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The X-linked histone demethylase Kdm6a in CD4+ T lymphocytes modulates autoimmunity

Yuichiro Itoh,1 Lisa C. Golden,1,2 Noriko Itoh,1 Macy Akiyo Matsukawa,1 Emily Ren,1 Vincent Tse,1 Arthur P. Arnold,2 and Rhonda R. Voskuhl1

1Department of Neurology, David Geffen School of Medicine, UCLA, Los Angeles, California, USA. 2Molecular Biology Institute, UCLA, Los Angeles, California, USA. 3Department of Integrative Biology and Physiology, UCLA, Los Angeles, California, USA.

Multiple sclerosis (MS) is a putative T cell–mediated autoimmune disease. As with many autoimmune diseases, females are more susceptible than males. Sexual dimorphisms may be due to differences in sex hormones, sex chromosomes, or both. Regarding sex chromosome genes, a small percentage of X chromosome genes escape X inactivation and have higher expression in females (XX) compared with males (XY). Here, high-throughput gene expression analysis in CD4+ T cells showed that the top sexually dimorphic gene was Kdm6a, a histone demethylase on the X chromosome. There was higher expression of Kdm6a in females compared with males in humans and mice, and the four core genotypes (FCG) mouse model showed higher expression in XX compared with XY. Deletion of Kdm6a in CD4+ T cells ameliorated clinical disease and reduced neuropathology in the classic CD4+ T cell–mediated autoimmune disease experimental autoimmune encephalomyelitis (EAE). Global transcriptome analysis in CD4+ T cells from EAE mice with a specific deletion of Kdm6a showed upregulation of Th2 and Th1 activation pathways and downregulation of neuroinflammation signaling pathways. Together, these data demonstrate that the X escapee Kdm6a regulates multiple immune response genes, providing a mechanism for sex differences in autoimmune disease susceptibility.

Introduction
Multiple sclerosis (MS) is a putative T cell–mediated autoimmune disease of the CNS. The etiology of MS is not completely understood, although genetic and environmental factors have been proposed (1, 2). Activated T lymphocytes in the peripheral blood of MS patients pass through the blood-brain barrier (BBB) to infiltrate the CNS, in turn activating resident microglia and astrocytes, resulting in white matter lesions characterized by inflammation, demyelination, and axonal damage. For many autoimmune diseases, females are more susceptible than males. Sexual dimorphisms may be due to differences in sex hormones, sex chromosomes, or both. In MS, the ratio of females to males is approximately three to one, and more robust immune responses have been shown in women compared with men (3–6). Based on investigations using mouse models, sex differences in autoimmune diseases can be caused by differences in sex hormones (estrogen vs. testosterone), sex chromosomes (XX vs. XY), or both (6–10). How differences in sex chromosomes contribute to sex differences in autoimmune diseases remains unclear.

In mammals, females (XX) have 2 copies of X genes, while males (XY) have only 1 copy. This 2-fold difference in genomic dose of X chromosome genes could potentially be deleterious. However, in females, this dosage difference is compensated for by random inactivation of 1 X chromosome, resulting in similar expression levels of X genes in both sexes (11). Kdm6a (lysine demethylase 6A, also known as Utx) is an X-linked gene, but unlike the majority of X genes, it escapes from X inactivation, potentially leading to higher expression of Kdm6a in immune cells of females as compared with males (12–15).

Histone demethylase KDM6A specifically mediates the removal of repressive trimethylation on histone H3 lysine 27 (H3K27me3) to expose a region of chromatin for transcription. KDM6A associates with the methyltransferase KMT2D in the same protein complex (16), and KMT2D adds the activating H3 lysine 4 (H3K4me3) histone mark (17). As a result, this complex activates gene expression by histone modifications that make the chromatin transcriptionally permissive in a cell lineage–specific way (18, 19). Whether sexually dimorphic expression of Kdm6a could be a causal factor in the female predominance of autoimmune diseases is unknown.

In this study, we examined Kdm6a expression in CD4+ T lymphocytes of female versus male mice, as well as in XX versus XY mice of the four core genotypes (FCG) model (20, 21). Then, the role of Kdm6a in autoimmune disease was determined by deleting Kdm6a in CD4+ T cells to ascertain the effect on the classic CD4+ T cell–mediated autoimmune disease experimental autoimmune encephalomyelitis (EAE), with subsequent global transcriptome analysis in mice devoid of Kdm6a in CD4+ T cells.

Results
Females (XX) have higher expression of Kdm6a RNA. Previously, we used the FCG model to show that XX mice were more susceptible than XY, irrespective of gonadal type, to both EAE and lupus (6, 8),

[Related Commentary: https://doi.org/10.1172/JCI130312]
Expression Omnibus database [GEO] GSE94671) (Figure 1, A and B, and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI126250DS1). This sex difference of KDM6A gene expression was confirmed in human naïve CD4+ T cells (Figure 1E). That other genes thought to escape X inactivation do not appear when comparing female versus male CD4+ T cell data sets may be due to a variety of factors, such as the tissue type, cell type, or hormonal status (28).

We then used the FCG mouse model (20, 21), which allows comparison of XX and XY− mice of the same gonadal sex (Y− denotes lack of the testis-determining Sry gene on the Y chromosome). In addition, XX and XY− gonadal females were ovariolectomized to remove activational effects of ovarian hormones. In autoantigen-stimulated CD4+ T cells from C57BL/6J and SJL mice, there was higher expression of Kdm6a and Kdm5c in XX compared with XY− mice (Figure 1, C and D, and Supplemental Figure 1, B and C). In contrast, other genes thought to escape X inactivation, namely, Ddx3x, Eif2s3x, Uba1, and Usp9x, were not differentially expressed between XX and XY− CD4+ T cells (Supplemental Figure 1, B and C).

Kdm6a was the most differentially expressed X escapee, we next investigated to determine whether Kdm6a expression in CD4+ T cells influences autoimmune disease. We crossed mice containing homozygous floxed Kdm6a alleles with mice expressing Cre under the CD4 promoter to produce a conditional KO of Kdm6a only in CD4+ T cells (cKO). Deletion of Kdm6a in CD4+ T cells was confirmed with genomic PCR (Figure 2A). As expected, expression of Kdm6a was decreased in cKO compared with WT in the CD4+ T cells from EAE mice that were stimulated with autoantigen (Figure 2B, FDR = 0.07). To examine the functional significance of Kdm6a expression in CD4+ T cells on disease, active EAE was induced in female cKO and WT (littermate controls; CD4-Cre negative) mice (20, 21). We then used the FCG mouse model (20, 21), which allows comparison of XX and XY− mice of the same gonadal sex (Y− denotes lack of the testis-determining Sry gene on the Y chromosome). In addition, XX and XY− gonadal females were ovariolectomized to remove activational effects of ovarian hormones. In autoantigen-stimulated CD4+ T cells from C57BL/6J and SJL mice, there was higher expression of Kdm6a and Kdm5c in XX compared with XY− mice (Figure 1, C and D, and Supplemental Figure 1, B and C). In contrast, other genes thought to escape X inactivation, namely, Ddx3x, Eif2s3x, Uba1, and Usp9x, were not differentially expressed between XX and XY− CD4+ T cells (Supplemental Figure 1, B and C).

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Figure 1. Differentially expressed X chromosome genes in mouse CD4+ T cells. (A) Using the high-throughput sequencing approach, sexually biased X chromosome genes in CD4+ T cells were determined for C57BL/6J naïve CD4+ T cells from spleen (GSE94671; 3 males and 3 females). Yellow dots within the bar representing the X chromosome indicate genes with higher expression in females than in males (threshold for significance was FDR < 0.1). Kdm6a showed increased expression in CD4+ T cells from females as compared with males. Xist and Tsix, which regulate X inactivation, and 4 predicted genes with unknown functions (Gm26992, Gm27733, Gm27927, and Gm27520) also showed increased expression in females. Shown below the bar representing the X chromosome are previously reported X escapees found in other tissues (black text) as well as X genes not thought to escape X inactivation, but involved in immunity (blue text) (58, 59). None had significantly different expression. (B-E) Expression of Kdm6a in (B) naïve CD4+ T cells from C57BL/6J spleen (GSE94671; 3 males and 3 females); (C) stimulated CD4+ T cells from C57BL/6J lymph nodes (GSE121292; FCG: 6 XX and 6 XY−); (D) stimulated CD4+ T cells from SJL lymph nodes (GSE121705; FCG: 6 XX and 5 XY−); and (E) human naïve CD4+ T cells from healthy control blood (GSE56033; 205 males and 294 females). In box-and-whisker plots, thick lines inside the boxes represent the median of the data. The lower and upper ends of boxes show quantiles (25% and 75%), and whiskers show the minimum and maximum values excluding outliers (circles). FDR was calculated using R package edgeR for A–D. For E, 1-way ANOVA was used.

indicating that a sex difference in X and/or Y gene expression can contribute to the sex difference in autoimmune diseases. Here, we focused on X genes over Y genes because of the much higher gene content of the X chromosome, including genes related to immune function, as compared with the Y chromosome, which during evolution has lost many genes not related to reproduction. First, we determined which X genes have sexually dimorphic gene expression in CD4+ T lymphocytes. Since the majority of X genes are randomly inactivated, here we focused on genes that escape X inactivation (X escapees). Possible sex differences in RNA expression of 6 known X escapees in mice (Ddx3x, Eif2s3x, Kdm5c, Kdm6a, Uba1, and Usp9x) (22–27) were examined in CD4+ T cells using high-throughput RNA-Seq data sets. In C57BL/6J naïve CD4+ T cells, expression of the Kdm6a gene was higher in females than in males and Kdm5c showed a trend of difference, while the other 4 X escapees were not significantly different (NCBI’s Gene
Figure 2. Deletion of Kdm6a in CD4+ T cells protects mice from EAE. (A) Genomic PCR for isolated CD4+ T cells from EAE mice showed successful deletion of Kdm6a in cKO mice homozygous for the KO allele. (B) In cKO mice, RNA expression of Kdm6a was downregulated in CD4+ T cells. RNA expression levels of Kdm6a were graphed with log2-transformed TPM normalization. In box-and-whisker plot, thick lines inside the boxes represent the median of the data. The lower and upper ends of boxes show quartiles (25% and 75%), and whiskers show the minimum and maximum values. FDR was calculated using edgeR. (C) cKO mice showed decreased EAE severity scores compared with WT littermates. n = 4 WT; n = 5 cKO. P < 0.0001, repeated measures 1-way ANOVA. Additional EAE experiments are shown in Table 1. (D) Representative ×10 images of the ventral spinal cord of EAE in WT and cKO stained with antibodies for CD3 (green), Iba1 (red), βAPP (red), and NF200 (red) costained with DAPI (blue). Scale bars: 100 μm. (E) Dot plots show a decrease in cKO for CD3+ cell count (P = 0.03276), globoid Iba1+ cell count (P = 0.02593), and βAPP+ percentage area for axonal damage (P = 0.01207), and show an increase in cKO for NF200+ intact axonal count (P = 0.03276) in the white matter. n = 8 WT; n = 11 cKO. The height of columns represents mean, and error bars represent SEM. For each stain, P value was determined by Mann–Whitney U test.
NF200⁺ intact axons, each as compared with those in WT littermates (Figure 2, D and E).

Since CD4⁺Cre deletion of Kdm6a could affect CD4⁺CD8⁺ cells during thymic development of CD8⁺ cells, CD8⁺ T cells were isolated and assessed for whether Kdm6a was deleted. Indeed, Kdm6a was deleted in CD8⁺ T cells as well (Supplemental Figure 2). This warrants further study of CD8⁺ T lymphocytes in EAE, but here we focused on CD4⁺ T lymphocytes in EAE.

**Deletion of Kdm6a changes the transcriptome in CD4⁺ T cells in EAE mice.** We next investigated downstream genes regulated by Kdm6a in CD4⁺ T cells. We induced EAE in cKO mice and their WT littermate controls. On EAE day 17, lymph node cells were isolated and cultured with autoantigen before CD4⁺ T cell sorting. Whole-transcriptome analyses for CD4⁺ T cells from cKO and WT mice with EAE were performed using RNA-Seq. Principal component analysis of the transcriptomes showed clear separation of cKO and WT genotypes (data not shown). We found that 1728 genes were significantly different between cKO mice and WT controls (FDR < 0.1; Supplemental Table 1): 859 genes were upregulated, and 869 genes were downregulated in cKO cells. Within these 1728 differentially expressed genes, 531 genes showed more than 2-fold difference: 349 upregulated and 182 downregulated genes (Figure 3A). Expression differences of several genes were validated with quantitative reverse-transcription PCR (RT-PCR) using an independent set of cKO and WT mice to generate samples (Figure 3B and Table 2).

To determine the biological relevance of differentially expressed genes between cKO and WT mice, canonical pathway analysis was performed. Genes with low expression were eliminated from this analysis using an additional threshold of log, scale counts per million greater than 1 (FDR < 0.1, logFC > 1 or logFC < –1, logCPM > 1), with the remaining 364 differentially expressed genes proceeding to pathway analysis (235 up- and 130 downregulated genes). Figure 3 shows the top 10 enriched canonical pathways for upregulated (Figure 3C) and downregulated (Figure 3D) genes, with levels of significance of enrichment in each pathway represented by –log(P value). The top pathway for upregulated genes in CD4⁺ T cells of mice with a selective deletion of Kdm6a, as compared with WT, was the Th1 and Th2 activation pathway, with the Th2 pathway second (Figure 3C). On the other hand, the top pathway for downregulated genes was the neuroinflammation signaling pathway (Figure 3D). Differentially expressed genes in the top up- and downregulated pathways were visualized for their fold changes (x axis, logFC) and significance levels (y axis, –log10(P value)) using volcano plots (Figure 3, E and F, respectively). A heatmap shows differential expression of individual genes within top pathways in CD4⁺ T cells from Kdm6a cKO versus WT mice (Figure 3G).

**Deletion of Kdm6a affects the transcriptome in CD4⁺ T cells in healthy mice.** CD4⁺ T cells from healthy (non-EAE) mice were stimulated with anti-CD3/CD28 antibodies, followed by RNA-Seq. Expression differences of 3 genes were validated with quantitative RT-PCR using an independent set of samples (Table 2). Approximately 70% of differentially expressed genes in the healthy group showed small fold changes (–0.5 < logFC < 0.5); thus differences between Kdm6a cKO and WT mice were less robust in healthy mice than differences observed in EAE mice (Supplemental Figure 3A and B). We then performed pathway analysis for RNA-Seq data from healthy mice. We used the same filtration threshold (FDR < 0.1, logCPM > 1, logFC > 1 or logFC < –1) as described for EAE data. However, in order to have enough genes to perform pathway analysis in the healthy group, we assessed enrichment of all genes in the cKO group (both up- or downregulated). Gene expression changes in the pathways shown in Supplemental Figure 3, C and D, show enrichment of both up- and downregulated pathways in cKO compared with WT in healthy mice. In both healthy and EAE mice, Th1 and Th2 cell–related pathways were top enriched pathways, although the enrichment in healthy mice was less robust than in EAE. We then compared the differentially expressed genes in cKO versus WT that were in common between healthy and EAE mice (Supplemental Figure 3E). The majority of differentially expressed genes changed in the same directions in both healthy and EAE mice; however, a small number of genes showed changes in opposite directions (Supplemental Figure 3E).

**Deletion of Kdm6a decreases CD44 expression in CD3⁺CD4⁺ T cells in healthy mice.** To ascertain whether the conditional KO of Kdm6a in CD4⁺ cells changed the composition of T cell subpopulations, we examined T cell populations in spleens from healthy (non-EAE) cKO and WT mice. While the percentage of CD3⁺ cells was unchanged, CD3⁺CD4⁺ T cells were decreased, while

### Table 1. EAE scores for WT and Kdm6a cKO mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sex</th>
<th>Group</th>
<th>Incidence</th>
<th>Day of onset</th>
<th>Peak clinical score</th>
<th>Cumulative disease index (all days)</th>
<th>Length of experiment (days)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>WT</td>
<td>4/4</td>
<td>17.3 ± 2.3</td>
<td>4.3 ± 0.3</td>
<td>107.1 ± 5.1</td>
<td>46</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cKO</td>
<td>3/5</td>
<td>15.0 ± 0.0</td>
<td>2.5 ± 0.5</td>
<td>25.8 ± 4.4</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>WT</td>
<td>5/5</td>
<td>15.4 ± 1.4</td>
<td>3.5 ± 0.4</td>
<td>79.1 ± 19.4</td>
<td>46</td>
<td>0.0242</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cKO</td>
<td>7/7</td>
<td>14.9 ± 0.4</td>
<td>2.8 ± 0.2</td>
<td>34.3 ± 4.3</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>WT</td>
<td>5/5</td>
<td>13.2 ± 0.9</td>
<td>3.9 ± 0.1</td>
<td>57.4 ± 2.8</td>
<td>29</td>
<td>0.00118</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cKO</td>
<td>4/4</td>
<td>16.3 ± 1.8</td>
<td>2.9 ± 0.5</td>
<td>15.5 ± 8.3</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>WT</td>
<td>9/9</td>
<td>11.5 ± 0.5</td>
<td>3.9 ± 0.2</td>
<td>18.8 ± 1.8</td>
<td>17</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cKO</td>
<td>11/11</td>
<td>14.2 ± 0.4</td>
<td>2.1 ± 0.3</td>
<td>6.5 ± 1.2</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

A protective effect of Kdm6a deletion in CD4⁺ T cells was observed in 4 independent EAE experiments. Experiments 1 (shown in Figure 2B) and 2 were conducted at the same time, using males and females. The RNAs from experiment 4 were used for the high-throughput sequencing experiment and quantitative RT-PCR validation. P values were from 1-way repeated measures ANOVA comparing cKO mice and their WT littermate controls.
Figure 3. Differences in Th1/Th2 activation and neuroinflammation signaling pathways in CD4 Kdm6a KO mice. (A) Scatterplot shows upregulated (red) and downregulated (blue) genes in CD4+ T cells in cKO as compared with WT. Differentially expressed genes were identified with edgeR (FDR < 0.1, logFC > 1 or logFC < -1, logCPM > 1). Other genes filtered out with this threshold are shown as gray dots. (B) The expression difference of 5 genes was validated with RT-PCR using biological replicates of 3 WT and 3 cKO: increased in cKO (Klra7: P = 0.0018, Ccl5: P = 0.0012), decreased in cKO (P2rx7: P = 0.0017, Tlr1: P = 0.0017, Cxcl10: P = 0.0204, t tests). In box-and-whisker plots, thick lines inside boxes represent the median of the data. Lower and upper ends of boxes show quantiles (25% and 75%), and whiskers show minimum and maximum values. P values were determined by t test. (C and D) Top 10 canonical pathways for the differentially expressed genes between WT and cKO CD4+ T cells. Significantly expressed genes (FDR < 0.1, logCPM > 1) were classified into 2 groups, and canonical pathway analysis was performed for each: (C) upregulated genes (logFC > 1) and (D) downregulated genes (logFC < -1), each in cKO as compared with WT. Genes within the top upregulated pathways in (C) Th1 and Th2 activation pathway and downregulated pathway in (D) neuroinflammation signaling pathway were visualized for their significance levels with volcano plots (E and F, respectively; pink dots represent Th1 and Th2 activation pathway genes, and green dots represent neuroinflammation signaling pathway genes). Gray circles represent all other genes not in these pathways, with the blue line as a threshold of significance (FDR = 0.1: any gene above this line considered significantly different). (G) Heatmaps for genes within the top canonical pathways: Th1 and Th2 activation pathway and neuroinflammation signaling pathway (red indicates upregulated, green represents downregulated).
CD3^+CD8^+ T cells and CD3^+NK1.1^+ cells were each increased in the cKO compared with WT (Figure 4A). There was no difference in CD4^+CD25^+FOXP3^+ T regulatory cells between WT and cKO (not shown, WT, 11.51% ± 0.2700%, cKO, 10.91% ± 0.2270%, P = 0.1075, t test). We further characterized T cells in Kdm6a cKO mice by analyzing the populations of naive and memory CD3^+CD4^+ T cells. The naive CD3^+CD4^+ population was increased, while the memory CD3^+CD4^+ population was decreased in the cKO compared with the WT (Figure 4B). Also, within the CD3^+CD4^+ T cell population, there was a decrease in CD44 expression in the cKO as measured by MFI (Figure 4, B and C). Together, these results were consistent with a shift toward more naive and fewer memory T cells in the cKO as compared with WT.

To ascertain whether the decreased expression of CD44 in CD3^+CD4^+ T cells from cKO mice could be due to direct effects of KDM6A's histone demethylase activity on the Cd44 gene, we examined H3K27me3 ChIP-Seq data from CD4 Kdm6a cKO mice (GSE70795) and found more H3K27me3 modification near the transcriptional start site of the Cd44 gene in the cKO compared with WT (Figure 4D), suggesting direct regulation of Cd44 expression by KDM6A. Further, we analyzed the expression of Cd44 mRNA in healthy WT versus cKO CD4^+ T cells stimulated with anti-CD3/CD28 antibodies and found lower expression of Cd44 in the cKO as compared with WT (Figure 4E). These results are consistent with previous reports that CD44 depletion promotes a Th2 phenotype upon stimulation (29), with decreased IL-2 production (30) and decreased T cell–mediated inflammatory responses in several animal models, including EAE (31).

**Influence of Kdm6a on CD4^+ T cell proliferation and cytokine production.** We assessed proliferation of naive CD4^+ T cells stimulated with anti-CD3/CD28. The proliferation index was no different between WT and cKO, but the division index was lower in the cKO (Figure 5, A and B) and the percentage of undivided cells was higher in the cKO, each as compared with WT (Figure 5, C–E). Next, we determined whether the cKO of Kdm6a affects cytokine levels. In healthy CD4^+ T cells stimulated with anti-CD3/CD28, there was a decrease in IL-2 and IL-17A, and an increase in IFN-γ, in the cKO compared with WT, while IL-5 was no different between the two (Figure 5F). Finally, when we analyzed cytokine production from lymph node cell cultures of EAE mice stimulated with autoantigen (Figure 5G), again there was a decrease in IL-2 and IL-17A in the cKO. However, in EAE, there was also a decrease in IFN-γ as well as an increase in IL-5. This was consistent with RNA-Seq data showing a shift to more of a Th2 phenotype during EAE.

**Discussion**

We hypothesized that an X chromosome gene that escapes X inactivation would have higher expression in females and may contribute to the sex difference in autoimmune disease susceptibility. By analyzing several high-throughput RNA-Seq data sets, we identified Kdm6a as the top sexually dimorphic gene that escapes X inactivation in CD4^+ T cells in humans and mice. When Kdm6a was deleted in CD4^+ T cells, EAE was ameliorated. Further, the CD4^+ T cell transcriptome was altered. High-throughput RNA-Seq and canonical pathway analysis showed upregulation of T helper cell–related pathway genes and downregulation of neuroinflammation pathway genes. Since the expression of Kdm6a in CD4^+ T cells is disease promoting in EAE, higher expression of Kdm6a from 2 alleles in females is consistent with increased female susceptibility to MS.

Since Kdm6a is an X-linked gene, there are 2 copies of Kdm6a in females (XX) and 1 copy in males (XY). In males, the Y chromosome has a homologous gene, Uty, that evolved from the same ancestor as the Kdm6a gene; however, the functionality of the UTY protein is controversial. It was suggested that there was no histone demethylase activity of UTY (18, 32–35). On the other hand, Walport et al. reported that UTY possesses histone demethylase activity, albeit with lower activity than that of KDM6A (36). Thus, UTY function in EAE may be similar to that of KDM6A, but the effect of UTY may be of a lesser degree. While mechanisms for balance and compensation between the sexes occur, absolute balance is often not the case (37). Also, a difference in the overall histone methylation state between males and females would not merely reflect sexually dimorphic expression of histone demethylases. For example, histone demethylases, as well as other histone modifiers, play a role in X inactivation in females, but not males.

KDM6A is a histone modifier that affects whole genome expression by removing the suppressive histone mark H3K27me3. Removal of H3K27me3 increases expression of genes regulated...
by this mark. Therefore, by deleting Kdm6a, the H3K27me3 mark remains and the expression of target genes is lowered. In our RNA-Seq data comparing cKO and WT, we observed both up- and downregulated genes in the cKO, which indicates that Kdm6a deficiency in CD4+ T cells has both direct (downregulation) and indirect (upregulation) effects. Genes that are upregulated in response to Kdm6a deletion may include an intermediate gene, such that KDM6A directly regulates a gene that inhibits expression of the target gene.

The most enriched pathways upregulated in cKO CD4+ T cells were the Th1 and Th2 activation pathway, followed by the Th2 pathway. Downregulated pathways included neuroinflammation signaling, Toll-like receptor signaling, and IL-17 signaling. This is consistent with EAE being a Th1/Th17-mediated disease. At the single gene level, the Th2 cytokine, Il5, had increased RNA expression in CD4+ T cells in the cKO. While a Th1 chemokine receptor, Cxcr3, had higher expression in cKO CD4+ T cells, there was a decrease of the CXCR3 ligand Cxcl10 in the cKO. The master Th17 transcription factor Rorc was also downregulated in cKO CD4+ T cells. Together, these data show broad immunomodulation in cKO mice with consistent alteration in histone modification across the genome in mice with a specific deletion of Kdm6a in CD4+ T cells in EAE.

KDM6A has a JmjC domain that is evolutionarily conserved in the jumonji family of transcription factors and has a function in the histone demethylation mechanism. KDM6A and other JmjC domain–containing proteins are known to play a role in the immune response (38). In macrophages, knockdown of Kdm6a inhibited the production of IL-6 and IFN-β, and the regulation of IL-6 production was JmjC domain dependent (39). Another JmjC domain–containing protein, JMJD3 (also known as KDM6B), which is an autosomal paralog of Kdm6a, regulates CD4+ T cell differentiation. Deletion of Jmjd3 promoted Th2/Th17 differentiation in the small intestine and colon and suppressed Th1 differentiation (40). In an in vitro differentiation experiment of CD4+ T cells from spleen and lymph node, Jmjd3 deficiency selectively reduced Th17 differentiation. Additionally, cKO of Jmjd3 in CD4+ T cells reduced EAE disease severity, likely due to decreased Th17 differentiation (41). Furthermore, another Jmjc protein family gene, Mina (also called Riox2), binds to the Il4 promoter and represses its expression, modulating Th2 differentiation (42).

Recently, KDM6A was identified as the molecular target of metformin. Metformin is an FDA-approved drug for the treatment of diabetes; it inhibits the histone demethylase activity of KDM6A to increase the global level of H3K27me3 histone modifications (43). Thus, in the context of this study, metformin could be viewed...
as a pharmacologic Kdm6a knockdown that is not cell specific. Metformin has been used in EAE studies in which it attenuated clinical disease scores and was immunomodulatory (44, 45). This supports our finding that deficiency of Kdm6a in CD4+ T cells is protective in EAE. While our study shows cell specificity, both studies support an important role of Kdm6a in EAE. Thus, metformin as a treatment for MS warrants investigation. Consistent with the importance of sex as a biological variable in therapeutic dosing, since Kdm6a is an X chromosome gene that has higher expression in females, the effective dose of metformin in MS may be different in females versus males.

Previous research indicated that sex differences in the MS mouse model EAE are caused both by gonadal hormones and by sex chromosomes. In this study, we determined the role of the X inactivation escapee gene Kdm6a in this sex difference. Kdm6a in CD4+ T cells plays a disease-promoting role in EAE and is immunomodulatory. Thus, expression of KDM6A from 2 alleles in women versus 1 allele in men may contribute to the higher susceptibility of women to MS. Modulation of KDM6A in CD4+ T cells is a therapeutic target for MS and potentially other autoimmune diseases with a female preponderance (46).

Methods

High-throughput gene expression analysis for X chromosome genes. RNA-Seq data sets for C57BL/6J naive CD4+ T cells from spleen (GSE94671; 3 males and 3 females), C57BL/6J stimulated CD4+ T cells from lymph nodes (GSE121292; FCG: 6 XX and 6 XY), and SJL stimulated CD4+ T cells from lymph nodes (GSE121705; FCG: 6 XX and 5 XY) were obtained from the GEO database. R package QuasR (47) was used for the read alignment to the mouse genome (mm10) followed by counting at the gene level. Significance of differences between male and female (or XX and XY) mice was determined using R package edgeR (48). FDR of 0.1 was used as the threshold of differentially expressed genes.

A microarray data set for human naive CD4+ T cells from healthy controls (GSE56033; 205 males and 294 females) was obtained from the GEO database. Statistical analyses and production of figures were performed in R (R Core Team, 2018, http://www.R-project.org/). The microarray data sets were quantile normalized before running any analysis. Differentially expressed genes were identified using 1-way ANOVA.

Animals. Mice of the FCG model were generated from males lacking the testis-determining Sry gene on the Y chromosome, called the Y* chromosome, but with an Sry transgene on chromosome 3 (20). XY mice (gonadal females) were compared with XX (gonadal females) to assess the effect of XX vs. XY sex chromosomes in mice without a confound of differences in gonadal hormones (49). Conditionally targeted Kdm6a mice were generated from “KO-first” mice containing the allele Kdm6a<sup>tm2a(EUCOMM)Wtsi</sup> (50–52). To remove the lacZ reporter cassette and generate a floxed Kdm6a allele, KO-first Kdm6a mice were crossed with B6.129S4-T(ROSA)26Sor<sup>tm1(FLP1)Dym</sup>/RainJ mice (Jackson Laboratory), which have global expression of the FLP1 recombinase, including expression in germline cells. This cross generated Kdm6a<sup>fl/WT</sup> mice with 1 floxed Kdm6a allele and 1 WT allele. Kdm6a<sup>fl/WT</sup> XX mice were backcrossed onto the C57BL/6J background for at least 13 generations. Then, Kdm6a<sup>fl/WT</sup> female mice were crossed with
B6.Cg-Tg(CD4-cre)1Cwi/BfluJ (Jackson Laboratory) male mice to generate CD4-Cre+/– Kdm6afl/y female mice and CD4-Cre+/– Kdm6afl/y female mice were then crossed with Kdm6afl/y males to generate CD4-Cre+/– Kdm6afl/y females, CD4-Cre+/– Kdm6afl/y males, and their littermate controls without CD4-Cre (CD4-Cre−/−). These mice were used for the experiments in this study.

**Active EAE induction.** cKO mice and WT littermate C57BL/6 mice were induced with active EAE using standard methods for induction of disease with myelin oligodendrocyte glycoprotein (MOG) amino acids 35–55 peptide (MOG35–55) (53). Briefly, EAE was induced in female and male mice at 12 to 16 weeks of age by subcutaneous injection of MOG35–55 (200 μg/animal, Mimotopes) emulsified in CFA supplemented with mycobacterium tuberculosis H37Ra (200 μg/animal, Difco Laboratories) over 2 sites drained by left inguinal and auxiliary lymph nodes on day 0 (0.1 mL/mouse). A booster immunization was applied subcutaneously on day 7 over contralateral lymph nodes. Pertussis toxin (500 ng/mouse) (List Biological Laboratories) was injected intraperitoneally on days 0 and 2 (54). The animals were monitored daily and scored based on a standard EAE 0–5 scale scoring system: 0, healthy; 1, complete loss of tail tonicity; 2, loss of righting reflex; 3, partial paralysis; 4, complete paralysis of one or both hind limbs; and 5, preterminal state, as described (55). EAE scores were performed blinded for cKO versus WT status.

**CD4⁺ T cell or CD8⁺ T cell isolation.** Mice were euthanized with a lethal dose of isoflurane. Auxiliary, brachial, and inguinal draining lymph nodes were collected from female (CD4⁺) and male (CD8⁺) WT and Kdm6a KO EAE mice (8 to 12 weeks old) and passed through 40 μm cell strainers to obtain single-cell suspensions. Lymphocytes were isolated via Lympholprep density gradient (Axis-Shield) and subsequently washed with 1× PBS. CD4⁺ T cells or CD8⁺ T cells were isolated by negative selection using the EasySep Mouse CD4⁺ T Cell Isolation Kit or EasySep Mouse CD8⁺ T Cell Isolation Kit, respectively, following the manufacturer’s instructions (STEMCELL Technologies).

**Lymphocyte culture.** Healthy CD4⁺ T cells from the lymph nodes were cultured in cRPMI medium 24-well plates at a concentration of 1 × 10⁶ cells per well with Mouse T-Activator CD3/CD28 Dynabeads (Gibco, Life Technologies) following the manufacturer’s instructions at 37°C with 5% CO₂ for 36 hours. Cells were harvested, spun down at 300 g, 4°C for 7 minutes, then resuspended in 600 μL TrisReagent (Zymo Research) and stored at −80°C until RNA isolation.

Single-cell lymphocytes from EAE-induced mice were resuspended in complete RPMI media: cRPMI; RPMI-1640 (Lonza), 5% FBS, 1% L-glutamine (200 mM, Gibco, Thermo Fisher Scientific), 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific), 1% NEAA (×100, Lonza), 1% Na pyruvate (100 mM, Lonza), 1.25% 1M HEPES (Lonza), and 0.1% 2-mercaptoethanol (55 mM, Gibco, Thermo Fisher Scientific). Cells were plated in 96-well plates at a concentration of 5 × 10⁴ cells/100 μL with 25 μg/mL of MOG35–55 autoantigen and were cultured at 37°C with 5% CO₂ for 36 hours. Cells were spun down at 350 g for 10 minutes at 4°C. Cells were harvested, washed with cRPMI media, resuspended in RoboSep Buffer (0.5 mL for up to 1 × 10⁵ cells, STEMCELL Technologies), and sorted for CD4⁺ T cells by negative selection using the manufacturer’s instructions (STEMCELL Technologies). Isolated CD4⁺ T cells were homogenized in 600 μL of TriReagent (Zymo Research) and stored at −80°C for RNA isolation.

**Cytokine measurements.** Supernatants from healthy female CD4⁺ cell cultures stimulated with Mouse T-Activator CD3/CD28 Dynabeads as well as from female EAE day 29 lymph node cultures stimulated with autoantigen were analyzed for cytokine production using the Mouse Th1/Th2/Th17 Cytokine Kit (catalog 560485, BD Biosciences) and the Mouse IL-5 Flex Set (catalog 558302, BD Biosciences) following the manufacturer’s instructions. Samples were run in duplicate, and the average for each sample was reported. Data were analyzed on a SORP BD LSRII Analytic Flow Cytometer using BD FACSDiva 8.0 and FCAP Array Software (version 3.0, BD Biosciences).

**Immunofluorescence staining.** After exposure to a lethal dose of isoflurane and subsequent lymph node collection, the mice were perfused transcardially with ice-cold 1× PBS for 8–15 minutes, followed by ice-cold 10% buffered formalin for 10 to 15 minutes. Spinal cords were dissected and submerged in 10% buffered formalin overnight at 4°C, followed by 30% sucrose in 1× PBS for 24 hours. Tissues were embedded in 75% gelatin/15% sucrose solution for cryostat sectioning, then post-fixed overnight in 10% buffered formalin and cryoprotected in 30% sucrose. The embedded tissues were flash frozen in dry ice and stored at −80°C. Tissues were cryosectioned at −25°C into 40 μm thick sections using a cryostat (Leica CM1860) and stored in 1× PBS with 0.1% sodium azide at 4°C before immunofluorescence staining.

Thoracic spinal cord sections were washed thoroughly using 1× PBS to remove residual sodium azide. Tissue sections were incubated with 5% glacial acetic acid, 95% ethanol solution for 15 minutes at room temperature (RT) and then washed with 1× PBS before blocking. For blocking, tissues were incubated in blocking solution (10% goat serum, 0.1% Tween 20) for 1 hour at RT. Primary and secondary antibody stainings were done at appropriate concentrations with 2% NGS, 1× PBS with 0.1% Tween-20 (1× PBST) overnight at 4°C and 2 hours at RT, respectively. After serial washes, sections were mounted onto slides (SuperfrostPlus, VWR), allowed to semidry, and covered-lipped (Fisher Scientific) with Fluoromount-G (SouthernBiotech) for confocal microscopy as described (56). The following primary antibodies were used: rabbit anti-NF200 (Sigma Aldrich, catalog N4142, 1:250), rabbit anti-IAPP (Life Technologies, catalog 51-2700, 1:200), rabbit anti-Iba1 (Wako Chemicals, catalog 019-19741, 1:1000), and rat anti-CD3 (BD Pharmingen, catalog 552573, 1:750). The following secondary antibodies were used: goat anti-rabbit IgG-cy5 conjugated (Invitrogen, catalog A32733, 1:500), goat anti-rat IgG-cy3 conjugated (Jackson Immunoresearch Laboratories Inc., catalog 112-165-167, 1:500).

Quantification of immunostaining results was performed on 3 sections of spinal cord (levels T1–T5) per animal. Images of immunostained sections were obtained using an Olympus BX51 fluorescence microscope with a DPSO digital camera, and 10× images were taken at 8 to 10 μm thickness with 2 μm stacks. Identical light intensity and exposure times were applied to all photographs from each set, and images were processed using the integrated software program Slidebook4.2 (Intelligent Imaging Innovations). ImageJ (NIH) was used for image analysis. The images for NF200 and IAPP were converted to binary images with fixed threshold and analyzed for the area fraction in ventral white matter of spinal cord. Globoid Iba1⁺ cells and CD3⁺ cells were each manually counted in ventral white matter, and the number was normalized by averaging with area size. All analyses were done in a blinded fashion.

**RNA isolation, high-throughput sequencing, and statistical analysis.** RNA purification was performed using the Direct-zol RNA MicroPrep Plus Kit following the manufacturer’s instructions (Zymo Research). The RNA-Seq library was made using the KAPA Stranded
RNA-Seq Kit (Kapa Biosystems), which consists of mRNA enrichment, cDNA generation, end repair, A-tailing, adaptor ligation, strand selection, and PCR amplification. Barcoded adaptors were used for multiplexing samples in 1 lane. Sequencing was performed on Illumina HiSeq3000 for a single end 1 × 50 run. Data quality check was done on Illumina SAV. Demultiplexing was performed with the Illumina Bcl2fastq v 2.17 program. These procedures were performed at the UCLA Technology Center for Genomics and Bioinformatics core facility.

Statistical analyses and production of figures were performed in R (R Core Team, 2018). Qualities of raw sequence data were examined using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and Trimmomatic (57) was used for cleaning. R package QuasR (47) was used for the read alignment to the mouse genome (mm10), followed by counting at the gene level. We assumed read counts followed a negative binomial distribution and constructed generalized linear models based on this negative binomial distribution assumption. To visualize the relationship of samples, principal component analysis was performed using transcripts per kilobase million (TPM). The genes with count numbers less than 1 in more than 3 samples were filtered out. Differentially expressed genes between cKO and WT mice were identified with R package edgeR (48). Heatmaps were created using the R package gplots. An FDR of 0.1 was used as the threshold of differentially expressed genes. Canonical pathway enrichment analysis was performed for the differentially expressed genes (FDR < 0.1, logFC > 1 or logFC < −1, logCPM > 1) using Ingenuity Pathway Analysis software (QIAGEN).

Quantitative nested RT-PCR. To validate the RNA-Seq result, we used quantitative nested RT-PCR, which is advantageous over standard quantitative RT-PCR in that it better amplifies the genes from a small amount of RNA and increases the gene specificity.

RNAs were treated with RNase-Free DNase (Promega) to eliminate possible genomic DNA contamination, followed by first-strand cDNA synthesis with SuperScript III (Invitrogen) using a mixture of nuclease possible genomic DNA contamination, followed by first-strand cDNA synthesis with SuperScript III (Invitrogen) using a mixture of dNTPs, 2 mM MgCl2, 0.4 μM each primer (outer primers), 5 ng cDNA, 0.075 μL of Ex Taq DNA Polymerase (Takara), and one-tenth volume of ×10 Ex Taq Buffer (Mg2+ free) (Takara). The PCR reaction was held at 94°C for 4 minutes before the beginning of 15 cycles of 94°C for 45 seconds/60°C for 30 seconds/72°C for 60 seconds, followed by a single 7-minute period at 72°C. To maintain the ratio between target and internal control (Actb) genes, this first round of amplification was performed as a multiplex PCR of each target (P2rx7, Tlr1, Cxcl10, Ccl5, or Kbfa1) and control (Actb) primers. PCR products were purified with DNA Clean & Concentrator-5 Capped Columns (Zymo Research).

Quantitative PCR was carried out on the purified DNA from first amplification with nested primers using PowerUp SYBR Green Master Mix (Applied Biosystems) in Applied Biosystems 7300 Real-Time PCR System. The differences between cKO and WT littermate RNAs were analyzed by t test. Primers were as follows: P2rx7 (outer: 5′-CCCTGTCACAGTGAAGCATCA-3′ and 5′-GAGGCTCTGCTGTTCCATCT-3′, Ccl5 (outer: 5′-CCCTACAGCATCCTTCCTACT-3′ and 5′-AGGCAAGCATGACAGGA-3′, P2rx7: 5′-GGTCCGACGTCAGGAT-3′ and 5′-CCACTCTGTTGCTGATTCCAC-3′ and 5′-AGTCCGAGAAACAAATGTGAG-3′, Kbfa1 (outer: 5′-CTTAGGGGCCACCTCTTCAT-3′ and 5′-TGAGATCCATGACACCTTTG-3′), and Actb (outer: 5′-TTGCTGACAGATGCAGAACAG-3′ and 5′-GGTGATAAACCGCTGCTGAC-3′, nested: 5′-GGCTCTAGCACCAGAGA-3′ and 5′-ACTCTGTTGCTGATTCCAC-3′). For the quantitative PCR validation for non-EAE (healthy) mice, primers were as follows: P2rx7 (5′-AGCCTCTGGGAAAAATGCT-3′ and 5′-TGCTGAGAGATGGACACA-3′), Rgc (5′-TGGACAGGACGATGTG-3′ and 5′-TAGTACTGTTCGACCAG-3′ and 5′-TGTGAGAATGAGGGCCATAGG-3′, Kbfa1 (5′-GTGCCCACGTCAAGGAGTAT-3′ and 5′-GTGCCCACGTCAAGGAGTAT-3′, nested: 5′-GTGCCCACGTCAAGGAGTAT-3′ and 5′-TGTGCCCACGTCAAGGAGTAT-3′), Actb (5′-GTGCCCACGTCAAGGAGTAT-3′ and 5′-TGTGCCCACGTCAAGGAGTAT-3′, and 5′-ACTCTGTTGCTGATTCCAC-3′). FACS buffer (1× PBS + 2% FBS) to a concentration of 1 × 106 cells/100 μl, and 16/32 blocking antibody (BioLegend, 101302) was added at 1:100 dilution and incubated at RT for 10 minutes. Surface proteins were stained in FACS buffer (1× PBS + 2% FBS) to a concentration of 1 × 106 cells/100 μl, and 16/32 blocking antibody (BioLegend, 101302) was added at 1:100 dilution and incubated at RT for 10 minutes. Surface proteins were stained with anti–CD3-BV421 (catalog 100027), anti–CD4-APC (catalog 100042), anti–CD8-PE (catalog 100707), anti–CD44-FITC (catalog 103006), and anti–CD62L-BV711 (catalog 104445), anti–NK1.1-PerCP (catalog 108725), (BioLegend 1:100), or the isotype controls; BV421 rat IgG2b,κ (catalog 400639), APC rat IgG2b,κ (catalog 400612), PE rat IgG2a,κ (catalog 400508), FITC rat IgG2b,κ (catalog 400506), BV711 rat IgG2a,κ (catalog 400511), or PerCP mouse IgG2a,κ (catalog 400250) (BioLegend 1:100) for 20 minutes at RT in the dark. Data are representative of 3 replicate experiments. NK1.1 staining was included in only the last 2 experiments.

For FOXP3 staining, surface proteins were stained with anti–CD4-FITC (catalog 103008) and anti–CD25-PE (catalog 102008), or FITC rat anti–IgG2b,κ (catalog 400806), and PE rat anti–IgG1,γ (catalog 401906) (1:100, BioLegend) for 20 minutes at RT. The cells were fixed and permeabilized overnight at 4°C in the dark using the FoxP3 Transcription Factor Staining Buffer Kit (Thermo Fisher Scientific) following the manufacturer’s instructions. Intracellular proteins were stained with anti–FOXP3-APC (17-5773-82, 1:100, Thermo Fisher Scientific) for 30 to 60 minutes at RT in the dark. Data are representative of 2 replicate experiments.

For all flow cytometric experiments, each animal was analyzed in triplicate and average values were reported per animal. Flow cytometric analyses were performed on the SORP BD LSRII Analytic Flow Cytometer using BD FACSDiva 8.0 and FlowJo (version 10.5.3) software. Positive gates were determined based on positive and isotype controls.

H3K27me3 ChIP-Seq analysis. ChIP-Seq data for H3K27me3 modification in mature CD4 cell single-positive thymocytes from WT and cKO were obtained from the GEO database (GSE70795; ref. 38). R package QuasR (47) was used for the read alignment to the mouse genome (mm10) followed by the visualization of H3K27me3 modification along the genome location.

CD4+ T cell proliferation assay. Healthy CD4+ T cells from the lymph nodes were resuspended at 1 × 106 cells/ml in prewarmed...


Data availability. Data sets generated during this study were deposited in the NCBI's Gene Expression Omnibus database (GEO GSE121703 and GSE128615).

Statistics. Differences of EAE scores between WT and cKO were measured by repeated measures 1-way ANOVA. Quantitative differences of CD3+ cell count, globoid Iba1+ cell count, βAPP+ percentage area for axonal damage, and NF200+ intact axonal count were examined by Mann-Whitney U test. The statistical tests for microarray and RNA-Seq were separately described in the each section. P values of less than 0.05 was considered significant.

Study approval. The present studies in animals were reviewed and approved by the Chancellor’s Animal Research Committee of the UCLA Office for the Protection of Research Subjects. Human data were from the GEO database; thus, no approval is necessary.

Author contributions

YI and RRV contributed to the study design. YI and NI contributed to the induction of EAE and scoring. LCG contributed to CD4+ T cell isolation and immune phenotyping. NI, MAM, ER, and VT contributed to tissue collection and processing, immunostaining, and histological analysis. YI contributed to mouse breeding, RNA isolation, quantitative RT-PCR, data mining and analysis of sourced data sets, and high-throughput sequencing analysis. YI, NI, LCG, APA, and RRV wrote the manuscript. All authors read and accepted the final version of the manuscript.

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Address correspondence to: Rhonda R. Voskuhli, Neuroscience Research Building 1, Room 475D, 635 Charles E. Young Drive South, Los Angeles, California 90095, USA. Phone: 310.206.4636; Email: rvoskuhli@mednet.ucla.edu.