The histone H3 lysine-27 demethylase Jmjd3 plays a critical role in specific regulation of Th17 cell differentiation

Zhi Liu1,4†, Wei Cao1,4†, Longxia Xu2, Xi Chen1, Yu Zhan1, Qian Yang1, Sanhong Liu1, Pengfei Chen1, Yuhang Jiang1, Xiaohua Sun1, Yu Tao1, Yiming Hu1, Cuifeng Li1, Qi Wang1, Ying Wang1, Charlie Degui Chen2,*, Yufang Shi1,*, and Xiaoren Zhang1,†,*  

1 Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine, Shanghai 200031, China  
2 State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China  
3 Collaborative Innovation Center of Systems Biomedicine, Shanghai Jiao Tong University School of Medicine, Shanghai 200240, China  
4 These authors contributed equally to this work.  
* Correspondence to: Xiaoren Zhang, E-mail: xzhang@sibs.ac.cn; Yufang Shi, E-mail: yufangshi@sibs.ac.cn; Charlie Degui Chen, E-mail: cdchen@sibcb.ac.cn

Interleukin (IL) 17-producing T helper (Th17) cells play critical roles in the clearance of extracellular bacteria and fungi as well as the pathogenesis of various autoimmune diseases, such as multiple sclerosis, psoriasis, and ulcerative colitis. Although a global transcriptional regulatory network of Th17 cell differentiation has been mapped recently, the participation of epigenetic modifications in the differentiation process has yet to be elucidated. We demonstrated here that histone H3 lysine-27 (H3K27) demethylation, predominantly mediated by the H3K27 demethylase Jmjd3, crucially regulated Th17 cell differentiation. Activation of naïve CD4+ T cells immediately induced high expression of Jmjd3. Genetic depletion of Jmjd3 in CD4+ T cells specifically impaired Th17 cell differentiation both in vitro and in vivo. Ectopic expression of Jmjd3 largely rescued the impaired differentiation of Th17 cells in vitro in Jmjd3-deficient CD4+ T cells. Importantly, Jmjd3-deficient mice were resistant to the induction of experimental autoimmune encephalomyelitis (EAE). Furthermore, inhibition of the H3K27 demethylase activity with the specific inhibitor GSK-J327 demethylase activity with the specific inhibitor GSK-J3 dramatically suppressed Th17 cell differentiation in vitro. At the molecular level, Jmjd3 directly bound to and reduced the level of H3K27 trimethylation (me3) at the genomic sites of Rorc, which encodes the master Th17 transcription factor Rorγt, and Th17 cytokine genes such as Il17, Il17f, and Il22. Therefore, our studies established a critical role of Jmjd3-mediated H3K27 demethylation in Th17 cell differentiation and suggest that Jmjd3 can be a novel therapeutic target for suppressing autoimmune responses.

Keywords: histone H3K27 demethylation, Jmjd3, Th17 cells, autoimmune disease

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Introduction

Interleukin (IL) 17-producing T helper (Th17) cells that produce IL-17, IL-17F, IL-21, and IL-22 are critical for mucosal defense against extracellular bacteria and fungi, as well as the development of autoimmune inflammatory diseases (Korn et al., 2009). Activation of CD4+ T cells by the combination of transforming growth factor β1 (TGF-β1) and IL-6 initiates Th17 cell differentiation, whereas IL-21 and IL-23 expand Th17 cell population and stabilize the phenotype of Th17 cells (Korn et al., 2007; McGeachy et al., 2009). The signals initiated by cytokine receptors in naïve CD4+ T cells induce and activate specific transcription factors that control the transcriptional program of T helper cells. Th17 cell differentiation is driven by the steroid receptor-type nuclear receptors RORγt and RORα, which are transcription factors that induce the transcripts of key cytokines, the IL-23 receptor, and the chemokine receptor CCR6 (Ivanov et al., 2006; Manel et al., 2008; Yang et al., 2008). HIF-1α, STAT3, IRF4, Batf, and IκBζ are required for RORγt induction and cytokine production (Brustle et al., 2007; Harris et al., 2007; Yang et al., 2007; Schraml et al., 2009; Durant et al., 2010; Okamoto et al., 2010; Dang et al., 2011). In addition, other transcription factors, including RUNX1, Ahr, and C-maf, also participate in Th17 cell differentiation (Quintana et al., 2008; Veldhoen et al., 2008; Zhang et al., 2008; Lazarevic et al., 2011; Rutz et al., 2011; Wang et al., 2014).

Epigenetic regulators of histone modification have been shown to integrate with transcription factors to regulate the program of
T cell differentiation by controlling chromatin structure and DNA accessibility (Wei et al., 2009; Zhu et al., 2010; Kanno et al., 2012). For histone modifications, trimethylation (me3) of H3K4 and H3K27 is associated with gene activation and gene repression, respectively (Barski et al., 2007), and the H3K27me3 level is dictated by the balance between histone methylation and active histone demethylation. H3K27 methylation is mediated by the polycomb repressive complex 2 (PRC2), composed of Ezh2, Suz12, and Eed (Schuettengruber et al., 2007), whereas H3K27 demethylation is mediated by Jumonji-C (JmjC) domain family members, such as Jmjd3 and Utx (Agger et al., 2007; Hong et al., 2007; Xiang et al., 2007). Global analysis of the histone modifications of H3K4me3 and H3K27me3 in CD4\(^+\) T cells has revealed a remarkable difference in the chromatin structures of distinct T helper cells and some of their signature transcription factors (Wei et al., 2009). Recently, a global transcriptional network of the Th17 cell lineage was delineated and it was suggested that H3K27me3 might be associated, with Th17 cell differentiation (Ciofani et al., 2012; Liu et al., 2012; Escobar et al., 2014). However, these studies only observed changes in H3K27 modification during Th17 cell differentiation as a concomitant phenomenon, but did not clarify the direct involvement of either H3K27 methyltransferases or demethylases in Th17 cell differentiation in vitro or in vivo. Here, we observed that Jmjd3 positively regulated the differentiation of Th17 cells, but not the differentiation of Th1, Th2, or regulatory T (Treg) cells. As a result, Jmjd3-deficient mice were resistant to experimental autoimmune encephalomyelitis (EAE). Our results demonstrate that Jmjd3 promotes Th17 cell differentiation through binding to and reducing the H3K27me3 level at genomic loci of key Th17-associated genes.

Results

T cell receptor signaling can induce Jmjd3 expression

An obligate first step in the activation of CD4\(^+\) T cells is the engagement of T cell receptor (TCR) and CD28. To determine the expression pattern of Jmjd3 after T cell activation, we purified naive CD4\(^+\) T cells and stimulated them with antibodies specific for the invariant signaling protein CD3 (anti-CD3) and the costimulatory receptor CD28 (anti-CD28). Quantitative PCR (qPCR) analysis showed that Jmjd3 was strongly induced by the stimulation via TCR and CD28. The upregulation of Jmjd3 mRNA occurred rapidly and peaked at 2 h after stimulation, and this expression was subsequently maintained at a high level. In contrast, the mRNA level of Utx, another H3K27 demethylase, did not increase and remained at a relatively low level (Figure 1A).

Due to the lack of a reliable commercial antibody to Jmjd3 for the examination and immunoprecipitation of Jmjd3 protein, we generated Jmjd3-Flag-tagged knock-in mice (Zhang et al., 2015). Consistent with the change in mRNA expression, Jmjd3 protein was expressed at low basal level and was strongly upregulated after TCR and CD28 stimulation, whereas Utx was undetectable (Figure 1B and data not shown). We also examined Jmjd3 expression in CD4\(^+\) T cell subsets and found comparable Jmjd3 mRNA levels among different CD4\(^+\) T cell subsets (Supplementary Figure S3).

We further analyzed the dynamic expression of Jmjd3 during Th17 cell differentiation. The expression of Jmjd3 mRNA peaked at ~2 h, before the initial increased expression of Rorc at ~4 h. After this initial peak, the expression of Jmjd3 mRNA decreased gradually, whereas Utx mRNA did not increase over the entire time course. The mRNA level of Jmjd3 was not upregulated more in Th17 cells compared with Th0 cells (Figure 1C). Restimulation with phorbol-12-myristate-13-acetate (PMA) and ionomycin also dramatically upregulated Jmjd3 mRNA in Th17 cells (Figure 1D). The above data indicate that T cell activation induces Jmjd3 expression.

T cell-specific deletion of Jmjd3

To elucidate the physiological and pathological roles of Jmjd3 in CD4\(^+\) T cell development and differentiation, we generated Jmjd3 conditional knockout (cKO) mice by targeted deletion of exons 14–20, and crossed them with mice with transgenic expression of Cre recombinase driven by the T cell-specific Cda4 promoter (CD4\(^+\)-cre) to ensure deletion of the loxp-flanked Jmjd3 alleles in CD4\(^+\) T cells. Genotyping and reverse-transcription PCR analysis showed that the expression of Jmjd3 was abrogated in CD4\(^+\) T cells from cKO mice (Supplementary Figure S1). Jmjd3 cKO mice were viable and phenotypically indistinguishable from wild-type (WT) littermates over a 6-month window of analysis. We examined the status of CD4\(^+\) and CD8\(^+\) T cells in the thymus and peripheral lymphoid organs of these mice. Jmjd3 deletion had no effect on T cell development in the thymus. Moreover, WT littermates and Jmjd3 cKO mice showed similar numbers and distributions of CD4\(^+\) and CD8\(^+\) T cells in the periphery. The activated/memory CD4\(^+\) T cells and CD8\(^+\) T cells in the periphery, as marked by the phenotype CD62L\(^low\)/CD44\(^hi\), were unaltered by deletion of Jmjd3 in 6–8-week-old mice. Proliferative response of Jmjd3-deficient CD4\(^+\) T cells to TCR stimulation was intact (Supplementary Figure S2).

Loss of Jmjd3 leads to impaired Th17 cell differentiation

To examine the function of Jmjd3 in CD4\(^+\) T cell differentiation in vitro, we purified naive CD4\(^+\)CD62L\(^+\) T cells from the spleen and lymph nodes of Jmjd3 cKO or WT littermates, and primed them to differentiate into Th1, Th2, Th17, and iTreg cells. After 72 h, we restimulated the cells with PMA and ionomycin for 5 h, and assessed the expression of cytokines by intracellular staining and flow cytometry. Under Th17-polarizing condition (TGF-β and IL-6) or pathogenic Th17-polarizing condition (TGF-β, IL-6, IL-1β, and IL-23), the frequency of IL-17-secreting cells was significantly lower in Jmjd3 cKO CD4\(^+\) T cells compared with WT controls. The mRNA level of Rorc, Rora, and other Th17-related genes, such as Il-17, Il-17f, Il-21, Il-22, and Il-23r, was also profoundly reduced in Jmjd3 cKO CD4\(^+\) T cells (Figure 2A and C). Consistent with a pervious study, there was no defect in the differentiation of IFNγ-producing Th1 cells or IL-4-producing Th2 cells (Satoh et al., 2010). iTreg cells from Jmjd3 cKO naive CD4\(^+\) T cells were comparable to that from WT controls (Figure 2A and B). Consistently, cytokine levels in the cell culture supernatants as determined by multiplexed bead immunoassay showed that the amount of IL-17 production was much less in both the standard...
and pathogenic Th17-polarizing conditions in Jmjd3 cKO cell cultures, whereas the amount of IFNγ or IL-4 production in Jmjd3 cKO Th1 or Th2 cell cultures was not altered (Figure 2D). Taken together, these results show that Jmjd3 selectively contributes to the differentiation of Th17 cells but not to the differentiation of Th1, Th2, or iTreg cells.

**Ectopic expression of Jmjd3 rescues Th17 cell differentiation in Jmjd3-deficient T cells**

The results described above suggest that Jmjd3 positively regulates the commitment of naïve CD4+ T cells to Th17 cells. To test whether ectopic expression of Jmjd3 could rescue Th17 cell differentiation in Jmjd3-deficient CD4+ T cells, we transduced Jmjd3 cKO CD4+ T cells with retrovirus expressing GFP or Jmjd3 under Th17-polarizing condition. After 4 days, we assessed IL-17 production in sorted GFP+ cells using intracellular staining and flow cytometry. Overexpression of Jmjd3 led to a much higher frequency of IL-17-secreting cells (Figure 3A), as well as dramatically increased expression of Rorc, Il-17, Il-17f, Il-21, Il-22, and Il23r mRNA (Figure 3B). The results here therefore confirm the essential role of Jmjd3 in Th17 cell differentiation.

**Jmjd3-deficient mice are resistant to EAE**

Given the critical role of Jmjd3 in Th17 cell differentiation in vitro, we next investigated the contribution of Jmjd3 to Th17 cell differentiation in vivo. We employed the EAE mouse model, a model of autoimmune disease mediated by Th1 and Th17 cells, to evaluate the pathophysiological relevance of Jmjd3 function in Th17 cell differentiation. We immunized sex- and age-matched Jmjd3 cKO mice and WT littermates with myelin oligodendrocyte glycoprotein (MOG) peptide emulsified in complete Freund’s adjuvant (CFA) followed by administration of pertussis toxin, and then monitored the mice for clinical signs of EAE. WT littermates displayed neurologic symptoms as early as on Day 12 post-immunization, and most animals developed severe disease (average clinical score of 3). However, the onset of EAE was substantially delayed in Jmjd3 cKO mice compared with WT littermates, with a marked reduction in severity (Figure 4A). Moreover, the incidence of EAE was significantly lower in Jmjd3 cKO mice, indicating that they were much more resistant to the disease (Figure 4B). Histopathological analysis of the spinal cord sections from WT littermates and Jmjd3 cKO mice on Day 27 post-immunization showed notably decreased inflammation and demyelination in Jmjd3 cKO tissues than in WT.
Figure 2 Jmjd3 deficiency selectively reduces Th17 cell differentiation. (A) Intracellular staining for IFNγ, IL-4, IL-17, and Foxp3 in WT or Jmjd3 cKO CD4+ T cells polarized under Th0, Th1, Th2, Th17, or Treg condition. Data are representative of at least three independent experiments. (B) RT–PCR analysis of Tbx21, Gata3, and Foxp3 expression in the indicated CD4+ T cell subsets from WT or Jmjd3 cKO mice. (C) RT–PCR analysis of Rorc, Rora, Il17, Il17f, Il21, Il22, and Il23r in WT or Jmjd3 cKO Th17 cell cultures. (D) IFNγ, IL-4, and IL-17 production in WT or Jmjd3 cKO CD4+ T cells activated under Th0-, Th1-, Th2-, or Th17-polarizing condition. Data are presented as mean ± SD of three independent experiments.
tissues (Figure 4C). Among a panel of pro- and anti-inflammatory cytokines tested, the production of IL-17, but not other cytokines, by MOG 35–55-stimulated T cells was significantly reduced in Jmjd3 cKO mice compared with WT littermates (Figure 4D).

In parallel, both the frequency and absolute number of effector (or infiltrating) Th17 cells were markedly lower in both the central nervous system (CNS) and lymph nodes of Jmjd3 cKO mice versus WT mice, whereas the percentage of Th1 cells was not affected (Figure 5A and B). We also analyzed CD4+ T cells for Foxp3 expression, and found no difference between Jmjd3 WT and cKO CD4+ T cells in either the spleen or the CNS, suggesting that the resistance of Jmjd3-deficient mice to EAE was not owing to a compensatory increase in Foxp3+ Treg cells (Figure 5C and D). Consistently, qPCR analysis also showed that Th17-associated genes, such as Il17f, Il22, and Il23r, were significantly reduced in CD4+ T cells from lymph nodes of Jmjd3-deficient EAE mice by magnetic-activated cell sorting (Figure 5E). Therefore, these results indicate that a profound defect in the differentiation of CD4+ T cells into Th17 cells in vivo gives rise to EAE resistance in Jmjd3 cKO mice.

**Jmjd3 controls H3K27me3 levels at target genes in Th17 cells**

Our results indicate that Jmjd3 regulates a series of Th17-associated genes, including Rorc, Rora, Il17f, Il17r, and Il21 (Figure 2C). Since Jmjd3 is well known for its demethylase activity, we hypothesize that Jmjd3 binds to the Rorc locus and modifies its H3K27me3 levels directly, and then regulates Th17 cell differentiation. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) studies under Th17- and Th1-biased conditions by using Jmjd3-Flag-tagged knock-in mice. After 72 h of culture, we observed considerable enrichment of Jmjd3 at the sites located ~1.5 kilobases (kb) (position 4, p-4) and 0.6 kb (p-6) upstream of the first exon of Rorc in Th17 cells. Jmjd3 was also detected to enrich at the binding sites for STAT3 located ~6 kb (BS1) downstream of the first exon of Rorc, which was previously defined (Durant et al., 2010). However, Jmjd3 was not enriched at the Rorc locus in Th1 cells. These data indicate that Jmjd3 binding to the Rorc regulatory elements is specific for Th17 cells (Figure 6A and C).

Because Jmjd3 functions as a H3K27 demethylase, we performed ChIP-qPCR analysis to assess whether Jmjd3 binding affects the level of H3K27me3 modification at the Rorc locus in Jmjd3 cKO Th17 cells and their WT controls. We found that the repressive histone marker H3K27me3 at the Jmjd3 binding sites (p-4, p-6, and BS1) of Rorc locus was significantly increased in Jmjd3 cKO Th17 cells compared with WT controls, whereas the level of H3K27me3 at p-3 was comparable between WT and
Jmjd3 cKO Th17, since Jmjd3 was not shown to bind at p-3. Levels of H3K27me3 at the loci of Il17, Il17f, and Il22 in cKO Th17 cells were also higher than that in WT controls (Figure 6B). Because Jmjd3 was rapidly induced after TCR stimulation (Figure 1), we examined whether the induced expression of Jmjd3 indeed resulted in the changes of H3K27me3 level during Th17 cell differentiation. Not surprisingly, H3K27me3 levels at Rorc, Il-17, and Il17f loci declined and maintained at a very low level after 4 h when Jmjd3 protein could be detected in WT Th17 cells. In contrast, H3K27me3 levels at Rorc, Il-17, and Il17f loci remained at a relatively high level in Jmjd3 cKO Th17 cells (Figure 6D and Supplementary Figure S4). Thus, these results suggest that Jmjd3 controls chromatin accessibility at many Th17-related loci, such as Rorc, Il17, Il17f, and Il22, and thereby promotes their expression.

Inhibition of H3K27 demethylase suppresses Th17 cell differentiation

We next used the small molecule inhibitor GSK-J4 (Kruidenier et al., 2012) to target the demethylase activity of Jmjd3/Utx to test whether this demethylase activity is required for Jmjd3 functional effects on the differentiation of naive CD4+ T cells into major subsets (Th1, Th2, Th17, and iTreg). Mouse CD4+CD62L+ naive T cells were purified from the spleen and lymph nodes, and differentiated under standard CD4+ T cell polarizing conditions in the presence of DMSO or 80 nM GSK-J4. After 3 days of culture, cells treated with GSK-J4 produced much less IL-17 and significantly decreased the expression of Th17-related genes in a dose-dependent manner under Th17-polarizing condition (Figure 7A and D), whereas GSK-J4 had no impact on the differentiation of Th1 (assessed as the proportion of IFN-γ+ cells and Tbx21 expression), Th2 (IL-4+ cells and Gata3 expression), or Treg (Foxp3+ cells and Foxp3 expression) cells (Figure 7B and C). Recently, GSK-J4 was reported to also target other members of JmjC family besides the H3K27me3/me2 demethylase subfamily (Heinemann et al., 2014). However, among ~30 JmjC genes annotated in the mouse genome, Jmjd3 was most highly expressed and strongly induced in Th17 cells, suggesting that GSK-J4 probably suppressed Th17 cell differentiation through inhibiting Jmjd3 activity (Supplementary Figure S5). These data indicate that targeting Jmjd3 with a small molecule inhibitor may be beneficial to patients suffering from autoimmune diseases.
Discussion

T cell activation under specific cytokine-biased conditions promotes the differentiation of naïve CD4⁺ T cells into different T helper cell subsets. Transcription factors have been shown to integrate with epigenetic regulators to drive T helper cell differentiation. In addition, inactivation of the histone methyltransferase Jmjd3-deficient mice exhibit defective Th17 development during EAE. (A) Intracellular staining for IL-17 and IFNγ in CD4⁺ T cells derived from the CNS (left) and lymph nodes (right) of WT (n = 4) or Jmjd3 cKO (n = 4) mice. (B) Total numbers and percentages of CD4⁺ T cell populations isolated from the CNS and lymph nodes of WT or Jmjd3 cKO mice. (C) Intracellular staining for Foxp3 in CD4⁺ T cells derived from the CNS (left) and the spleen (right) of WT (n = 4) and Jmjd cKO (n = 4) mice. (D) Total numbers and percentages of CD4⁺Foxp3⁺ T cell populations isolated from the spleen of WT and Jmjd cKO mice. (E) Real-time PCR analysis of MACS-sorted CD4⁺ T cells derived from lymph nodes of WT and Jmjd cKO EAE mice. Data are presented as mean ± SD of three independent experiments.
Ezh2 was shown to specifically enhance Th1 and Th2 cell differentiation and plasticity, whereas limited effects were observed for Th17 cell differentiation (Tumes et al., 2013). It was also recently shown that miR-155 contributes to Th17 cell differentiation by suppressing the inhibitory effects of Jarid2, which recruits PRC2 to chromatin and increases H3K27 methylation, coinciding with a

![Figure 6](image-url)

Figure 6 Jmjd3 controls H3K27me3 levels at genomic sites of Th17-related genes. (A) ChiP-qPCR analysis of the binding of Jmjd3 to the Rorc promoter (numbers along horizontal axis correspond to Rorc at bottom) in Jmjd3-Flag-tagged Th17 cells. (B) ChiP-qPCR analysis for anti-H3K27me3 along with control IgG at the Rorc, Il17, Il17, and Il22 loci in WT and Jmjd3 cKO Th17 cells. (C) ChiP-qPCR analysis of the binding of Jmjd3 to the Rorc promoter in Jmjd3-Flag-tagged Th1 cells. (D) ChiP-qPCR analysis of the level of H3K27me3 at Rorc and Il-17 loci in CD4+ T cell differentiated for 0, 4, 12, 24, or 48 h under Th17 condition. Data are representative of two or three independent experiments.
Figure 7 Jmjd3 inhibition suppresses Th17 cell differentiation. (A) Intracellular staining for IL-17 in Th17 cells in the presence of indicated concentration of GSK-J4 or DMSO as a control. (B) Intracellular staining for IFNγ, IL-4, and Foxp3 in the indicated CD4+ T cell subsets in the presence of 80 nM GSK-J4 or DMSO as a control. (C) RT–PCR analysis of Tbx21, Gata3, and Foxp3 in the indicated CD4+ T cell subsets in the presence of 80 nM GSK-J4 or DMSO. (D) RT–PCR analysis of Rorc, Rora, Il17, Il17f, Il21, Il22, and Il23r in Th17 cells in the presence of indicated concentration of GSK-J4. Data are presented as mean ± SD of three independent experiments.
failure to express IL-22, IL-10, or IL-9 (Escobar et al., 2014). However, whether H3K27 methyltransferase and/or demethylase is involved in Th17 cell differentiation still remains unknown. Our results demonstrate that the TCR-induced H3K27 demethylase Jmjd3 functions as a critical positive regulator of Th17 cell differentiation both in vitro and in vivo. Jmjd3 is induced after T cell activation, and inhibition of Jmjd3 activity, either by a small molecular inhibitor or by Jmjd3 genetic deficiency, results in impaired Th17 cell differentiation. Mechanically, Jmjd3 binds to the promoters of many Th17-associated genes, such as Rorc, Il-17, Il-17f, and Il-22, and reduces the H3K27me3 level at these genomic loci, thus resulting in the activation of these genes and consequently promoting Th17 cell differentiation. Due to defective Th17 cell differentiation in vivo, Jmjd3 cKO mice exhibit resistance to EAE in comparison with WT controls.

It has recently been reported that Jmjd3 negatively regulates CD4+ T cell differentiation into Th2 and Th17 cells in the small intestine and colon, and promotes T cell differentiation into Th1 cells (Li et al., 2014). In contrast, and we others did not observe the defect in Th1 and Th2 cell differentiation in Jmjd3-deficient CD4+ T cells (Satoh et al., 2010). One possible explanation for this discrepancy is that Jmjd3 and Utx may have redundant roles in Th1 cell differentiation, as both Jmjd3 and Utx have been shown to function as a Tbx21 partner in primary Th1 cells (Miller et al., 2010). More importantly, Jmjd3 and/or Utx promotes IFNγ expression independent of H3K27 demethylase potentials (Miller et al., 2010). In this study, only exons 14–20 of Jmjd3 coding the JmJC domain was deleted in Jmjd3-deficient CD4+ T cells, which may also account for the normal Th1 differentiation in Jmjd3-deficient mice. Here we showed that loss of Jmjd3 led to impaired Th1 cell differentiation, and Jmjd3 was involved in H3K27me3 modification at master regulator Rorc and Th17-related genes. These findings were supported by previous reports (Ciofani et al., 2012; Liu et al., 2012; Escobar et al., 2014). In addition, Th17 cell differentiation was markedly suppressed by an inhibitor of H3K27 demethylase, GSK-J4, suggesting that Jmjd3 may serve as a potent therapeutic target in autoimmune conditions.

As an essential transcription factor for Th1cell differentiation, interferon regulatory factor 4 (IRF4) was identified to be regulated by Jmjd3 in M2 macrophages, suggesting that Jmjd3 might control Th17 cell differentiation through regulating IRF4 expression (Brustle et al., 2010; Satoh et al., 2010). However, we did not observe significant differences in the mRNA level and H3K27me3 modification of irf4 gene between WT and Jmjd3 cKO Th17 cells at different time points (Supplementary Figure S6), revealing that Jmjd3 did not directly regulate IRF4 during Th17 cell differentiation.

There are several explanations for the question why Jmjd3 selectively affects Th17 cell differentiation. A possible reason is that Jmjd3 interacts with the master transcription factor Rorγt and STAT3, thus favorably changing the chromatin accessibility of their downstream genes, as indicated by a previous report (Ciofani et al., 2012). Another possibility is that other H3K27me3-involved CD4+ T cell differentiation is mediated by histone methyltransferase Ezh2, as previously reported (Tumes et al., 2013).

In summary, this study demonstrates that, besides transcription factors that are known to regulate Th17 cell differentiation, the histone H3K27 demethylase Jmjd3 plays an essential role in Th17 cell differentiation. Further studies are needed to clarify how Jmjd3 selectively promotes Th17 cell differentiation.

**Materials and methods**

**Mice**

Jmjd3 cKO mice on the C57BL/6 background were generated by targeted deletion of exons 14 through 20, which contain the JmJC H3K27 demethylase domain. Jmjd3 knock-in mice were generated by insertion of a Flag tag downstream of the stop codon of the Jmjd3 gene. Both Jmjd3 cKO and knock-in mice were generated in Charlie Degui Chen’s lab. CD4-cre mice were a gift from Dr Zichun Hua (Nanjing University). All mice were maintained in specific pathogen-free conditions. All animal studies were approved by the Institutional Animal Care and Use Committee of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

**CD4+ T cell purification and in vitro differentiation**

Naive CD4+CD26– T helper cells were prepared by MACS from the spleen and lymph nodes of Jmjd3 WT and cKO mice. The purity of sorted naive cells was >90%. Naive CD4+ T cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2.5 μg/ml) for 3 days under the following conditions for different T helper cells: 10 μg/ml anti-IL-4 and 10 μg/ml anti-IFNγ for Th0; 10 μg/ml anti-IL-4 and 10 ng/ml IL-12 for Th1; 10 μg/ml anti-IFNγ and 10 ng/ml IL-4 for Th2; 3 ng/ml TGF-β, 30 ng/ml IL-6, 10 ng/ml IL-1β, 10 ng/ml IL-23, 10 μg/ml anti-IL-4, and 10 μg/ml anti-IFNγ for Th17; 10 μg/ml anti-IL-4, 10 μg/ml anti-IFNγ, 5 ng/ml TGFβ, and 50 ng/ml IL-2 for iTreg.

**Retrovirus production**

The Jmjd3 cDNA was amplified and cloned into pMSCV (MSCV-IRES-GFP). Plat-E cells were transfected with 8 μg of the indicated plasmid using Lipofectamin 2000 (Invitrogen). Viral supernatant was collected or concentrated by ultracentrifuge and supplemented with 8 μg/ml polybrene (Sigma).

**Measurement of cytokines**

Cytokine levels in the cell culture supernatants were determined by multiplexed bead immunoassay using the Luminex Technology (Bio-Plex; Bio-Rad Laboratories).

**Immunoblotting**

*In vitro* cultured CD4+ T cells were lysed in ice-cold buffer containing a protease inhibitor cocktail (Roche). The lysates were fractionated by SDS–PAGE and analyzed by immunoblotting with specific antibodies to Flag (F316; Sigma) and GAPDH.

**Antibodies and flow cytometry**

Mouse anti-CD4 (RM4-5), anti-CD8 (53–67), anti-CD25 (PC61), anti-CD44 (G44–26), and anti-CD62L (MEL-14) antibodies were purchased from BD Biosciences. For intracellular staining, cells were restimulated with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 5 h with the addition of GolgiPlug (BD). After restimulation, the cells were washed and stained for...
extracellular markers, followed by fixation, permeabilization using the Cytofix/Cytoperm Kit (554714; BD), and intracellular staining to detect mouse IL-17A (12-7177-81; eBioscience), IFNγ (554411; BD Biosciences), and IL-4 (562044; BD Biosciences). For Foxp3 intracellular staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Kit (00-5523-00; eBioscience). Dead cells were stained with Fixable Viability Stain 450 (562247; BD Biosciences). Cells were acquired on a FACSCalibur (BD Biosciences), and the data were analyzed using the FlowJo Software.

RNA isolation and real-time qPCR
Total RNA was extracted with TRIzol Reagent according to the manufacturer’s instructions (Invitrogen). For cDNA synthesis, RNA was reverse-transcribed with a PrimeScript RT Reagent Kit (TaKaRa), and then cDNA was amplified by real-time PCR with a SYBR Premix ExTag Kit (TaKaRa) using an ABI Prism 7500 Fast Cycler (Applied Biosystems). The primer sequences are described in Supplementary Table S1. The level of mRNA was normalized to that of Gapdh.

ChIP
Naïve CD4+ T cells sorted from the spleen and lymph nodes of Jmjd3 cKO and WT mice or Jmjd3 knock-in mice were differentiated under Th0-, Th1-, and Th17-polarizing conditions as described above. Differentiated T helper cells were stimulated with PMA and ionomycin for 5 h and then cross-linked with 1% formaldehyde at a density of 1 × 10^6 cells/ml for 10 min at room temperature. Chromatin was sonicated with UCD-300 (Bioruptor), and ChIP was performed with the Chromatin Immunoprecipitation Kit (17–371; Millipore) according to the manufacturer’s instructions, followed by real-time PCR for quantification of ChIP-enriched DNA. The antibodies used for ChIP included polyclonal rabbit anti-H3K27me3 (07–449; Millipore) and anti-Flag (F316; Sigma). Primer sequences are presented in Supplementary Table S2.

Induction of EAE
For induction of EAE, mice were immunized subcutaneously on Day 0 with 300 mg/mouse MOG 35–55 peptide (MEGVYGRSP SRVVHLRYNGK) emulsified in CFA (Sigma-Aldrich) and injected intravenously on Days 0 and 2 with 200 ng/mouse of pertussis toxin (List Biological Laboratories). The following scoring system was used: 0 — no disease; 1 — limp tail; 2 — weak/partially paralyzed hind legs; 3 — completely paralyzed hind legs; 4 — complete hind leg paralysis and partial front leg paralysis; and 5 — complete paralysis/death. Disease incidence and scores were recorded daily. For analysis of CNS infiltrate, both the brain and spinal cord were collected from perfused mice, and mononuclear cells were prepared with a 37% Percoll gradient and pelleted for 20 min at 2000 rpm. The cell pellets were resuspended in FACS buffer or T cell medium and used for subsequent experiments.

Statistical analysis
Data were analyzed using the Student’s t-test with GraphPad 4.0 software. *P < 0.05 was considered statistically significant (*P < 0.05, **P < 0.01).

Supplementary material
Supplementary material is available at Journal of Molecular Cell Biology online.

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Conflict of interest: none declared.

References


