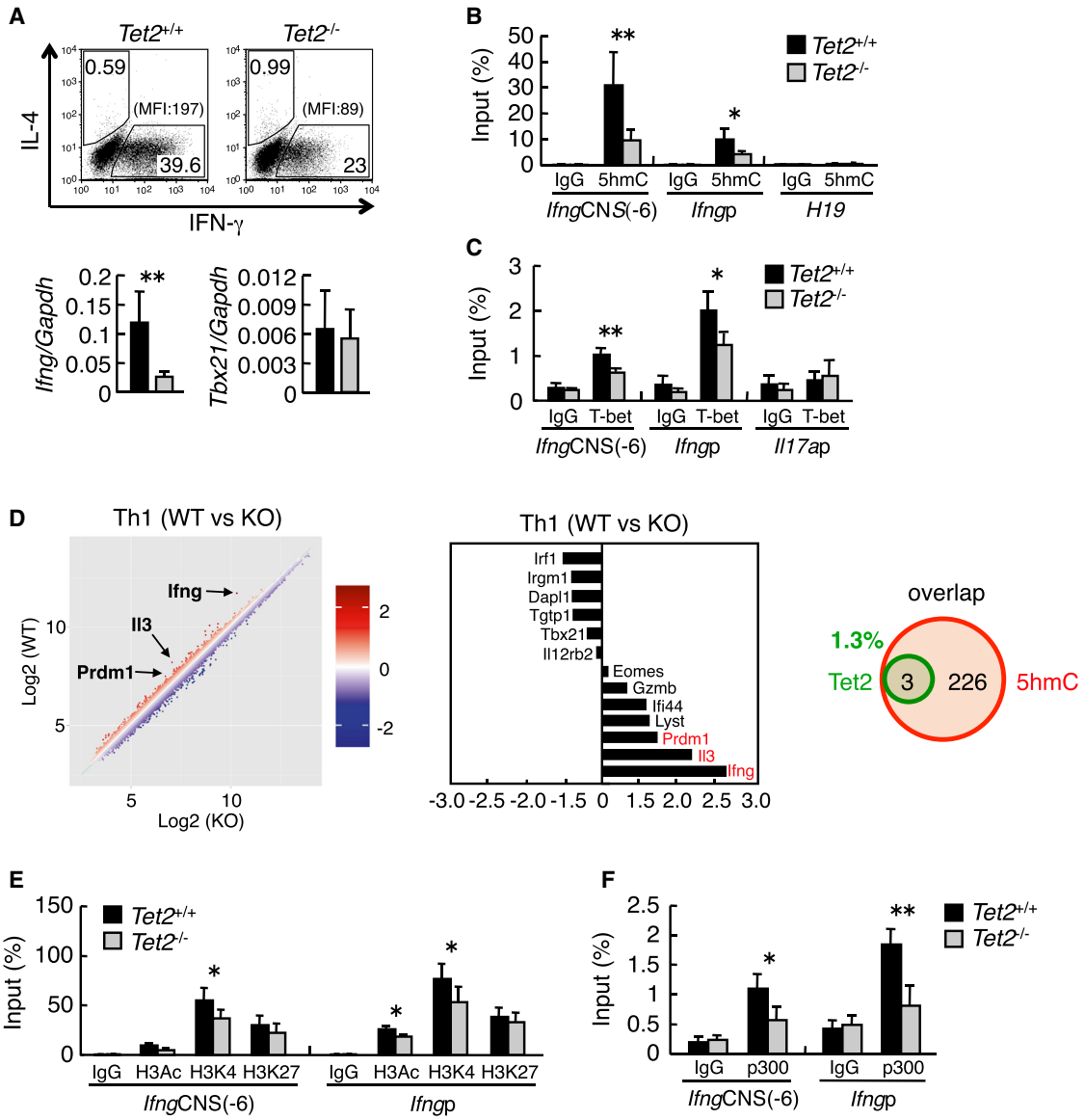


Figure 5. Tet2 Is Important for Th1 Cell Differentiation In Vitro

(A) Top: intracellular cytokine staining after 5 days of Th1 cell differentiation from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice. Data are representative of at least three individual experiments. Bottom: the expression of *Ifng* and *Tbx21* mRNA by *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ Th1 cells.

(B and C) The hMeDIP (B) and ChIP (C) on *Ifng* were performed in Th1 cells from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice.

(D) Microarray analysis comparing *Tet2*$^{+/+}$ versus *Tet2*$^{-/-}$ Th1 cells. The bar graph showed representative genes. Venn diagram showing the number of 5hmC-containing genes affected or unaffected by Tet2 deficiency. 5hmC-containing genes were identified if at least one peak of α-5hmC binding was present on their promoter plus gene body regions (−3K to TTS).

(E and F) The ChIP of *Ifng* was performed in Th1 cells from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice.

(A–C, E, F) The data represent the average of at least three independent experiments. All the data are shown as mean ± SD. See also Figures S5 and S6 and Table S4.

tag density as a result of Tet2 deficiency was more profound in Th17 cells than in Th1 cells, suggesting that Tet2 deficiency might have more global effect on Th17 cells than on Th1 cells.

Under Th1 cell polarization condition, *Tet2*$^{-/-}$ T cells showed a marked reduction in IFN-γ at mRNA and protein levels, but *Tbx21* expression and cell proliferation were not affected (Figures 5A and S6B–S6D). Furthermore, 5hmC marks in *Ifng* were decreased by Tet2 deficiency in Th1 cells (Figure 5B). In contrast, we found that 5hmC status in *Tbx21* was not changed by Tet2 deficiency (Figure S6E). Moreover, Tet2 did not bind to this region in Th1 cells (Figure S6F), suggesting the involvement of other Tet family proteins. The recruitment of T-bet in *Ifng* was also substantially decreased by Tet2 deficiency in Th1 cells (Figure 5C). Because Tet2 recruitment to the *Ifng* locus is dependent on T-bet (Figure 4B), Tet2 and T-bet might reciprocally regulate the function of each other in *Ifng* expression. To globally identify

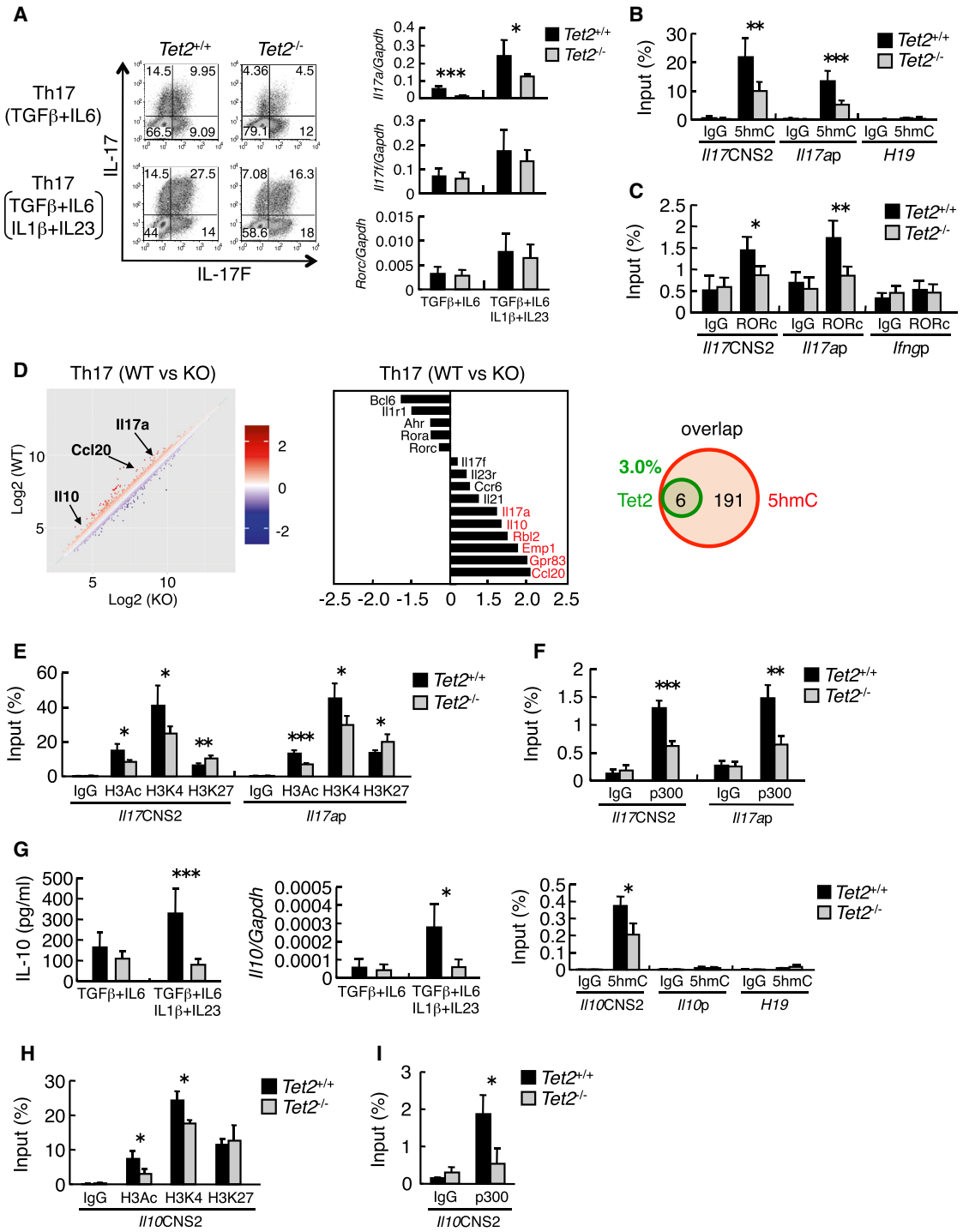


Figure 6. Tet2 Controls Th17 Cell Development In Vitro

(A) Left: intracellular cytokine staining after 4 days of Th17 cell differentiation from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice. Data are representative of at least three individual experiments. Right: the expression of *Il17a*, *Il17f*, and *Rorc* mRNA in Th17 cells from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice.

(B and C) The MeDIP (B) and ChIP (C) on *Il17* were performed in Th17 cells from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice.

(D) Microarray analysis comparing *Tet2*$^{+/+}$ versus *Tet2*$^{-/-}$ Th17 cells. The bar graph shows representative genes. Venn diagram showing the number of 5hmC-containing genes affected or unaffected by Tet2 deficiency. 5hmC-containing genes were identified if at least one peak of α-5hmC binding was present on their promoter plus gene body regions (−3K to TTS).

(E and F) The ChIP of *Il17* was performed in Th17 cells from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice.

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the target genes of Tet2 in Th1 cells, we next performed DNA microarray analysis by using *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ Th1 cells. Tet2 target genes were then identified based on microarray and hMeDIP-seq data. A 1.5-fold change (WT versus KO) was used as a cutoff for significance. Although a total of 229 Th1-cell-specific genes (genes expressed at least 2-fold higher in Th1 than Th17 cells) had 5hmC in Th1 cells, surprisingly, only the expression of *Ifng*, *Il3*, and *Prdm1* were changed due to Tet2 deficiency (1.3%, 3/229) (Figure 5D). On the other hand, we found 51 genes with at least 1.5-fold difference in gene expression between *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ Th1 cells, 26 of which have 5hmC peaks on their promoters and/or gene bodies (Table S4), suggesting that Tet2 might regulate the expression of genes without 5hmC modification.

As mentioned above, 5hmC partly correlates with H3K4me3 (Figure S4). Recently, it has been reported that Tet2 facilitates GlcNAcylation and H3K4 methylation (Deplus et al., 2013). Therefore, to clarify whether Tet2 regulates the chromatin modification in Th cells, we analyzed H3Ac, H3K4me3, and H3K27me3 modifications at *Ifng* in *Tet2*$^{-/-}$ Th1 cells by ChIP assay. As shown in Figure 5E, in CNS(−6) and *Ifng* promoter regions, permissive histone markers H3Ac and H3K4me3 were modestly reduced in *Tet2*$^{-/-}$ Th1 cells. Additionally, to further understand how Tet2 might regulate chromatin accessibility, we also evaluated the recruitment of p300 to the *Ifng* locus. The deficiency of Tet2 resulted in marked inhibition of the binding of p300 to CNS(−6) and the promoter region of *Ifng* (Figure 5F).

Next, we sought to examine the role of Tet2 in Th17 cells in the same manner. Under Th17 cell differentiation conditions, *Tet2*$^{-/-}$ T cells showed a marked suppression in IL-17 mRNA and protein expression compared to *Tet2*$^{+/+}$ T cells, but the expression of IL-17F, IL-2, and RORγt as well as cell expansion were normal (Figures 6A and S6G–S6I). In addition, we examined the effect of Tet2 on 5hmC peaks and RORγt recruitment in the *Il17* locus. As expected, the deficiency of Tet2 led to a reduction of 5hmC and RORγt binding at CNS2 and *Il17a* promoter regions in Th17 cells (Figures 6B and 6C), implying a reciprocal regulation of Tet2 and RORγt function in *Il17a* expression. To assess the target genes of Tet2 in Th17 cells, we also performed microarray analysis with *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ Th17 cells. Based on the analysis of microarray and MeDIP-seq results, only six genes (*Ccl20*, *Il10*, *Gpr83*, *Emp1*, *Rbl2*, and *Il17a*) were found as Tet2-regulated genes from a total of 197 Th17-cell-specific genes (genes that are expressed at least 2-fold higher in Th17 than Th1 cells) with 5hmC in Th17 cells (3%, 6/197) (Figure 6D). Similar to the finding in Th1 cells, about 50% of genes with at least 1.5-fold difference in gene expression between *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ Th17 cells had 5hmC peaks on their promoter and/or gene body regions (Table S4). To evaluate the effect of Tet2 on chromatin remodeling at *Il17* locus, we examined histone modifications and p300 recruitment in *Tet2*$^{-/-}$ Th17 cells. As shown in Figure 6E, H3Ac and H3K4me3 were reduced in *Tet2*$^{-/-}$ Th17 cells along with increased repressive histone marker H3K27me3 at CNS2 and *Il17a* promoter. Importantly, the deficiency of Tet2 led to considerable inhibition in binding of p300 to CNS2 and *Il17a* promoter regions (Figure 6F).

Microarray and hMeDIP-seq analysis suggested that Tet2 regulates IL-10 production in Th17 cells. Indeed, under Th17 cell conditions, *Tet2*$^{-/-}$ T cells displayed a considerable repression of IL-10 production compared with *Tet2*$^{+/+}$ T cells (Figure 6G). In addition, we also evaluated 5hmC status of *Il10* in *Tet2*$^{-/-}$ T cells. As expected, the ablation of Tet2 resulted in a reduction of 5hmC at the CNS2 region but not the *Il10* promoter (Figure 6G). To elucidate the impact of Tet2 on the chromatin structure of the *Il10* locus, we analyzed histone modifications and p300 recruitment in the CNS2 region of *Il10* in *Tet2*$^{-/-}$ Th17 cells. H3Ac and H3K4me3 marks as well as p300 recruitment were diminished in the absence of Tet2 (Figures 6H and 6I). As previously reported (Ouyang et al., 2011), IL-10-producing IFN-γ^{+} Th1 cells are present in a variety of infections and they play an important task of protecting against severe immune-mediated pathology. Therefore, we also assessed the role of Tet2 in IL-10 production from Th1 cells. Similar to Th17 cells, the induction of IL-10 and 5hmC in the CNS2 region of *Il10* was strongly abrogated by Tet2 deficiency in Th1 cells (data not shown).

Collectively, our results demonstrate that Tet2 promotes the development of Th1 and Th17 cells by regulating specifically the expression of signature cytokines through active DNA demethylation and that its deficiency had some effect on chromatin remodeling, especially p300 recruitment to the target genes.

Tet2 Controls IL-10, IFN-γ, and IL-17 Production in Autoimmunity

To examine whether Tet2 is critical for Th cell differentiation in vivo, we first immunized mice with MOG_{35-55} peptide and analyzed the spleens of *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice on day 7. Similar to in vitro results, we found that the MOG-specific production of IFN-γ and IL-10 were decreased in *Cd2*^{Cre}*Tet2*$^{f/f}$ mice (Figure S7A). There was no or slight difference in the production of MOG-specific IL-17F and IL-2 between *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice (data not shown). However, in contrast to in vitro results, the production of MOG-specific IL-17 was comparable between *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice (Figure S7A). It has been known that IL-10 plays a critical role in suppressing autoimmunity and inflammatory responses. A previous study demonstrated that IL-10 produced by Th17 cells restrains the pathologic effects of Th17 cells (McGeachy et al., 2007). Therefore, to clarify whether the unexpected high production of IL-17 in *Cd2*^{Cre}*Tet2*$^{f/f}$ mice was caused by the reduced IL-10 production, we treated mice with IL-10 receptor 1-specific blocking antibody (α-IL-10R Ab). After treating with α-IL-10R Ab, *Cd2*^{Cre}*Tet2*$^{f/f}$ mice showed a marked reduction of MOG-specific IL-17 and IFN-γ production (Figure 7A), implying that Tet2 plays a similar role in Th1 and Th17 cell differentiation in vitro and in vivo.

To further address the function of Tet2 in autoimmune responses, both *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice were subjected to

(G) Left: ELISA analysis of supernatants from Th17 cell differentiation on day 4. All groups were analyzed in triplicate. Middle: the expression of *Il10* mRNA in Th17 cells from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice. Right: a hMeDIP of *Il10* and *H19* loci was performed in Th17 cells from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice.
(H and I) The ChIP of *Il10* was performed in Th17 cells from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice.
(A–C, E–I) The data represent the average of at least three independent experiments. All the data are shown as mean ± SD. See also Figure S6 and Table S4.

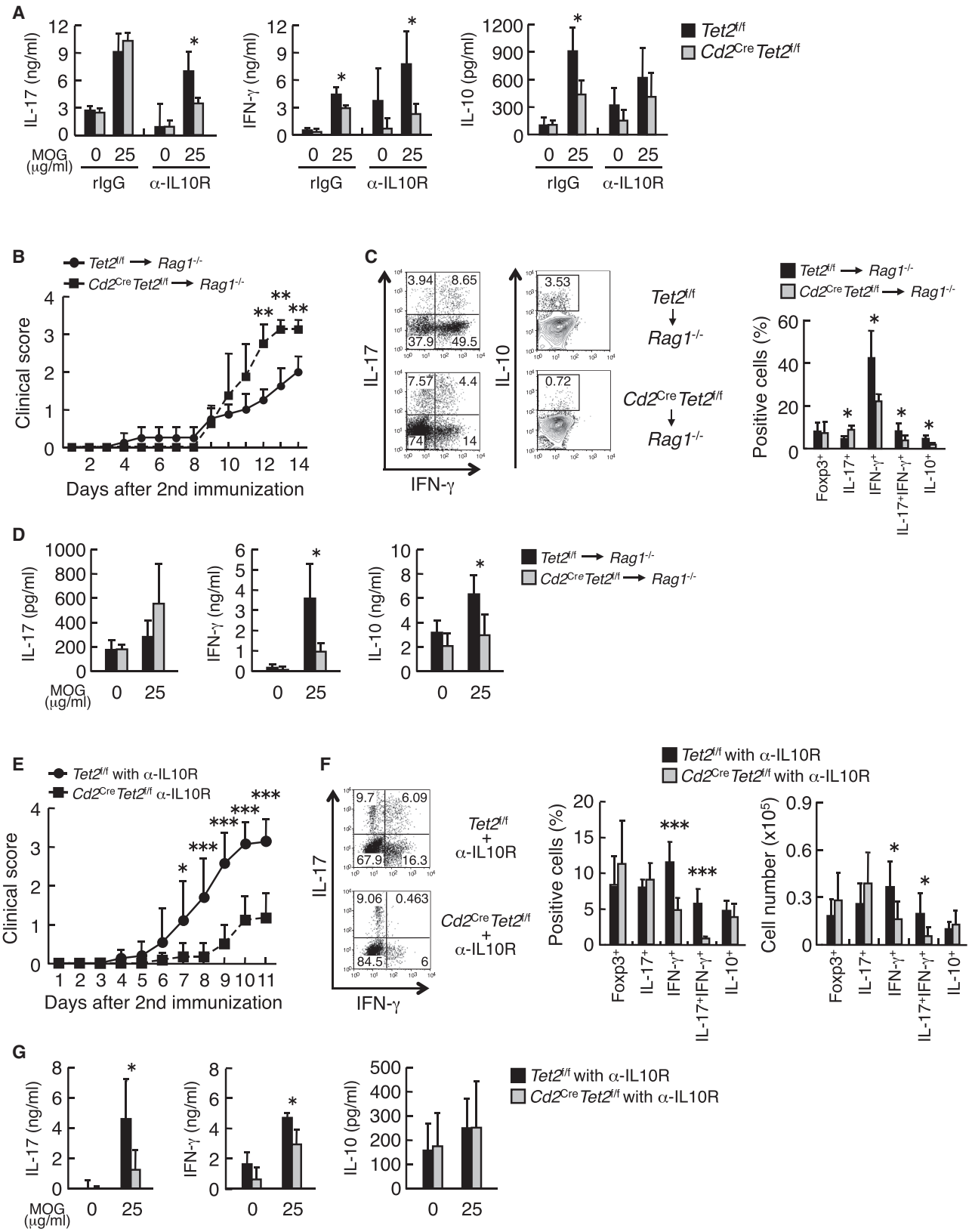

Figure 7. Tet2 Regulates Cytokine Production in Autoimmunity

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MOG peptide-induced experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis (Baxter, 2007). The severity of EAE diseases was enhanced in *Tet2*-deficient mice (Figure S7B). The frequency and absolute number of IL-17$^+$ T cells in the central nervous system on day 13 after the second immunization were strongly elevated in *Cd2*^{Cre}*Tet2*$^{f/f}$ mice, whereas the frequencies and absolute numbers of IFN-γ^+, IFN-γ^+IL-17$^+$, IL-10$^+$, and IL-10$^+$IL-17$^+$ T cells in the central nervous system were decreased in *Cd2*^{Cre}*Tet2*$^{f/f}$ mice (Figures S7C and S7D). In agreement with in vitro findings, the frequency and absolute number of Foxp3$^+$ T cells in the central nervous system were similar between *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice (Figure S7D). The *Cd2*^{Cre} transgenic mice are useful for generating conditional mutations not only in T cells but also in B cells (de Boer et al., 2003). To confirm the CD4$^+$ T-cell-intrinsic function of Tet2 in vivo, we transferred *Tet2*$^{f/f}$ or *Cd2*^{Cre}*Tet2*$^{f/f}$ CD4$^+$ T cells into *Rag1*$^{-/-}$ mice and immunized them with MOG peptide. The mice transferred with *Tet2*$^{f/f}$ CD4$^+$ T cells developed disease score around 2.0, whereas the mice transferred with *Cd2*^{Cre}*Tet2*$^{f/f}$ CD4$^+$ T cells had more severe symptoms (score around 3.0) (Figure 7B). The frequencies of IFN-γ^+, IFN-γ^+IL-17$^+$, and IL-10$^+$ T cells in the central nervous system on day 14 after the second immunization, but not IL-17$^+$ and Foxp3$^+$ T cells, were much lower in the mice that received *Cd2*^{Cre}*Tet2*$^{f/f}$ CD4$^+$ T cells than *Tet2*$^{f/f}$ CD4$^+$ T cells (Figure 7C). Furthermore, we found that a marked reduction of IFN-γ and IL-10 secretion, but not IL-17, were also observed by ELISA assays in the splenocytes of mice that received *Cd2*^{Cre}*Tet2*$^{f/f}$ CD4$^+$ T cells (Figure 7D). These results indicate that Tet2 expression in CD4$^+$ T cells plays an important role in the suppression of disease severity of EAE.

To address the mechanism whereby Tet2 restrains the disease severity in EAE, we did EAE experiments in *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice with α-IL-10R Ab treatment at every 4 days during EAE. As a result of the treatment, the severity of EAE diseases was reduced in α-IL-10R Ab-treated *Cd2*^{Cre}*Tet2*$^{f/f}$ mice (Figure 7E). The enhancement of IL-17$^+$ T cells in *Cd2*^{Cre}*Tet2*$^{f/f}$ mice was abolished by neutralization of IL-10 (Figure 7F). Furthermore, the frequencies and absolute numbers of IFN-γ^+ and IFN-γ^+IL-17$^+$ T cells but not Foxp3$^+$ and IL-10$^+$ T cells in the central nervous system on day 11 after the second immunization were decreased in α-IL-10R Ab-treated *Cd2*^{Cre}*Tet2*$^{f/f}$ mice (Figure 7F). In addition, the strong reduction of IFN-γ and IL-17 secretion was also observed in splenocytes of α-IL-10R Ab-treated *Cd2*^{Cre}*Tet2*$^{f/f}$ mice (Figure 7G).

Taken together, we conclude that Tet2 plays an important role in regulating the expression of IL-10, IL-17, and IFN-γ in vivo and in regulating T-cell-mediated autoimmune diseases.

DISCUSSION

Epigenetic mechanisms have been proposed to regulate the specification and plasticity of Th cell lineages (Wei et al., 2009). Active DNA demethylation by Tet proteins has been recently identified with critical roles in many physiological processes. Here we provide evidence that 5hmC and Tet2 play important roles in T cell function, particularly cytokine expression.

In this study, we first generated genome-wide maps of 5mC and 5hmC modifications in Th cell subsets differentiated in vitro by use of high-throughput DIP-seq approach. Consistent with other studies (Tsagaratou et al., 2014), we found that 5hmC is enriched in gene body and enhancer regions. However, a decrease of the overall amount of 5hmC was found when naive CD4$^+$ T cells differentiated into effector cells. 5hmC cannot be recognized by DNMT1, and therefore cannot be maintained during DNA replication and cell proliferation (Valinluck and Sowers, 2007). A proliferation-dependent passive DNA demethylation, suggested by the decrease of 5hmC during differentiation of naive CD4$^+$ T cells into effectors, might be crucial for the relief of methylation-dependent transcriptional repression in naive T cells. More interestingly, the lineage-specific cytokine as well as some of the master transcription factors were tightly regulated by DNA methylation in naive T cells. The repression of these genes' expression by 5mC was removed only in the lineage specified by the factors, but was maintained in other lineages, strongly suggesting that the transition from 5mC to 5hmC was an active (replication-independent) DNA demethylation process.

It is unclear whether DNA demethylation-dependent gene regulation is controlled by lineage-defining transcription factors. Our data suggest that a proportion of key transcription factor as well as p300 binding sites co-localize with 5hmC but not 5mC in Th1 and Th17 cells, as well as Th2 cells, but not iTreg cells (data not shown). More importantly, the recruitment of Tet2 as well as DNA demethylation is dependent upon key transcription factors such as T-bet, RORγt, and Stat3, indicating a critical role of

(A) Age-matched *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice (n = 4) were immunized with MOG$_{35-55}$ peptides on day 0 and then analyzed on day 7 for IL-17, IFN-γ, and IL-10 production. For neutralization of IL-10, α-IL-10R or α-Rat IgG1 was intraperitoneally injected on day 0 and day 4. Data are representative of at least two individual experiments.

(B) *Rag1*-deficient mice were reconstituted with CD4$^+$ T cells derived from *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice. Clinical scores were monitored daily after EAE induction with MOG$_{35-55}$.

(C) Left: IL-17, IFN-γ, and IL-10 were determined in the infiltrates of central nervous system of immunized mice. Right: statistic of the frequency.

(D) IL-17, IFN-γ, and IL-10 were measured by ELISA in the splenocytes of immunized mice, after restimulation with indicated concentration of MOG peptide (μg/ml).

(E) Age-matched *Tet2*$^{f/f}$ and *Cd2*^{Cre}*Tet2*$^{f/f}$ mice were immunized twice with MOG$_{35-55}$. For neutralization of IL-10, 500 μg α-IL-10R or isotype control antibody was administered intraperitoneally every 4 days after immunization. Shown here is the combinational result of two individual EAE experiments (the total number of mice used: WT = 8, KO = 10).

(F) Left: IL-17 and IFN-γ were determined in the infiltrates of central nervous system of immunized mice with α-IL-10R. Right: statistic of the frequency and absolute number.

(G) IL-17, IFN-γ, and IL-10 were measured by ELISA in the splenocytes of immunized mice with α-IL-10R, after restimulation with indicated concentration of MOG peptide (μg/ml). Data are representative of at least two individual experiments.

All the data are shown as mean ± SD. See also Figure S7.

these transcription factors in the active DNA demethylation and chromatin restructuring process during T cell differentiation. Although the regulation of gene expression by Tet2 seems to be gene specific, the molecular mechanism by which these transcription factors control gene-specific recruitment of Tet2 remains to be determined. The key transcription factors for Treg cells does not colocalize with 5hmC or 5mC (data not shown), suggesting a differential mechanism by which iTreg cells are epigenetically regulated at the methylation level.

Increasing evidence suggests that DNA methylation is intimately linked to histone modifications. However, precisely how Tet-mediated DNA demethylation and chromatin modifications cooperatively contribute to gene expression has not yet been clearly defined. Here, the integrative analysis of our DIP-seq results and published histone modification data indicated that 5hmC and H3K4me3 modifications were partly colocalized at the regulatory elements of signature genes in Th cells. In support of our findings, recent studies reported that 5hmC and Tet proteins are enriched in euchromatin (Mellén et al., 2012; Deplus et al., 2013). In addition, we found that Tet2 deficiency decreased the recruitment of p300 to promoters and enhancer regions. These results suggest possible regulation of chromatin configuration by Tet proteins.

Our genome-wide analysis revealed a 80%–83% reduction of 5hmC modification in *Tet2*$^{-/-}$ Th1 and Th17 cells as compared to control, suggesting that in addition to signature cytokines, Tet2 might also control 5hmC on many other loci across the whole genome in Th1 and Th17 cells. In contrast, about 20% 5hmC were not affected by Tet2 deficiency. Thus, other Tet family members might control demethylation process on these loci in Th1 and Th17 cells. Indeed, despite the possession of lineage-specific 5hmC, Tbx21 in Th1 cells and IL-17F in Th17 cells were unaffected by Tet2 depletion. Furthermore, the integrated analysis of microarray and hMeDIP-seq data indicated that the vast majority of expressed genes bearing 5hmC in Th1 and Th17 cells are not affected by Tet2 deficiency in vitro. In addition, we found that the alteration in cytokine expression in Tet2-deficient cells was partial. These results led to a hypothesis that Tet2 function might be compensated by other Tet family members. Moreover, we also found that Tet2 deficiency did not affect Th2 and iTreg cell differentiation or 5hmC peaks on their signature genes. Thus, further assessment with genetically modified mice of other Tet proteins might yield additional insight into the roles of Tet-mediated active DNA demethylation in Th cell differentiation.

Among all genes with at least 1.5-fold differences in their expression between *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ Th1 and Th17 cells, 50% of them do not have 5hmC peaks associated with their proximal promoters and gene body. Therefore, these results suggest that Tet2 might regulate these genes indirectly. Alternatively, several reports recently demonstrated that 5hmC could be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by Tet proteins (Pastor et al., 2013). Moreover, active DNA demethylation pathways through these modifications have actually been shown in mESCs (Pastor et al., 2013). Therefore, we do not exclude the possibility that 5hmC in these genes might already be oxidized to 5fC/5caC. The roles of these oxidants in Th cell differentiation will need to be studied.

We observed that Tet2-mediated active DNA demethylation controls cytokines production in vivo. Importantly, IL-10 production from CD4$^+$ T cells by Tet2 plays a crucial role in prevention of excessive inflammation in EAE. Type 1 regulatory T (Tr1) cells predominantly produce IL-10 and are differentiated by IL-27 and TGF-β (Awasthi et al., 2007). We found that development of Tr1 cells, which express IFN-γ and IL-10, were inhibited in the absence of Tet2 (data not shown), suggesting that Tet2 plays a crucial role in Tr1 cell development. Although the source of IL-10 production among Th cell subsets in vivo remains to be elucidated, several papers have proposed that IL-10 from Th1, Tr1, and Th17 cells play a crucial role in limiting the pathologic effects of Th17 cells during EAE (Ouyang et al., 2011). Therefore, we believe that Tet2-mediated IL-10 production from Th1, Tr1, and Th17 cells is a key mechanism for prevention from exacerbation of EAE symptoms.

In summary, our results not only provide a global view of a novel epigenetic modification that occurs during Th cell differentiation, but also uncover a functional role for Tet2-mediated active DNA demethylation in the function of Th cells both in vitro and in vivo. Further assessment of TET proteins and 5mC oxidants will advance our understanding of the molecular mechanisms underlying dynamic changes of DNA methylation in T cell development and will provide new therapeutic targets for innovative treatments of autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice

Cd2^{Cre}*Tet2*$^{f/f}$ mice have been described previously (Moran-Crusio et al., 2011). *Cd2*^{Cre}*Tet2*$^{f/f}$ mice and wild-type littermates on the mixed background were used in experiments. *CD4*^{Cre}*Rorc*$^{f/f}$ mice and *Il17f*^{Cre} mice were generated in our lab (unpublished data). *Il17f*^{Cre} mice and *Rosa26*^{yfp} mice were crossed to generate *Il17f*^{Cre}*Rosa*^{yfp} mice. *Ifng*^{yfp} mice were generously provided by R.M. Locksley (University of California, San Francisco). C57BL/6 mice, *Tbx21*$^{-/-}$ mice (on the B6 background), *Il4*^{gfp} mice, *Rosa26*^{yfp} mice, and *Rag1*$^{-/-}$ mice (on the B6 background) were from the Jackson Laboratory. All the mice were housed in the SPF animal facility at the M.D. Anderson Cancer Center and the animal experiments were performed at the age of 6–12 weeks with the use of protocols approved by the Institutional Animal Care and Use Committee.

Naive T Cell Preparation and Differentiation

Naive T cells were isolated by sorting CD4$^+$CD25$^-$CD62L^{hi}CD44^{lo} cells from spleens and lymph nodes, differentiated under several Th cell conditions, and analyzed as described (Yang et al., 2008). The naive T cells (5 × 10^5 cells/well) were stimulated with the plate-bound α-CD3 (1 μg/ml) and the soluble α-CD28 (1 μg/ml). For Th0 cell differentiation, the cells were treated with 5 μg/ml α-IFN-γ (XMG1.2; BioXCell), 5 μg/ml α-IL-4 (11B11; BioXCell), and 30 U/ml IL-2. For Th1 cell differentiation, the cells were treated with 10 ng/ml mIL-12 (Peprotech), 5 μg/ml α-IL-4, and 30 U/ml IL-2. For Th2 cell differentiation, the cells were treated with 10 ng/ml mIL-4 (Peprotech), 5 μg/ml α-IFN-γ. For iTreg cell differentiation, the cells were treated with 1 ng/ml TGF-β1 (Peprotech), 5 μg/ml α-IFN-γ, 5 μg/ml α-IL-4, and 30 U/ml IL-2. For Th17 cell differentiation, the cells were treated with 0.5 ng/ml TGF-β1, 10 ng/ml IL-6 (Peprotech), 5 μg/ml α-IFN-γ, and 5 μg/ml α-IL-4. When indicated, 10 ng/ml IL-23 and 10 ng/ml IL-1β (Peprotech) were used for optimal Th17 cell differentiation.

DIP Sequencing and Data Analysis

The genomic DNA was purified and sonicated. DNA fragments (4 μg) were denatured and incubated with antibody against 5mC (Eurogentec), 5hmC (Active Motif), or control IgG at 4°C overnight. The IPed DNA fragments were prepared with Dynabeads Protein G (Life Technologies) and amplified with the Illumina ChIP-seq DNA preparation kit (IP-202-1012) and sequenced on the Illumina GA II and HiSeq2500 sequencing platforms.

The unique reads for 5mC and 5hmC were mapped into non-overlapping 200 bp windows of mouse genome (mm9). The peaks were called with SICER (Zang et al., 2009), with the Input DNA sample as a control and a FDR of 0.01 as the cutoff. To determine whether the sequencing depth was enough to cover 5mC and 5hmC libraries, we carried out a scaling analysis. The number of peaks identified from a fraction of randomly sampled reads in each library via SICER was plotted. An estimation of the total reads beyond which no more peaks would be identified was determined. To calculate the tag density of 5mC and 5hmC, the number of reads was first summed in 5-bp or 100-bp windows within the regions of 5 kb upstream and downstream of TSSs or CpGIs or within the regions of 2 kb upstream and downstream of the centers of all p300/TF binding sites. All tag counts were then normalized by the total number of bases within the windows and the total number of reads in the given library.

Statistical Analysis

The statistical significance was determined by Student's paired two-tailed t test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$). All error bars shown in this article represent SDs.

ACCESSION NUMBERS

The DIP-seq and microarray data are deposited in GEO, accession numbers GSE66944 and GSE66268.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, four table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.03.005.

AUTHOR CONTRIBUTIONS

C.D. and K.I. designed the research and analyzed the data. P.Z. co-supervised some of the experiments. K.I. performed most of the experiments, and X.W., B.-S.K., and S.T. participated in specific experiments. T.C., Y.D., and Y.Z. carried out Illumina sequencing experiments. D.N.-L. and I.A. provided *Cd2*^{Cre}*Tet2*$^{f/f}$ mice. X.Y., Q.T., T.C., and L.W. analyzed microarray and DIP-seq data. K.I., L.W., and C.D. prepared the manuscript.

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Note Added in Proof

Since the submission of this manuscript, an additional paper has demonstrated dynamic changes in 5hmC during Th1 and Th2 cell differentiation and thymic T cell development. This includes the following:

Tsagaratou, A., Äijö, T., Lio, C.W., Yue, X., Huang, Y., Jacobsen, S.E., Lähdesmäki, H., and Rao, A. (2014). Dissecting the dynamic changes of 5-hydroxymethylcytosine in T-cell development and differentiation. Proc. Natl. Acad. Sci. USA. *111*, E3306–E3315.