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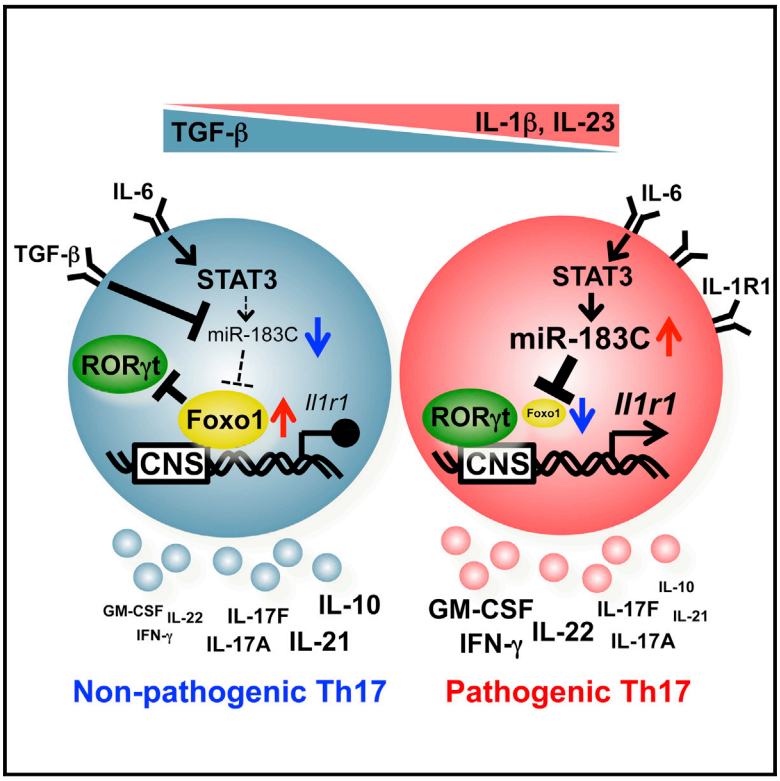
Immunity

The MicroRNA-183-96-182 Cluster Promotes T Helper 17 Cell Pathogenicity by Negatively Regulating Transcription Factor Foxo1 Expression

Graphical Abstract



Highlights

- miRNAs are essential for the pathogenic function of Th17 cells
- miR-183C induced by IL-6-STAT3 signaling promotes Th17 cell pathogenicity
- Foxo1 is a direct target of miR-183C and suppresses Th17 cell pathogenic function
- IL-1R1 regulation by miR-183C is involved in the pathogenicity of Th17 cells

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In Brief

Epigenetic regulation is important for T cell differentiation. However, the roles of miRNAs in Th17 cell development and function are unclear. Dong and colleagues identify miR-183C as a pathogenic Th17-cell-specific miRNA and demonstrate that miR-183C promotes Th17 cell pathogenicity via suppression of Foxo1 expression.

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The MicroRNA-183-96-182 Cluster Promotes T Helper 17 Cell Pathogenicity by Negatively Regulating Transcription Factor Foxo1 Expression

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SUMMARY

T helper 17 (Th17) cells are key players in autoimmune diseases. However, the roles of non-coding RNAs in Th17 cell development and function are largely unknown. We found that deletion of the endoribonuclease-encoding *Dicer1* specifically in Th17 cells protected mice from experimental autoimmune encephalomyelitis. We found that the *Dicer1*-regulated microRNA (miR)-183-96-182 cluster (miR-183C) was highly expressed in Th17 cells and was induced by cytokine IL-6-STAT3 signaling. miR-183C expression enhanced pathogenic cytokine production from Th17 cells during their development and promoted autoimmunity. Mechanistically, miR-183C in Th17 cells directly repressed expression of the transcription factor Foxo1. Foxo1 negatively regulated the pathogenicity of Th17 cells in part by inhibiting expression of cytokine receptor IL-1R1. These findings indicate that the miR-183C drives Th17 pathogenicity in autoimmune diseases via inhibition of Foxo1 and present promising therapeutic targets.

INTRODUCTION

After antigen stimulation, naive $CD4^+$ T cells differentiate into effector T cells with different effector phenotypes to regulate the adaptive immune response (Abbas et al., 1996). T helper 17 (Th17) cells, an effector subset that produces the cytokines IL-17A, IL-17F, and IL-22, play an important role in inducing tissue inflammation during autoimmune diseases as well as in host defense against extracellular bacteria and fungi (Korn et al., 2009). Th17 differentiation can be induced from naive T cells by a combination of cytokines TGF-β, IL-6, and IL-1 (Korn et al., 2009). These cytokines induce the expression of two nuclear orphan receptors, RORγt and RORα, which have also been shown to be indispensable for the development of Th17 cells (Ivanov et al., 2006; Yang et al., 2008a). In addition, both cytokines IL-21 and IL-23 are thought to be critical for the maturation and/or maintenance of Th17 cells (Nurieva et al., 2007; McGeachy et al., 2009).

Accumulating data indicate that the pathogenicity of Th17 cells might vary depending on the induction conditions. Th17 cells induced by IL-1β, IL-6, and IL-23 are thought to be more pathogenic than those induced by TGFβ and IL-6 in Th17-mediated inflammatory diseases (Peters et al., 2011). Furthermore, two papers have found that granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by Th17 cells is a crucial factor for the pathogenicity of Th17 cells during experimental autoimmune encephalomyelitis (EAE) (Codarri et al., 2011; El-Behi et al., 2011). Although the past few years have seen substantial progress in defining the functional regulators for Th17 differentiation, the precise molecular mechanism by which Th17 cells acquire pathogenic function has not been well defined.

Th17 differentiation is negatively regulated by various transcription factors. For example, forkhead box P3 (Foxp3), growth factor independent 1 (Gfi1), signal transducer and activator of transcription 5 (STAT5), and Ets1 have been identified as negative regulators of Th17 differentiation (Kurebayashi et al., 2013). The forkhead box O (Foxo) family of transcription factors is important in controling diverse cellular responses. Their function is tightly regulated by the phosphoinositide 3-kinase (PI3K) and Akt pathway, which in turn induces the phosphorylation of Foxo family proteins and their nuclear exclusion (Brunet et al., 1999). In the immune system, the functions of Foxo family proteins in the control of T cell responses have begun to be clarified with T cell-specific Foxo-deficient mouse models (Hedrick et al., 2012). For example, Foxo transcription factors promote the transcription of the *Foxp3* gene in induced T regulatory (Treg) cells, and Foxo1 negatively controls the differentiation of follicular helper T cells (Hedrick et al., 2012; Xiao et al., 2014). Furthermore, previous studies reported that Foxo1 negatively regulates IL-17A production from mouse and human memory T cells (Wu et al., 2013; Wan et al., 2011). However, the roles of Foxo family proteins in the differentiation as well as pathogenicity of Th17 cells remain unclear.

MicroRNAs (miRNAs) are a large class of non-coding RNAs that negatively modulate gene expression at the post-transcriptional level (Bartel, 2009). miRNAs have been reported to participate in the regulation of autoimmunity: deficiency of *Dicer1* or *Drosha* results in the development of autoimmune diseases in mice (O'Connell et al., 2010). Moreover, published works have verified that certain miRNAs play a role as pivotal regulators of the differentiation and function of Th cells (Baumjohann and Ansel, 2013). Regarding the role of miRNAs in Th17 cells, Cobb et al. reported that IL-17A production from Th17 cells induced by TGF-β and IL-6 was reduced in $Cd4^{Cre}Dicer1^{f/f}$ cells (Cobb et al., 2006). Furthermore, miR-155 and miR-326 have been shown to promote Th17 differentiation as well as the severity of EAE disease (O'Connell et al., 2010; Du et al., 2009). However, the role of miRNAs in pathogenic Th17 cells induced by IL-1β, IL-6, and IL-23 has not been elucidated so far.

Here, we show that pathogenic Th17 cell function was regulated by the miR-183-96-182 cluster (miR-183C) (Xu et al., 2007). Furthermore, the miR-183C targeted the 3′ untranslated region (3′ UTR) of *Foxo1* messenger (m)RNA. Foxo1 negatively regulated pathogenic Th17 cell function and miR-183C promoted pathogenic cytokine expression of Th17 cells and repressed Foxo1 expression. Collectively, our results demonstrate a critical role for miR-183C in Th17-cell-mediated autoimmune diseases.

RESULTS

miRNAs Are Necessary for Th17 Cell Function in Autoimmunity

Previously, several papers demonstrated the important roles of miRNAs in regulatory T cell function by using $Cd4^{Cre}Dicer1^{f/f}$ and $Foxp3^{Cre}Dicer1^{f/f}$ mice (O'Connell et al., 2010). To address the role of miRNAs in Th17 cells, we first investigated the role of miRNAs in Th17 development by using T-cell-specific *Dicer1* knockout ($Cd4^{Cre}Dicer1^{f/f}$) mice. As shown in Figure 1A, we found that when naive $CD4^+$ T cells from these and control mice were activated under the Th17 condition in vitro, the production of both IL-17A and IL-17F were significantly decreased in Dicer1-deficient T cells. Furthermore, real-time RT-PCR analysis revealed that *Il17a* and *Il17f* expression were diminished by deficiency of Dicer1 (Figure 1B). Importantly, lack of Dicer1 did not affect *Rorc* expression in Th17 cells (Figure 1B).

To investigate the Th17-cell-intrinsic function of target molecules, we next generated $Il17f^{Cre}$ mice in which Cre was specifically induced in Th17 cells producing IL-17F. $Il17f^{Cre}$ was generated by insertion of an IRES-Cre-polyA cassette into *IL-17F* exon 2 (Figure S1A in the Supplemental Information available with this article online). After removal of the puromycin-resistant gene in the mouse germline, heterozygous $Il17f^{Cre}$ mice were bred with C57BL/6 mice, and PCR analysis of tail genomic DNA was used for genotype analysis (Figure S1B). Furthermore, $Il17f^{Cre}$ mice were crossed with $Rosa^{yfp}$ mice so that Cre expression could be monitored by flow cytometry. To evaluate $Il17f^{Cre}$ system, we differentiated naive T cells from $Il17f^{Cre}Rosa^{yfp}$ mice into the Th1 and Th17 cells in vitro. As shown in Figure S1C, Th17 cells were detectable by intracellular staining for IL-17A and IL-17F as well as yellow fluorescent protein (YFP) expression. In contrast, there was no induction of YFP expression under the Th1 condition (Figure S1C). Similar to findings from a previous report that analyzed $Il17a^{Cre}Rosa^{yfp}$ mice (Hirota et al., 2011), about 50% of Th17 cells failed to express YFP (Figure S1C). We speculate that restimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin might induce IL-17F production before *Cre* expression leads to deletion of loxp-flanked sequences. In addition, we found that $IL\text{-}17F^+YFP^-$Th17 cells have less intracellular IL-17F protein than do $IL\text{-}17F^+YFP^+$ Th17 cells (Figure S1D), implying that *Il17f* transcription and therefore protein concentrations of Cre recombinase were below a threshold critical for the induction of recombination. Moreover, we immunized $Il17f^{Cre}Rosa^{yfp}$ mice with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 ($MOG_{35\text{-}55}$) to evaluate $Il17f^{Cre}$ system in vivo. Most Th17 cells (more than 90%) expressed YFP in the central nervous system (CNS) in immunized mice (Figure S1E). Thus, these experiments indicate that $Il17f^{Cre}$ system works effectively, especially in vivo.

To clarify the Th17 cell-intrinsic function of miRNAs in vivo, we next generated $Il17f^{Cre}Rosa^{yfp}Dicer1^{f/f}$ mice in which Dicer was specifically deleted in Th17 cells producing IL-17F. Previous studies have reported that $Cd4^{Cre}Dicer1^{f/f}$ mice showed a marked reduction of the mature T cell compartment (O'Connell et al., 2010). In contrast, we observed no abnormality in T cell development in $Il17f^{Cre}Rosa^{yfp}Dicer1^{f/f}$ mice (Figures S2A and S2B). Furthermore, we confirmed that the expression of Dicer was efficiently deleted in Th17 cells but not in Th1 cells (Figure S2C) and that miRNAs were also markedly reduced in Th17 cells (Figure S2D). To analyze these mice in vivo, we immunized $Il17f^{Cre}Rosa^{yfp}Dicer1^{f/f}$ mice with $MOG_{35\text{–}55}$ to induce EAE. We found that $Il17f^{Cre}Rosa^{yfp}Dicer1^{f/f}$ mice were completely protected against the development of EAE disease (Figure 1C). The absolute cell numbers and frequency of $CD4^+YFP^+$ T cells in the CNS on day 14 after the second immunization were significantly decreased in $Il17f^{Cre}Rosa^{yfp}Dicer1^{f/f}$ mice, implying an important role of miRNAs in the survival and proliferation of Th17 cells (Figure 1D). Moreover, the production of IL-17A from $CD4^+YFP^+$ cells in the CNS was completely absent in $Il17f^{Cre}Rosa^{yfp}Dicer1^{f/f}$ mice (Figure 1E). Previously, Hirota et al. used fate-mapping mice and found that IFN-γ producing cells in the spinal cord are exclusively derived from Th17 cells (Hirota et al., 2011). In correlation with their results, we found that the production of IFN-γ from $CD4^+YFP^+$ cells in the CNS was also abolished in $Il17f^{Cre}Rosa^{yfp}Dicer1^{f/f}$ mice (Figure 1E). Taken together, these results indicate that miRNAs play an important role in the function of Th17 cells in vitro and in vivo.

IL6-STAT3 Strongly Induces miR-183C Expression in Th17 Cells

To identify the miRNA species involved in Th17 cell development, we performed RNA sequencing by using highly purified in-vitro-differentiated T cells from several transcriptional reporter mice ($Ifng^{yfp}$, $Il17f^{fp}$, and $Foxp3^{gfp}$), eliminating the influence of non-polarized cells. As shown in Table S1, we identified 122 differentially regulated miRNAs. Interestingly, among them we found only five miRNAs (miR-183, miR-96, miR-182, miR-10b, and miR-351) that were highly expressed (more than 3-fold change relative to other Th subsets) in Th17 cells, and three of these (miR-183, miR-96, and miR-182) belonged to a single miRNA cluster, miR-183C. Previously, Xu et al. found that

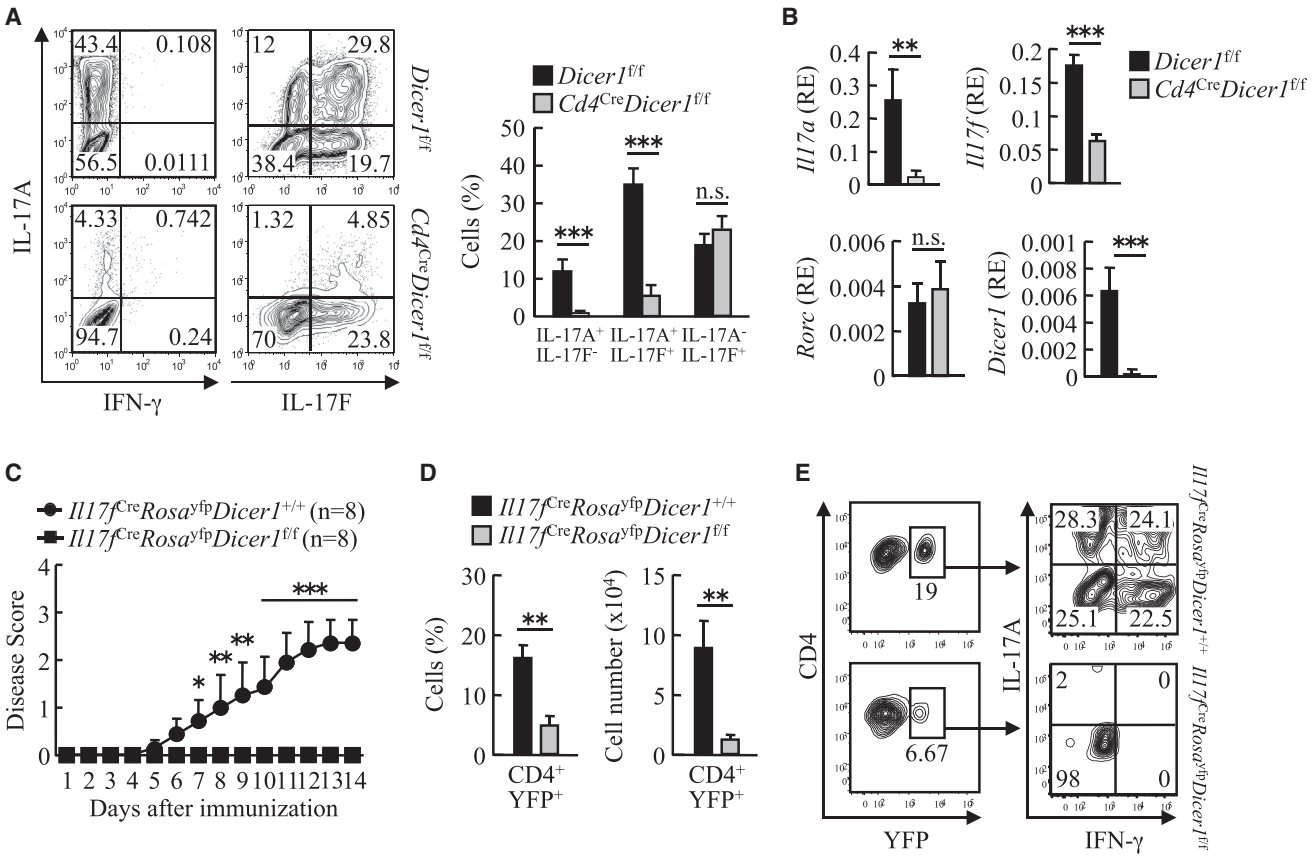

Figure 1. miRNAs Are Indispensable for the Pathogenic Function of Th17 Cells

(A) Naive CD4$^+$ from *Dicer1*$^{f/f}$ and *Cd4*^{Cre}*Dicer1*$^{f/f}$ mice were cultured under Th17 condition for 4 days. The production of IL-17A, IL-17F and IFN-γ were examined by intracellular staining. The bar graph shows the statistics of intracellular staining.

(B) Relative *Il17a, Il17f, Rorc*, and *Dicer1* mRNA expression in Figure 1A. The expression levels were monitored by real-time RT-PCR, and the data were normalized to *Gapdh* expression (RE: relative expression).

(C) Clinical scores of EAE in age-matched *Il17f*^{cre}*Rosa*^{yfp}*Dicer1*$^{+/+}$ (n = 8) and *Il17f*^{cre}*Rosa*^{yfp}*Dicer1*$^{f/f}$ (n = 8) mice.

(D) The cell frequencies as well as absolute cell numbers of CD4$^+$YFP$^+$ in the infiltrates of CNS from EAE mice.

(E) Intracellular staining showing IL-17A and IFN-γ production in CD4$^+$YFP$^+$ cells of CNS from EAE mice.

*p < 0.05; **p < 0.01; ***p < 0.005 (unpaired Student's t test). Data are a representative of two (E) independent experiments or are pooled from three (A and B) or two (C and D) independent experiments. The data are shown as mean + SEM (C and D) or SD (A and B). See also Figures S1 and S2.

miR-183C is specific to sensory organs (Xu et al., 2007), and one of its members, miR-182, is essential for proper clonal expansion of activated helper T cells (Stittrich et al., 2010). We decided to focus on miR-183C because its role in Th17 cells is not known.

To confirm the results of RNA sequencing, we investigated the expression of miR-183C in each Th-cell subset. As expected, miR-183, miR-96, and miR-182 were all highly expressed in Th17 cells compared to other Th-cell subsets (Figure 2A). Th17 cells can be induced by two different conditions. One subset is generated by differentiation of naive CD4$^+$ T cells in the presence of TGF-β1 plus IL-6 and is referred to here as of Th17(β). The other subset is generated in the presence of IL-1β, IL-6, and IL-23 and is referred to here as Th17(23). Th17(23) cells are considered "pathogenic" and are thought to enhance the pathogenesis of Th17-related inflammatory diseases (Peters et al., 2011). Of note, there might have been endogenous levels of TGF-β1 in our Th17(23) culture system because we used serum-containing media. We found that Th17(23) cells expressed significantly more miR-183C than did Th17(β) cells (Figure 2B), and this expression was increased between days 2 and 3 (Figure 2C). Th17 cells in the CNS from mice with EAE possess pathogenic function, whereas Th17 cells in the intestine show immunosuppressive properties in vivo (Symons et al., 2012). Compared to intestinal Th17 cells, YFP$^+$ Th17 cells in the CNS from *Il17f*^{Cre}*Rosa*^{yfp} mice that developed EAE disease highly expressed miR-183C (Figure 2D), indicating that miR-183C was expressed in pathogenic Th17 cells in vivo as well. Furthermore, we confirmed that Dicer was also indispensable for the development of Th17(23) cells (Figure S2E). Therefore, these results suggested that miR-183C was involved in the pathogenicity of Th17 cells.

To clarify the mechanism for the induction of miR-183C in Th17(23) cells, we next stimulated naive T cells with each of the cytokines composing the Th17(23) condition for 4 days. We verified that only IL-6 stimulation was able to induce miR-183C, and IL-1β and/or IL-23 enhanced the expression of

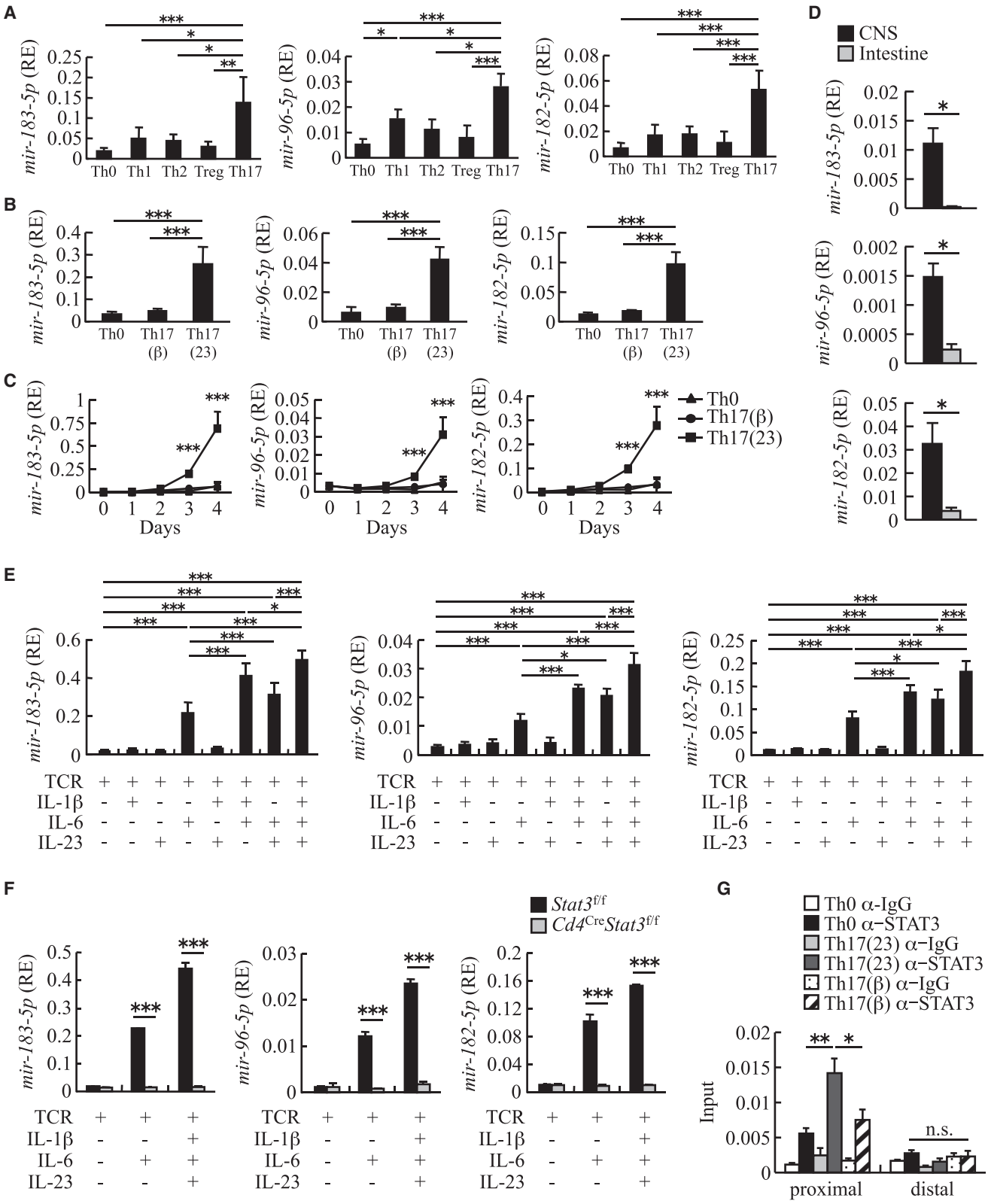

Figure 2. miR-183C Is Highly Expressed in Pathogenic Th17 Cells as a Result of IL-6-STAT3 Signaling

(A–C and E) The expression of miR-183C in primary CD4$^+$ T cells. Naive T cells were cultured under the indicated conditions for 4–6 days.

(D) The expression of miR-183C in Th17 cells from CNS and intestine. Age-matched *Il17f*^{cre}*Rosa*^{yfp} (n = 4) mice were immunized twice with MOG_{35-55}. After EAE disease developed, YFP$^+$CD4$^+$ T cells in the CNS were sorted. The steady-state intestinal Th17 cells were sorted from *Il17f*^{cre}*Rosa*^{yfp} (n = 4) mice.

(legend continued on next page)

miR-183C in the presence of IL-6 (Figure 2E). Furthermore, IL-6 was able to induce the expression of miR-183C even in Th1 and Th2 conditions, implying a crucial role of IL-6 in the induction of miR-183C expression in T cells (Figure S3A). Because IL-6 is present in both Th17(23) and Th17(β) skewing conditions, the suppression of miR-183C in the Th17(β) condition can be explained by the presence of TGF-β in culture (Figure 2B). Indeed, TGF-β treatment to IL-6-stimulated T cells resulted in significant repression of miR-183C induction (Figure S3B). Moreover, the inhibition of miR-183C expression by TGF-β was completely abrogated by the addition of anti-TGF-β antibody or TGF-β receptor kinase inhibitor (Figure S3B). Thus, these results suggest that the TGF-β pathway suppresses miR-183C expression in Th17(β) cells and antagonizes IL-6 effects. Signal transducer and activator of transcription 3 (STAT3) is a major molecule downstream of IL-6, IL-21, and IL-23 signaling (Shuai and Liu, 2003). To address whether STAT3 was involved in the induction of miR-183C, we examined the expression of miR-183C by using *Cd4*^{Cre}*Stat3*$^{f/f}$ mice. As shown in Figure 2F, the expression of miR-183C was completely diminished in *Stat3*-deficient Th17(23) cells. Therefore, we investigated whether STAT3 directly binds to the gene locus of miR-183C. MiR-183, miR-96, and miR-182 are located within 4 kb from each other on mouse chromosome 6qA3. The transcriptional start site (TSS) of miR-183C has already been reported (Chien et al., 2011; Lumayag et al., 2013). We found a STAT3 binding site in the proximal conserved non-coding sequence (CNS) of miR-183C's TSS. Chromatin immunoprecipitation (ChIP) analysis revealed that STAT3 strongly bound to the proximal CNS, but not to the distal CNS, in Th17(23) cells (Figure 2G). Moreover, TGF-β treatment resulted in significant reduction of STAT3 binding to the proximal CNS in Th17(β) cells, indicating a correlation between STAT3 binding and miR-183C expression (Figure 2G). Together, these results demonstrate that IL-6-STAT3 signaling is essential for the induction of miR-183C in Th17 cells.

miR-183C Promotes Pathogenic Function of Th17 Cells

To determine the role of miR-183C in Th17(23) differentiation, we overexpressed each miRNA in primary CD4$^+$ T cells by retroviral transduction. We designed retroviral vectors encoding primary (pri)-miR-183 (miR-183), pri-miR-96 (miR-96), and pri-miR-182 (miR-182), respectively, and confirmed by real-time PCR that these miRNAs were indeed overexpressed in primary CD4$^+$ T cells (Figure S4A). As shown in Figures 3A–3C, overexpression of miR-96 resulted in enhancement of IL-17A, IL-17F, IL-22, and GM-CSF production in the transduced (GFP$^+$) T cells at the protein and mRNA levels, whereas miR-183 and miR-182 only increased a subset of the cytokines we tested. Compared to miR-183 and miR-182 overexpression, miR-96 overexpression significantly increased the pathogenic cytokine production of Th17(23) cells. In contrast, the expression of *Rorc* was not affected by overexpression of these miRNAs (Figure 3C). Previous studies associated IL-23R, TGF-β3, T-bet, and IFN-γ expression with the pathogenicity of Th17 cells (Peters et al., 2011; Lee et al., 2012). Consistently, miR-96 significantly increased the expression of *Il23r*, *Tbx21*, and *Ifng*, but not *Tgfb3*, implying an important role for miR-96 in the pathogenicity of Th17 cells (Figure S4B). These results demonstrate the overlapping function of each member of miR-183C and show that miR-96 is the most potent element to promote pathogenic function of Th17 cells.

Next, we set out to confirm the pathogenicity of miR-183C in Th17(23) cells by adoptively transferring MOG-specific TCR transgenic (2D2) T cells transduced with miR-96 to wild-type recipient male mice, then inducing active EAE via $MOG_{35–55}$ immunization (only half of what is normally used). The transfer of 2D2 Th17(23) cells transduced with miR-96 resulted in more severe EAE (score around 2.0), whereas the transfer of 2D2 Th17(23) cells transduced with control vector led to very mild symptoms (score around 0.5) (Figure 3D). Correlating with disease score, the frequencies of IL-17A$^+$ GM-CSF$^+$ and IL-17A$^-$ GM-CSF$^+$ T cells in the CNS were much higher in the mice that received miR-96-transducing 2D2 Th17(23) cells than in control mice (Figure 3E). Taken together, these findings suggest that miR-183C is an important positive regulator that modulates the pathogenicity of Th17 cells in vitro and in vivo.

Deficiency of miR-183C Reduces Pathogenic Function of Th17 Cells

To investigate the function of miR-183C directly, we next analyzed miR-183C-deficient (*miR-183C*$^{-/-}$) mice (Lumayag et al., 2013). We confirmed the specific deletion of miR-183C in *miR-183C*$^{-/-}$ Th17(23) cells by real-time PCR analysis (Figure S4C). As shown in Figures 4A and 4B, *miR-183C*$^{-/-}$ Th17(23) cells showed significant reduction of IL-17A, IL-17F, IL-22, and GM-CSF at the protein level. Consistent with protein level, real-time RT-PCR revealed that *Il17a*, *Il17f*, *Il22*, and *Csf2* mRNA expression were markedly diminished when there was a lack of miR-183C (Figure 4C). To examine whether miR-96 alone can rescue the differentiation of *miR-183C*$^{-/-}$ Th17(23) cells, we induced overexpression of miR-96 in *miR-183C*$^{-/-}$ Th17(23) cells. miR-96 was not able to rescue Th17(23) cytokine production in *miR-183C*$^{-/-}$ cells (Figure S4D). In contrast, overexpression of miR-183C resulted in the complete recovery of Th17(23) cytokine expression in *miR-183C*$^{-/-}$ cells (Figure S4D). Therefore, these observations suggest that miR-96 function requires other two family members (miR-183 and miR-182) of miR-183C.

To address the CD4$^+$-T-cell-intrinsic function of miR-183C in vivo, we transferred *miR-183C*$^{+/+}$ or *miR-183C*$^{-/-}$ CD4$^+$ T cells into *Rag1*$^{-/-}$ mice and immunized them with $MOG_{35–55}$ peptide. As shown in Figure 4D, the mice receiving *miR-183*$^{-/-}$ CD4$^+$ T cells showed reduced severity of EAE disease (score around 2.0) in comparison to mice receiving *miR-183C*$^{+/+}$ CD4$^+$ T cells (score around 3.5). Correlating with disease score, the frequencies of IL-17A$^+$GM-CSF$^-$, IL-17A$^+$GM-CSF$^+$, and

(F) The expression of miR-183C in primary CD4$^+$ T cells from *Stat3*$^{f/f}$ and *Cd4*^{Cre}*Stat3*$^{f/f}$ mice. Naive T cells were cultured under the indicated conditions for 4 days. (G) A ChIP assay on a *mir-183C* locus (proximal and distal) was performed in Th0, Th17(23), and Th17(β) cells. The results were normalized to the input control. (A–E) miR-183, miR-96, and miR-182 expression were monitored by real-time PCR, and the data were normalized to U6 expression (RE: relative expression). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$ (ordinary one-way ANOVA and Tukey's multiple comparisons test [A–C and E] or unpaired Student's t test [D, F, and G]). Data are pooled from four (A and B) or three (C and E–G) experiments or are representative of two (D) independent experiments. The data are shown as mean + SD (A–G). See also Figures S2 and S3 and Table S1.

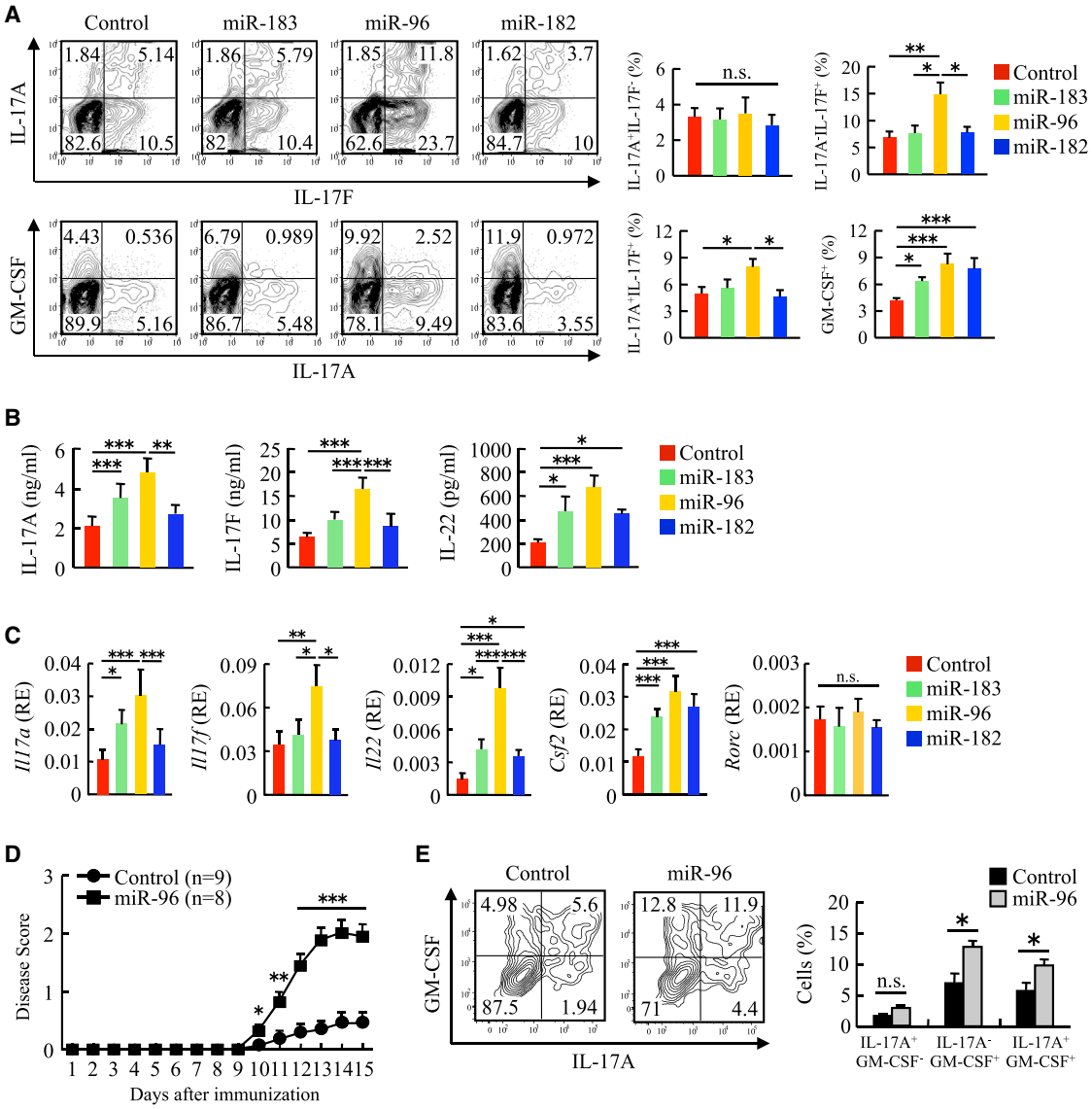

Figure 3. miR-183C Positively Regulates Pathogenic Function of Th17 Cells

(A) Retroviral transduction of IRES-GFP (control), miR-183-IRES-GFP (miR-183), miR-96-IRES-GFP (miR-96), and miR-182-IRES-GFP (miR-182) in Th17(23) cells. The production of IL-17A, IL-17F, and GM-CSF in the transduced (GFP^+) cells was examined by intracellular staining.

(B) The protein levels of IL-17A, IL-17F and IL-22 in Figure 3A.

(C) Relative *Il17a, Il17f, Il22, Csf2*, and *Rorc* mRNA expression in Figure 3A.

(D) Clinical scores of EAE in wild-type recipient male mice given adoptive transfer of 2D2 $CD4^+CD45.1^+$ Th17(23) cells (2×10^6) transduced with control and miR-96 vector.

(E) Left: IL-17A and GM-CSF production in transferred cells ($CD45.1^+$) of CNS from EAE mice. Right: frequency statistics.

*p < 0.05; **p < 0.01; ***p < 0.005 (ordinary one-way ANOVA and Tukey's multiple-comparisons test (A–C) or unpaired Student's t test [D and E]). Data are pooled from six (A and C), four (B), or two (D and E) independent experiments. The data are shown as mean + SEM (A, D, and E) or SD (B and C). See also Figures S4 and S5 and Table S2.

$IL\text{-}17A^-GM\text{-}CSF^+$ T cells in the CNS were much lower in the mice receiving *miR-183*$^{-/-}$ $CD4^+$ T cells than in the mice receiving *miR-183*$^{+/+}$ $CD4^+$ T cells (Figure 4E). Although Dicer1 deficiency affected the absolute number of Th17 cells in the CNS from EAE-induced mice (Figure 1D), miR-183C deficiency had no effect on Th17 cell number (Figures S4E and S4F). Therefore, these results imply that miR-183C promotes pathogenic function of Th17(23) cells independent of cell survival and proliferation. Collectively, these observations confirmed that endogenous miR-183C positively regulates cytokine production of Th17(23) cells and is associated with pathogenesis of autoimmunity.

miR-183C Regulates Expression of Pathogenic Signature Genes in Th17 Cells

A previous study defined the gene signature that distinguishes pathogenic Th17 cells from non-pathogenic Th17 cells (Lee

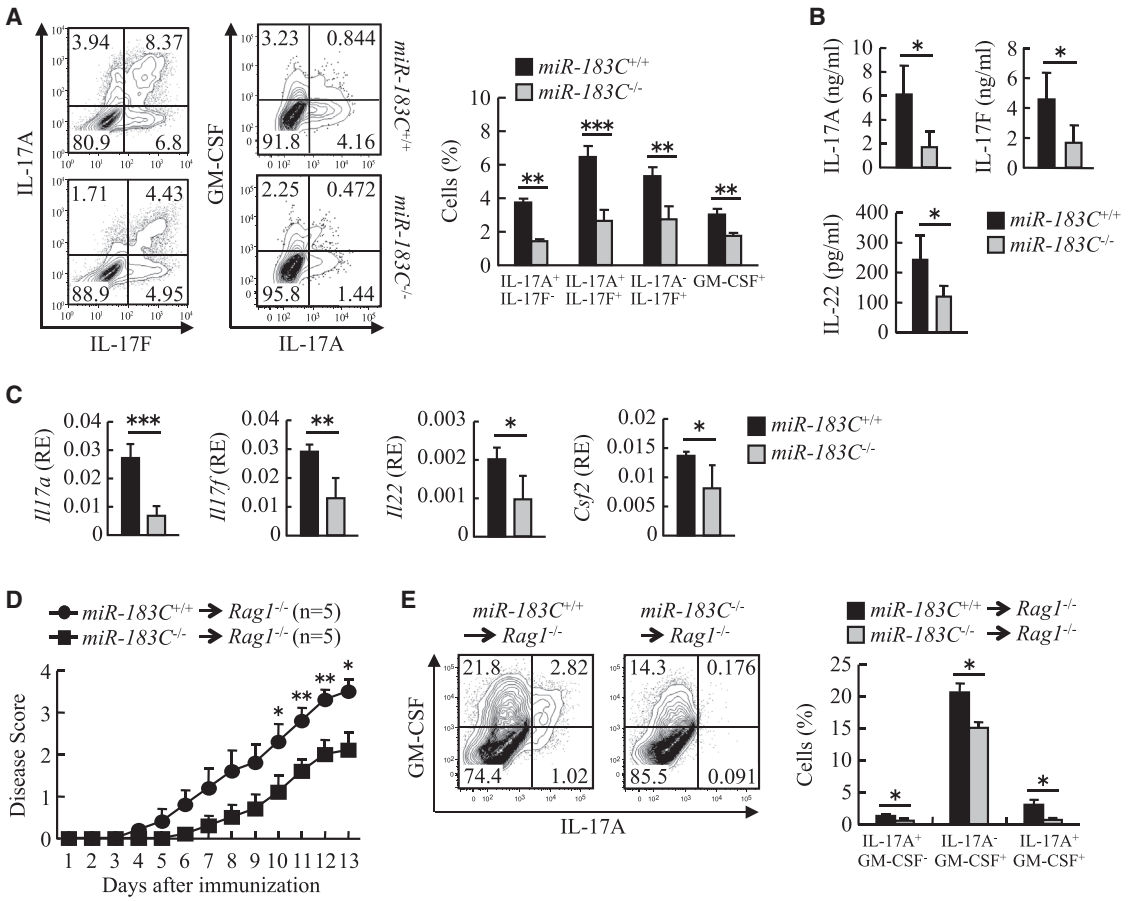

Figure 4. miR183C Deficiency Reduced the Pathogenicity of Th17 Cells

(A) Naive CD4$^+$ T cells from *miR-183C*$^{+/+}$ and *miR-183C*$^{-/-}$ mice were cultured under the Th17(23) condition for 4 days.

(B) The protein levels of IL-17A, IL-17F, and IL-22 in Figure 4A.

(C) Relative *Il17a, Il17f, Il22*, and *Csf2* mRNA expression in Figure 4A.

(D) Clinical scores of EAE in *Rag1*-deficient mice reconstituted with CD4$^+$ T cells derived from *miR-183C*$^{+/+}$ and *miR-183C*$^{-/-}$ mice.

(E) Left: IL-17A and GM-CSF production in transferred cells of CNS from EAE mice. Right: frequency statistics.

*p < 0.05; **p < 0.01; ***p < 0.005. (unpaired Student's t test). Data are a representative of two (D and E) independent experiments or are pooled from four (A–C) independent experiments. The data are shown as mean + SEM (A, D, and E) or SD (B and C). See also Figures S4 and S5 and Table S2.

et al., 2012). To estimate the contribution of miR-183C to the regulation of pathogenic genes in Th17 cells, we performed RNA-sequencing by using control vector-overexpressing Th17(23) cells and comparing them to miR-96-overexpressing Th17(23) cells, and by using *miR-183C*$^{+/+}$ Th17(23) cells and comparing them to *miR-183C*$^{-/-}$ Th17(23) cells. First, we investigated the differentially regulated genes in miR-96-overexpressing and *miR-183C*$^{-/-}$ Th17(23) cells. As shown in Figure S5A and Table S2, we found that 216 genes were positively regulated by miR-183C in Th17(23) cells. They were markedly upregulated in miR-96-overexpressing Th17(23) cells but downregulated when miR-183C was deleted, in comparison to what was observed in wild-type Th17(23) cells. Furthermore, gene-ontology analysis revealed that these 216 genes were mainly associated with "immune system process" (GO: 0002376, p value = 2.8E−23) and "immune response" (GO: 0006955, p value = 2.32E−22), suggesting that miR-183C specifically controls the expression of immune regulatory genes in Th17(23) cells (Figure S5B). We next examined whether miR-183C is involved in the regulation of previously defined 16 pathogenic signature genes (i.e., *Casp1*, *Ccl3*, *Ccl4*, *Ccl5*, *Csf2*, *Cxcl3*, *Gzmb*, *Icos*, *Il22*, *Il3*, *Il7r*, *Lag3*, *Lgals3*, *Lrmp*, *Stat4* and *Tbx21*)(Lee et al., 2012). We found that the expression of these genes was positively correlated with miR-183C expression (Figure S5A). Moreover, the overexpression of miR-96 promoted the expression of some pathogenic signatures (7/16 genes; about 44%), whereas the deletion of miR-183C suppressed the expression of some pathogenic signatures (9/16 genes; about 56%) (Figures S5C and S5D). Taken together, these observations support the idea that miR-183C plays a key role in the immune function of Th17(23) cells and positively regulates a set of pathogenic signature genes in Th17(23) cells. In addition to the defined 16 pathogenic genes, we found that miR-183C also promotes *Il23r*, *Il1r1*, and *Ifng* expression, considered to be other pathogenic signatures (Peters et al., 2011) (Figures S4B and S5A and Table S2), supporting the importance of miR-183C to the pathogenicity of Th17 cells.

Foxo1 Is Directly Regulated by the miR-183C in Pathogenic Th17 Cells

To understand how miR-183C regulates Th17 cell function, we applied the computational prediction programs TargetScan algorithm (Lewis et al., 2003) and miRDB algorithm (Wang, 2008) to identify the potential miR-183C targets. Among the predicted targets, we found that *Foxo1* mRNA had potential target sequences of the miR-183C in the 3′ untranslated region (3′ UTR) and that these target sequences are evolutionarily conserved (Figures S6A and S6B). To confirm *Foxo1* mRNA as a target of miR-183C, we cloned regions of the wild-type or mutated 3′ UTR (60–180) of *Foxo1* mRNA containing the predicted conserved binding sites for miR-183C into an expression vector downstream of a reporter gene encoding red fluorescent protein (RFP) (Figure 5A and Figure S6C). As shown in Figure 5B, we found that miR-183, miR-96, and miR-182 each inhibited the fluorescence intensity of a RFP reporter containing the wild-type Foxo1 3′ UTR but not that of a RFP reporter with a mutated 3′ UTR unable to bind any of them, indicating direct repression of Foxo1 expression by miR-183C. Similar to the effect on Th17(23) differentiation in Figure 3, miR-96 showed the strongest reduction of fluorescence intensity among the three species, although the expression level of miR-96 was lower than that of other miRNAs (Figure 5B and Figure S6D), implying an association between Foxo1 abundance and Th17(23) differentiation. Consistent with the strong induction of miR-183C expression in Th17(23) cells (Figure 2B), we found concurrent suppression of Foxo1 protein expression in these cells (Figure 5C). Conversely, Foxo1 expression in Th17(23) cells was markedly de-repressed by TGF-β addition in a dose-dependent manner, accompanied by repression of miR-183C expression (Figures 5D and 5E). These results demonstrate an inverse correlation between the expression of miR-183C and Foxo1 in Th17 cells and suggest that Foxo1 is a direct target of miR-183C in Th17 cells. To ascertain this, we next manipulated miR-183C expression in Th17(23) cells. Enforced expression of miR-96 in Th17(23) cells suppressed the expression of Foxo1 protein (Figure 5F). Conversely, T cells derived from *miR-183C*$^{-/-}$ mice exhibited enhanced Foxo1 expression in Th17(23) cells (Figure 5G).

Next, we sought to test the functional relevance of Foxo1 suppression by miR-183C in Th17(23) cells. We performed a Foxo1-knock-down experiment in *miR-183C*$^{-/-}$ Th17(23) cells by using a retroviral short hairpin RNA (shRNA) vector for Foxo1 (Harada et al., 2010). The treatment of shRNA vector for Foxo1 rescued Th17(23) cytokine production in *miR-183C*$^{-/-}$ T cells, similar to the extent of *miR-183C*$^{+/+}$ T cells transduced with control vector (Figure 5H). Collectively, our data suggest that Foxo1 is a functional key target of miR-183C in Th17(23) cells.

miR-183C Promotes Pathogenic Function of Th17 Cells in Part via Reduction of Foxo1 Expression

Foxo1 has been shown to play important roles in the differentiation of regulatory T cells as well as follicular helper T cells (Xiao et al., 2014; Ouyang et al., 2010). Furthermore, previous studies reported that Foxo1 negatively regulates IL-17A production from mouse and human memory T cells (Wu et al., 2013; Wan et al., 2011). To determine the role of Foxo1 in Th17 cells, we overexpressed Foxo1 in primary CD4$^+$ T cells under the Th17(23) condition. As shown in Figure 6A, the overexpression of Foxo1 resulted in the repression of IL-17A, IL-17F, and GM-CSF expression in transduced T cells. Consistent with flow-cytometry results, real-time RT-PCR analysis confirmed that enforced expression of Foxo1 significantly reduced the transcription of *Il17a*, *Il17f*, *Il22*, and *Csf2* mRNA (Figure 6B). In contrast, the induction of *Rorc* expression was not affected by overexpression of Foxo1 (Figure 6B). These data suggest that Foxo1 is a negative regulator of the pathogenic function of Th17(23) cells.

Next, we examined the effect of *Foxo1* deletion on Th17(β) differentiation by using a retroviral Cre-mediated gene defect. We confirmed the effective reduction of Foxo1 expression in primary CD4$^+$ T cells from *Foxo1*$^{f/f}$ mice transduced with a retroviral vector expressing Cre (Figure 6D). T cells transduced with Cre exhibited the significantly enhanced IL-17A, IL-17F, IL-22, and GM-CSF expression at the protein and/or mRNA levels (Figures 6C and 6D), suggesting an important role of Foxo1 in the pathogenicity of Th17 cells. Therefore, to investigate this, we performed an EAE experiment with *Il17f*^{Cre} *Foxo1*$^{f/f}$ mice. Consistent with our in vitro results, *Il17f*^{Cre} *Foxo1*$^{f/f}$ mice showed more severe symptoms than *Foxo1*$^{f/f}$ mice (Figure 6E). Correlating with disease score, the frequencies of IL-17A$^+$ GM-CSF$^+$ and IL-17A$^+$ GM-CSF$^-$ T cells in the CNS were much higher in the *Il17f*^{Cre} *Foxo1*$^{f/f}$ mice than in *Foxo1*$^{f/f}$ mice (Figure 6F). Thus, these observations indicate that Foxo1 negatively controls the pathogenicity of Th17 cells.

To analyze the function of the miR-183C-targeted sequence in the 3′ UTR of Foxo1, we constructed a retroviral expression vector encoding Foxo1 together with a wild-type 3′ UTR (Foxo1-WT 3′ UTR) or a mutated 3′ UTR that is unable to bind miR-183C (Foxo1-Mut 3′ UTR) and overexpressed these vectors in Th17(23) cells from *miR-183C*$^{+/+}$ and *miR-183C*$^{-/-}$ mice. Overexpression of Foxo1-WT 3′ UTR failed to suppress the pathogenic cytokine expression in *miR-183C*$^{+/+}$ Th17(23) cells, whereas Foxo1-Mut 3′ UTR significantly inhibited their expression in *miR-183C*$^{+/+}$ Th17(23) cells, similar to what occurred in *miR-183C*$^{-/-}$ Th17(23) cells (Figure 6G), implying that miR-183C-binding-site mutation of Foxo1 is sufficient to abrogate miR-183C function on Th17 cells. In contrast, the reduction of pathogenic cytokines by Foxo1-WT 3′UTR was comparable to that by Foxo1-Mut 3′ UTR in *miR-183C*$^{-/-}$ Th17(23) cells (Figure 6G). Furthermore, we confirmed that the enhanced Foxo1 expression by Foxo1-WT 3′ UTR overexpression was strongly repressed in *miR-183C*$^{+/+}$ Th17(23) cells but not in *miR-183C*$^{-/-}$ Th17(23) cells (Figure 6H). On the other hand, the forced expression of Foxo1-Mut 3′ UTR was successful in increasing of Foxo1 expression in *miR-183C*$^{+/+}$ Th17(23) cells (Figure 6H). Therefore, these data demonstrate that endogenous miR-183C promotes pathogenic cytokine expression of Th17(23) cells through its direct binding to 3′ UTR of Foxo1 and subsequent Foxo1 degradation.

Taken together, these results suggest that the repression of Foxo1 expression by miR-183C is one of the key mechanisms for regulating pathogenic function in Th17(23) cells.

IL-1R1 Regulation by miR-183C Is Involved in the Pathogenic Function of Th17 Cells

IL-1 signaling in T cells is required for GM-CSF production in Th17 cells and for Th17-cell-mediated autoimmunity (Sutton et al., 2006; Lukens et al., 2012). We previously reported that

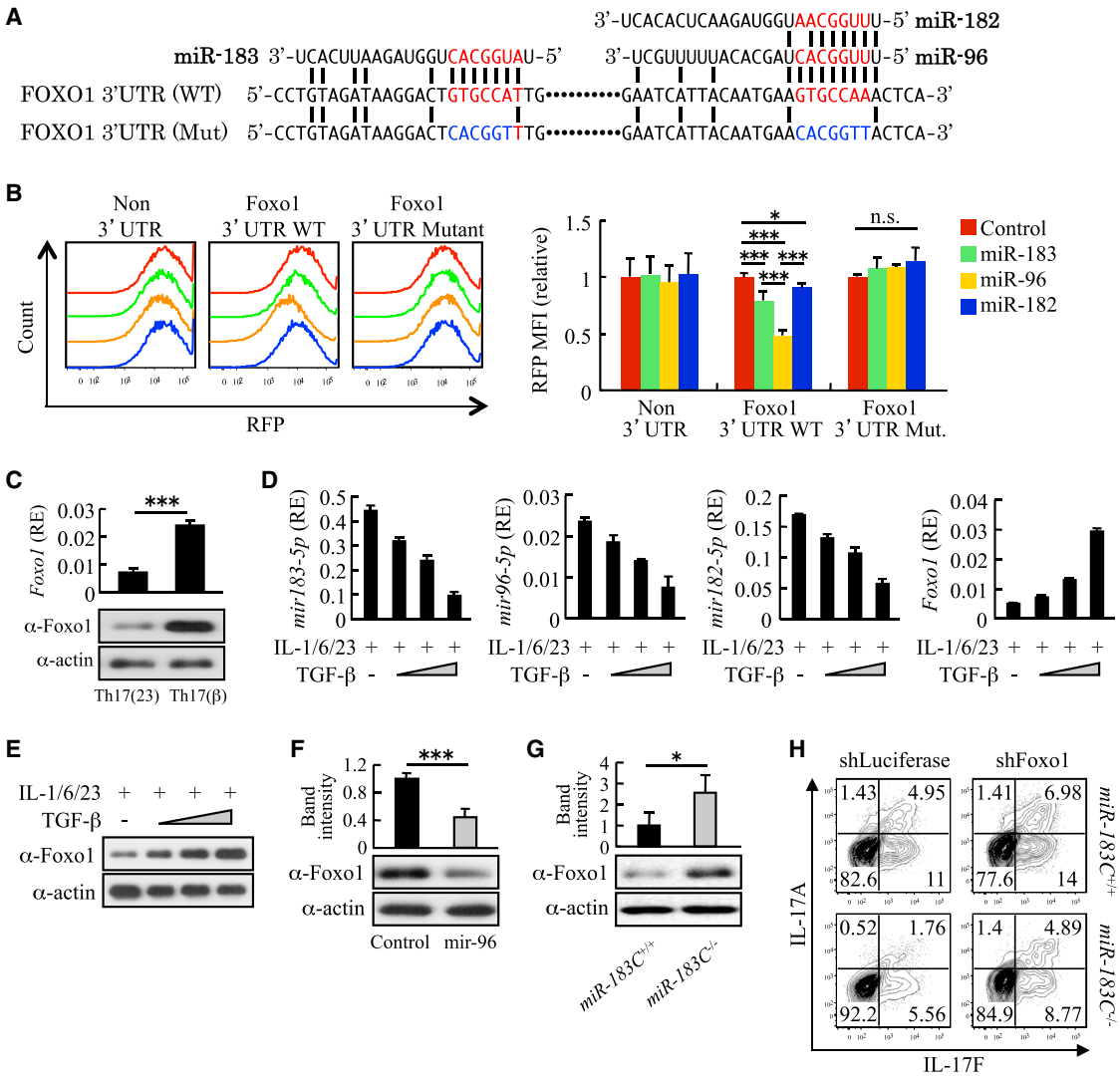

Figure 5. Foxo1 Is Regulated by miR-183C in Pathogenic Th17 Cells

(A) Sequence alignment between miR-183C and its putative binding sites (in red letters) in the FOXO1 3′ UTR. Mutation of the miR-183C target sites (in blue letters) is shown below.

(B) HEK293T cells were transfected with a target reporter containing the FOXO1 3′ UTR with wild-type (WT) or mutated target sites (Mut) (shown in Figure 5A), along with miR-183, miR-96, or miR-182. RFP expression was analyzed by flow cytometry 24 hr after transfection. The bar graph shows the statistics of mean fluorescence intensity (MFI) of RFP.

(C) The expression levels of Foxo1 in Th17(β) and Th17(23) cells by real-time RT-PCR (upper) and immunoblot analysis (lower).

(D) The expression of *miR-183*, *miR-96*, *miR-182*, and *Foxo1* by real-time RT-PCR.

(E) The expression of Foxo1 protein in Figure 5D by immunoblot analysis.

(F) Activated T cells were transduced with control and miR-96 and cultured under the Th17(23) condition for four days. The bar graph (upper) indicates band intensity compared to the control.

(G) The expression of Foxo1 in *miR-183C*$^{+/+}$ and *miR-183C*$^{-/-}$ Th17(23) cells as indicated by immunoblot analysis.

(H) Retroviral transduction of shRNA specific for luciferase (shLuciferase) or Foxo1 (shFoxo1) in *miR-183C*$^{+/+}$ and *miR-183C*$^{-/-}$ Th17(23) cells.

*p < 0.05; ***p < 0.005 (ordinary one-way ANOVA and Tukey's multiple-comparisons test [B] or unpaired Student's t test [C and F–G]). Data are representative of three (E) or two (D and H) independent experiments or are pooled from three experiments (B, C, F, and G). The data are shown as mean + SD (B–D, F, and G). See also Figure S6.

Il1r1 expression was increased by IL-6 stimulation in primary CD4$^+$ T cells and that IL-1 signaling is important in the maintenance as well as the induction of Th17-cell phenotypes (Chung et al., 2009). Moreover, Lee et al. showed by microarray analysis that IL-1R1 is more highly expressed in Th17(23) cells than in Th17(β) cells (Lee et al., 2012). In addition, Ouyang et al. indicated that *Il1r1* expression is upregulated in *Foxo1*$^{-/-}$ *Foxo3*$^{-/-}$ double-knockout Treg cells (Ouyang et al., 2010). To define the molecular mechanism by which Foxo1 suppresses Th17(23) cell function, we thus investigated the role of Foxo1 in IL-1R1 expression. Of note, overexpression of Foxo1 in Th17(23) cells significantly inhibited *Il1r1* expression, whereas the ablation of

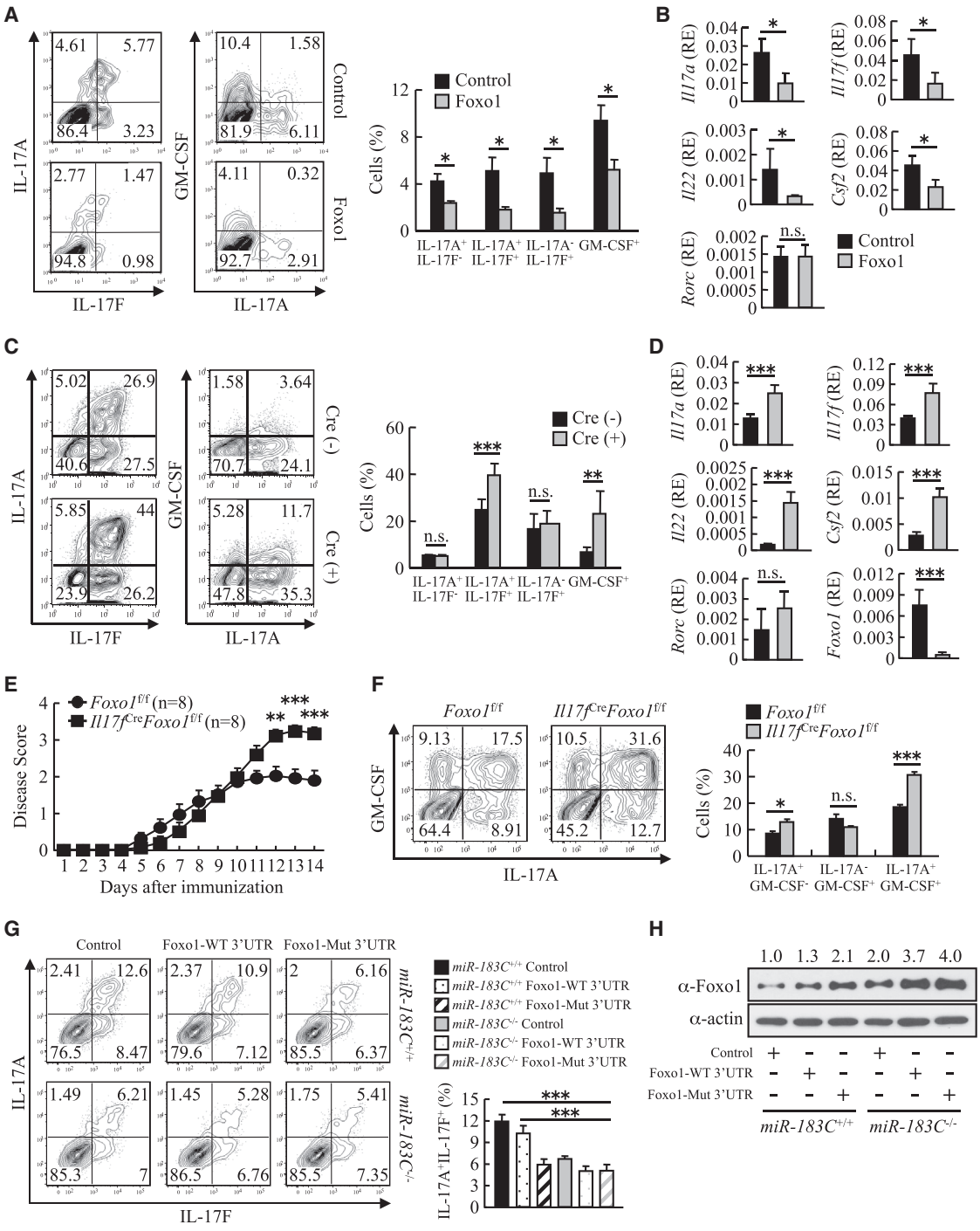

Figure 6. miR-183C Promotes the Pathogenic Function of Th17(23) Cells, in Part via Repression of Foxo1 Expression

(A) Retroviral transduction of IRES-GFP (control) and Foxo1-IRES-GFP (Foxo1) in Th17(23) cells.

(B) Relative *Il17a, Il17f, Il22, Csf2*, and *Rorc* mRNA expression levels in Figure 6A.

(C) Retroviral transduction of IRES-GFP (Cre(−)) and Cre-IRES-GFP (Cre(+)) in *Foxo1*$^{f/f}$ Th17(β) cells.

(D) Relative *Il17a, Il17f, Il22, Csf2, Rorc*, and *Foxo1* mRNA expression levels in Figure 6C.

(E) Clinical scores of EAE in age-matched *Foxo1*$^{f/f}$ (n = 8) and *Il17f*^{cre}*Foxo1*$^{f/f}$ (n = 8) mice.

(F) Left: IL-17A and GM-CSF production in CD4$^+$ cells of CNS from EAE mice. Right: frequency statistics.

(G) Retroviral transduction of IRES-GFP (control), Foxo1-WT 3′ UTR-IRES-GFP (Foxo1-WT 3′ UTR), and Foxo1-Mut 3′UTR-IRES-GFP (Foxo1-Mut 3′ UTR) in Th17(23) cells.

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Foxo1 resulted in a marked enhancement of *Il1r1* expression (Figure 7A). Consistent with mRNA data, we found that *Foxo1*-deleted Th17(β) cells show enhancement of IL-1R1 protein expression as compared to control Th17(β) cells (Figure 7B). We also analyzed IL-1R1-overexpressing Th17(β) cells as a positive control for IL-1R1 staining (Figure 7B). Together, these results indicate that Foxo1 suppresses IL-1R1 expression in Th17 cells.

RORγt is required for the induction of IL-1R1 expression in Th17 cells (Chung et al., 2009). We found three Foxo1 binding regions close to the ROR binding elements on the mouse *Il1r1* locus (Figure 7C). To investigate whether Foxo1 affects RORγt functions, we next examined the recruitment of Foxo1 and RORγt to these three regions of the *Il1r1* locus under both Th17(23) and Th17(β) conditions. ChIP analysis revealed that Foxo1 was effectively recruited to region 1 in Th17(β) cells but not in Th17(23) cells (Figure 7C). In contrast, RORγt failed to bind to region 1 in Th17(β) cells (Figure 7C). These findings led us to the hypothesis that Foxo1 might negatively regulate *Il1r1* transactivation by inhibiting RORγt binding in this region. To confirm this hypothesis, we generated a luciferase reporter containing the promoter and region 1 of *Il1r1* to analyze the transcriptional activity of IL-1R1. As shown in Figure 7D, Foxo1 efficiently inhibited RORγt-induced IL-1R1 reporter activity in HEK293T cells in a dose-dependent manner. On the other hand, Foxo1 did not affect the luciferase activity of a reporter containing only the IL-1R1 promoter region (Figure 7D). These data suggest that Foxo1 inhibits the transcription of IL-1R1 by interfering with RORγt recruitment to the enhancer region. To confirm the interference of RORγt activity by Foxo1 further, we next examined RORγt binding to region 1 in the *miR-183C*$^{-/-}$ cells as well as Foxo1-deleted cells. As shown in Figure 7E, RORγt binding was significantly enriched in region1 in Th17(β) cells as a result of Foxo1 deficiency. Additionally, *miR-183C*$^{-/-}$ Th17(23) cells showed a significant reduction of RORγt binding to region 1, accompanied by a strong increase in Foxo1 recruitment (Figure 7F). The above data together imply that Foxo1 might negatively regulate *Il1r1* expression via interference of RORγt activity in Th17 cells.

As shown in Figures S7A and S7B, IL-1R1 expression was significantly reduced in *miR-183C*$^{-/-}$ Th17(23) cells. In contrast, miR-96-overexpressing Th17(23) cells showed strong enhancement of IL-1R1 protein expression in comparison to control cells (Figure S7C). Moreover, the knock-down of Foxo1 rescued *Il1r1* expression in *miR-183C*$^{-/-}$ Th17(23) cells, similar to the extent of rescue in *miR-183C*$^{+/+}$ Th17(23) cells (Figure S7D). To test whether the increase in *Il1r1* expression by miR-183C occurs through the repression of Foxo1, we overexpressed Foxo1 together with its WT 3′ UTR or a mutant 3′ UTR, which lacked the target sequence for miR-183C, in Th17(23) cells from *miR-183C*$^{+/+}$ and *miR-183C*$^{-/-}$ mice. As a result, overexpression of the Foxo1-WT 3′ UTR failed to suppress the *Il1r1* expression in *miR-183C*$^{+/+}$ Th17(23) cells, although the Foxo1-Mut 3′ UTR significantly inhibited it (Figure S7E). In contract, overexpression of the Foxo1-WT 3′ UTR strongly suppressed *Il1r1* expression in *miR-183C*$^{-/-}$ Th17(23) cells, similar to the extent of suppression by the Foxo1-Mut 3′ UTR (Figure S7E). These results suggest that the repression of Foxo1 by miR-183C is one of the key mechanisms for regulating *Il1r1* expression in Th17(23) cells.

We next assessed whether enforced expression of IL-1R1 could rectify the defective function of *miR-183C*$^{-/-}$ Th(23) cells. We transduced *miR-183C*$^{+/+}$ or *miR-183C*$^{-/-}$ CD4^{+} T cells with a retroviral vector expressing IL-1R1 or empty vector (RVKM). The overexpression of IL-1R1 drove more robust cytokine production in *miR-183C*$^{+/+}$ Th17(23) cells than in control transductants (Figure 7G). Enforced expression of IL-1R1 also substantially enhanced the cytokine expression in *miR-183C*$^{-/-}$ Th17(23) cells, nearly to the extent of expression in *miR-183C*$^{+/+}$ Th17(23) cells transduced with control vector (Figure 7G). Consistent with FACS analysis, real-time RT-PCR analysis revealed that *Il17a*, *Il17f, Il22*, and *Csf2* expression in *miR-183C*$^{-/-}$ Th17(23) cells was restored after overexpression of IL-1R1 (Figure S7F). To confirm the pathogenic function of IL-1R1-overexpressing *miR-183C*$^{-/-}$ Th17(23) cells, we induced EAE disease by adoptively transferring such cells to recipient mice. The mice receiving control vector-overexpressing *miR-183*$^{-/-}$ Th17(23) cells showed significant reduction of EAE severity (score around 0.5) in comparison to the mice receiving control-vector-overexpressing *miR-183C*$^{+/+}$ Th17(23) cells (score around 2.0) (Figure 7H). On the other hand, mice receiving IL-1R1-overexpressing *miR-183C*$^{-/-}$ Th17 cells showed an EAE disease severity (score around 1.5) similar to that of mice receiving control-vector-treated *miR-183C*$^{+/+}$ Th17 cells (Figure 7H). Thus, these results demonstrate that the overexpression of IL-1R1 rectifies the defective function of *miR-183C*$^{-/-}$ Th17(23) cells physiologically. Collectively, these observations indicate that the reduction of IL-1R1 as a result of miR-183C deficiency is partly responsible for the decreased pathogenic cytokine production in Th17(23) cells.

DISCUSSION

Th17 cells are closely associated with autoimmune diseases; functional regulators of Th17 cells are thus of great interest with regard to clinical applications in the treatment of immune diseases (Miossec et al., 2009). Here, we reported that miRNAs were important for the function of Th17 cells in vitro and in vivo and that miR-183C was critical for pathogenic cytokine production in Th17 cells. Mechanistically, the miR-183C targeted transcription factor Foxo1 in Th17 cells. Moreover, Foxo1 directly bound to the *Il1r1* enhancer region and inhibited *Il1r1* transactivation by interfering with RORγt activity.

In this study, we first used *Cd4*^{Cre}*Dicer1*$^{f/f}$ mice to demonstrate that miRNAs play a crucial role in the production of IL-17A in T cells. Dicer1-deficient Th17 cells expressed about half the IL-17F expressed by WT Th17 cells, although IL-17A was

(H) The expression of Foxo1 protein in Figure 6G as indicated by immunoblot analysis. The numbers indicate Foxo1 band intensity compared to that in *miR-183C*$^{+/+}$ cells transduced with control vector.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$ (ordinary one-way ANOVA and Tukey's multiple-comparisons test [G] or unpaired Student's t test [A–F]). Data are representative of two (H) independent experiments or are pooled from five (A and C), four (B and D), three (G), or two (E and F) independent experiments. The data are shown as mean + SEM. (A, C, E, and F) or SD (B, D, and G).

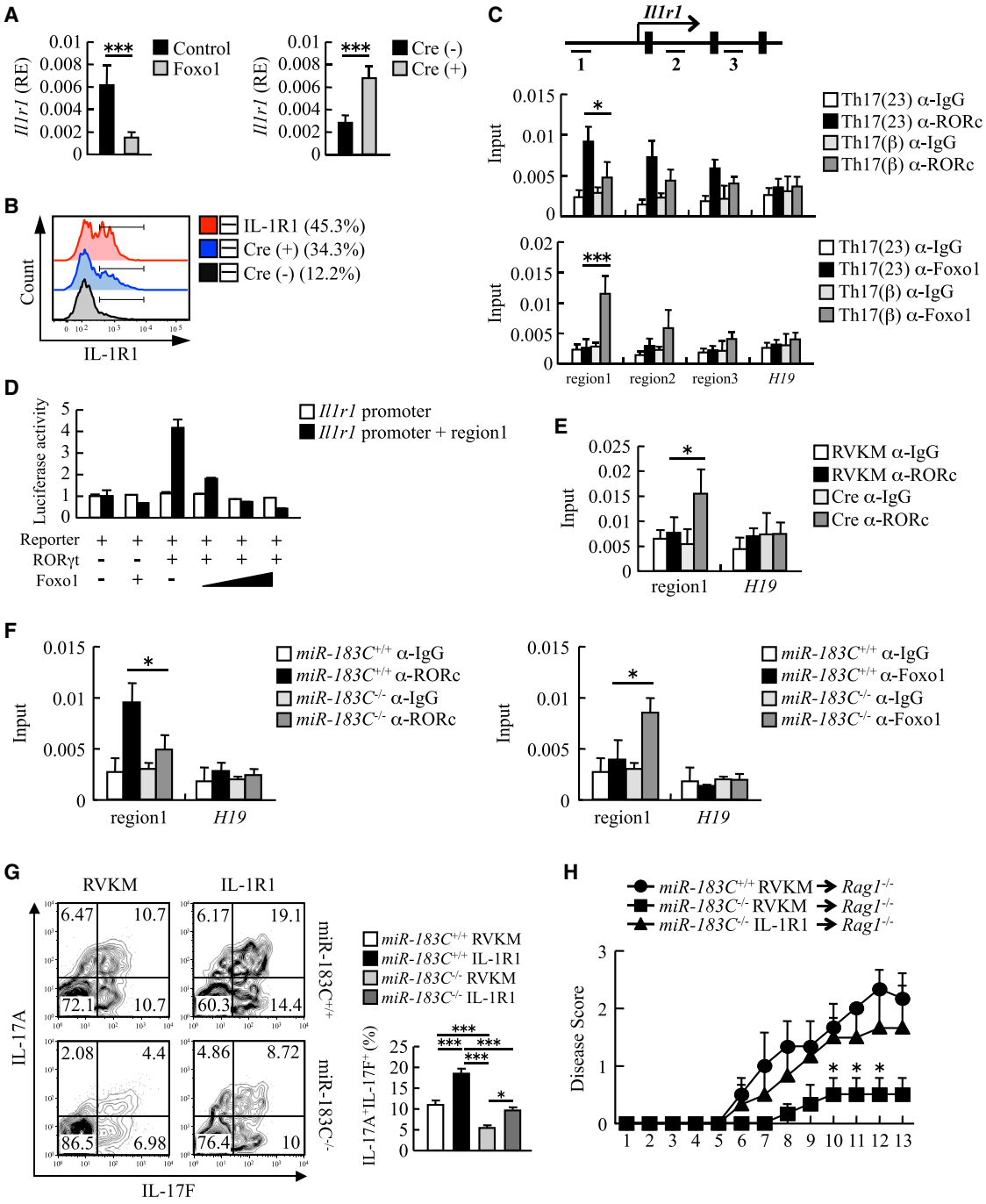

Figure 7. IL-1R1 Promotes the Pathogenic Function of Th17(23) Cells

(A) Retroviral transduction of IRES-GFP (control) and Foxo1-IRES-GFP (Foxo1) (left) or Cre-IRES-GFP (Cre (+)) (right) in Th17(23) (left) or Th17(β) (right) cells. The expression levels were monitored by real-time RT-PCR.

(B) IL-1R1 protein expression in Foxo1-deleted Th17(β) cells as indicated by intracellular staining. The panels were gated on GFP$^+$IL-17A$^+$ cells.

(C) A ChIP assay of RORγt and Foxo1 on the *Il1r1* locus were performed in Th17(β) and Th17(23) cells. Three horizontal bars represent the locations of the RORc and Foxo1-binding sites on the *Il1r1* locus as detected by real-time PCR. Three thick vertical bars represent exons 1, 2, and 3 *Il1r1*.

(D) HEK293T cells were transfected with the indicated plasmids and a luciferase reporter driven by the *Il1r1* promoter or *Il1r1*-promoter-containing region 1.

(E) A ChIP assay of RORγt on region1 of the *Il1r1* locus was performed in Foxo1-deleted Th17(β) cells.

(F) A ChIP assay of RORγt and Foxo1 on region1 of the *Il1r1* locus was performed in *miR-183C*$^{-/-}$ Th17(23) cells.

(G) Retroviral transduction of IRES-GFP (RVKM) and IL1R1-IRES-GFP (IL1R1) in *miR-183C*$^{+/+}$ and *miR-183C*$^{-/-}$ Th(23) cells.

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almost diminished, implying the existence of miRNAs that specifically regulate IL-17A expression in Th17 cells. Similarly, we previously reported that the deletion of the CNS2 region on the *Il17* locus affected IL-17A production more than it affected IL-17F production (Wang et al., 2012). Thus, IL-17A and IL-17F production are differentially regulated in Th17 cells, although the precise mechanism for the regulation of their expression remains unclear.

Here, we found that miR-183C was highly induced in Th17(23) cells via IL-6-STAT3 signaling and STAT3 directly bound to the promoter of the miR-183C. Furthermore, we have shown that TGF-β strongly suppresses STAT3 recruitment and miR-183C expression. The major signaling pathway of TGF-β is the Smad pathway. Similar to what occurs in the JAK-STAT pathway, after activation via TGF-β receptor, the activated Smad transcriptional factors translocate into the nucleus and bind to the target genes to regulate their transcriptional activity (Travis and Sheppard, 2014). Because we found many Smad binding elements (SBEs) around the promoter region of miR-183C (data not shown), it is possible that TGF-β might repress miR-183C expression through the direct binding of Smad to its promoter. Further assessment will be necessary to clarify the precise molecular mechanism underlying miR-183C expression.

In mammals, the Foxo family consists of four members: Foxo1, Foxo3, Foxo4, and Foxo6. Foxo family proteins are thought to function in a redundant manner because they bind to the same consensus sequence. In fact, many reports indicate that Foxo1 and Foxo3 cooperatively enhance Foxp3 expression and induce the differentiation of Treg cells (Harada et al., 2010; Ouyang et al., 2010). Although we identified *Foxo1* mRNA as a functional target of miR-183C in Th17 cells, miRNAs are generally believed to have multiple targets. Furthermore, we found that the reduction of Foxo1 expression partially restored the pathogenic cytokine production in *miR-183C*$^{-/-}$ Th17(23) cells. Thus, miR-183C probably targets other mRNAs in addition to *Foxo1* mRNA. Interestingly, it has been predicted that miR-183C would target all Foxo transcription factors (Eijkelenboom and Burgering, 2013). Collectively, these studies suggest the functional participation of Foxo3 and Foxo4 in the regulation of Th17 phenotypes by miR-183C. Therefore, further assessment with genetically modified mice of other Foxo family proteins will be pursued in future studies.

In the present study, we focused on IL-1R1 as one of the key targets of Foxo1 to explain the regulation of Th17-cell pathogenic function. However, the overexpression of IL-1R1 could not completely rescue Th17(23) cell function in *miR-183C*$^{-/-}$ CD4$^+$ T cells to the levels in IL-1R1-treated *miR-183C*$^{+/+}$ CD4$^+$ T cells, suggesting the existence of other targets for Foxo1 and/or miR-183C. IL-23 and IL-23R are also known as key components of the pathogenic Th17 signature. IL-23R-deficient mice are highly resistant to EAE disease (Awasthi et al., 2009). Furthermore, McGeachy et al. demonstrated that the generation of pathogenic Th17 cells is dependent on IL-23 exposure (McGeachy et al., 2007). Importantly, a recent study suggested that Foxo1 interacts with RORγt and acts as a repressor of IL-23R expression by binding directly to the *Il23r* promoter in Th17 cells (Wu et al., 2013). Therefore, Foxo1 might control the pathogenicity of Th17 cells to act upon the expression of not only IL-1R1 but also IL-23R or others.

In conclusion, we have demonstrated that the repression of Foxo1 via miR-183C is an important mechanism by which Th17 cells acquire pathogenicity. Thus, the miR-183C-Foxo1 axis might be a valuable therapeutic target in the treatment of many autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice

Il17f^{Cre} was generated by insertion of an IRES-Cre-polyA cassette into *IL-17F* exon 2. An Frt-flanked puromycin resistance gene and a diphtheria toxin gene served as positive and negative selection markers, respectively. For creation of chimeras, targeted embryonic stem clones were selected and injected into C57BL/6 blastocysts. High-percentage chimeras were bred with C57BL/6 for germline transmission. *Il17f*$^{Cre+/-}$ mice were obtained after excision of the PuroR cassette via a cross with the FLPeR strain. Heterozygous *Il17f*^{Cre} mice were bred with C57BL/6 at least seven times and used in experiments. The genotyping primers for *Il17f*^{Cre} were as follows: (F) 5′-ACATTGCC CACCACCAGGGCTC-3′, (R1) 5′-CCCATGGGGAACTGGAGCGGTTC-3′, and (R2) 5′-TTCGGCCAGTAACGTTAGG-3′. The primers F and R1 surrounding the insertion breakpoint in exon 2 amplify a 263 bp WT band, whereas the primers F and R2 flanking the FRT site amplify a 378 bp *Il17f*$^{Cre+/-}$ band. *Il17f*^{yfp} mice, *Stat3*$^{f/f}$ mice (on the C57BL/6 background), and *miR-183C*$^{-/-}$ mice (on the mixed background) were previously reported (Yang et al., 2008b; Yang et al., 2007; Lumayag et al., 2013). *Foxo1*$^{f/f}$ mice were kindly provided by R.A. DePinho (MD Anderson Cancer Center, Houston) (Paik et al., 2007). C57BL/6 mice, *Dicer*$^{f/f}$ mice, *Rosa26*^{yfp} mice, and *Rag1*$^{-/-}$ mice (on the C57BL/6 background) were from the Jackson Laboratory. Crossing *Il17f*^{Cre} mice, *Rosa26*^{yfp} mice, and *Dicer*$^{f/f}$ mice generated *Il17f*^{Cre}*Rosa*^{yfp}*Dicer*$^{f/f}$ mice (on the C57BL/6 background). Crossing *Il17f*^{Cre} mice and *Foxo1*$^{f/f}$ mice generated *Il17f*^{Cre}*Foxo1*$^{f/f}$ mice (on the mixed background). *Ifng*^{yfp} mice and *Foxp3*^{gfp} mice were generously provided by R.M. Locksley (University of California, San Francisco) (Stetson et al., 2003) and by A.Y. Rudensky (Memorial Sloan-Kettering Cancer Center, New York), respectively. All genetically modified mice and their littermate controls were used in experiments. All the mice were housed in the SPF animal facility, and the animal experiments were performed when animals were 7–12 weeks of age with the use of protocols approved by the Institutional Animal Care and Use Committee.

Naive T Cell Preparation and Differentiation

CD4$^+$CD25$^-$CD62L^{high}CD44^{low} cells were sorted from spleens and lymph nodes so that Naive T cells could be isolated. Naive T cells were differentiated under several Th cell conditions and were analyzed as described (Yang et al., 2008a). In brief, naive T cells (5 × 10^5 cells/well) were cultured at 37°C (5% CO_2) in complete medium. The cells were stimulated with the plate-bound α-CD3 (1 μg/ml; BioXCell) and the soluble α-CD28 (1 μg/ml; BioXCell). For Th0, the cells were treated with 30 U/mL IL-2, 5 μg/mL α-IFN-γ (XMG1.2; BioXCell), and 5 μg/mL α-IL-4 (11B11; BioXCell). For Th1, the cells were treated with 30 U/mL IL-2, 10 ng/mL IL-12 (Peprotech), and 5 μg/mL α-IL-4. For Th2, the cells were treated with 30 U/mL IL-2, 10 ng/mL IL-4 (Peprotech), and 5 μg/mL α-IFN-γ. For Treg cells, the cells were treated with 30 U/mL IL-2, 1 ng/mL TGF-β1 (Peprotech), 5 μg/mL α-IFN-γ, and 5 μg/mL α-IL-4. For Th17 cells, the cells were treated with 0.5 ng/mL TGF-β1, 10 ng/mL IL-1β

(H) Clinical scores of EAE in *Rag1*-deficient mice reconstituted with CD4$^+$ T cells derived from *miR-183C*$^{+/+}$ and *miR-183C*$^{-/-}$ mice.
*p < 0.05, ***p < 0.005 (ordinary one-way ANOVA and Tukey's multiple-comparisons test [G] or unpaired Student's t test [A and C–F]). Data are representative of two (B and D) independent experiments or are pooled from four (A and C) or three (E–G) independent experiments. The data are shown as mean + SD (A and C–G) or SEM. (H). See also Figure S7.

(Peprotech), 10 ng/mL IL-6 (Peprotech), 10 ng/mL IL-23, 5 μg/mL α-IFN-γ, and 5 μg/mL α-IL-4. For Th17(β) cells, the cells were treated with 0.5 ng/mL TGF-β1, 10 ng/mL IL-6, 5 μg/mL α-IFN-γ, and 5 μg/mL α-IL-4. When indicated, 10 μg/mL α-TGF-β (1D11; BioXCell) or 10 μM TGF-β receptor kinase inhibitor (SB431542; Calbiochem) was added. For Th17(23) cells, the cells were treated with 10 ng/mL IL-1β, 10 ng/mL IL-6, 10 ng/mL IL-23, 5 μg/mL α-IFN-γ, and 5 μg/mL α-IL-4.

ACCESSION NUMBERS

miRNA sequencing and mRNA sequencing data are deposited in GEO under accession numbers GSE67481 and GSE78505, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information include Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.05.015.

AUTHOR CONTRIBUTIONS

C.D., P.Z., and K.I. designed the research and analyzed the data. K.I. performed most of experiments, and B.-S.K., W.J., and W.X. participated in specific experiments. M.S.G. and C.X. performed small-RNA deep-sequencing analysis of Th subsets prepared by K.I., analyzed the data, and identified the miR-183-96-182 cluster as the most interesting one for Th17 cells. A.G.-M. and C.X. imported *miR-183C*$^{-/-}$ mice from S.X., bred them at TSRI, and prepared and shipped samples of lymphoid organs to K.I. for all his experiments involving this mutant mouse strain. S.X. created the *miR-183C*$^{-/-}$ mice and revised the manuscript. H.Y.J. analyzed RNA-sequencing data of Th17 cells generated by K.I. and A.G.-M. Manuscript preparation was by K.I. and C.D.

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REFERENCES

Abbas, A.K., Murphy, K.M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. Nature *383*, 787–793.

Awasthi, A., Riol-Blanco, L., Jäger, A., Korn, T., Pot, C., Galileos, G., Bettelli, E., Kuchroo, V.K., and Oukka, M. (2009). Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. J. Immunol. *182*, 5904–5908.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215–233.

Baumjohann, D., and Ansel, K.M. (2013). MicroRNA-mediated regulation of T helper cell differentiation and plasticity. Nat. Rev. Immunol. *13*, 666–678.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857–868.

Chien, C.H., Sun, Y.M., Chang, W.C., Chiang-Hsieh, P.Y., Lee, T.Y., Tsai, W.C., Horng, J.T., Tsou, A.P., and Huang, H.D. (2011). Identifying transcriptional start sites of human microRNAs based on high-throughput sequencing data. Nucleic Acids Res. *39*, 9345–9356.

Chung, Y., Chang, S.H., Martinez, G.J., Yang, X.O., Nurieva, R., Kang, H.S., Ma, L., Watowich, S.S., Jetten, A.M., Tian, Q., and Dong, C. (2009). Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity *30*, 576–587.

Cobb, B.S., Hertweck, A., Smith, J., O'Connor, E., Graf, D., Cook, T., Smale, S.T., Sakaguchi, S., Livesey, F.J., Fisher, A.G., and Merkenschlager, M. (2006). A role for Dicer in immune regulation. J. Exp. Med. *203*, 2519–2527.

Codarri, L., Gyülvészi, G., Tosevski, V., Hesske, L., Fontana, A., Magnenat, L., Suter, T., and Becher, B. (2011). RORγt drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. Nat. Immunol. *12*, 560–567.

Du, C., Liu, C., Kang, J., Zhao, G., Ye, Z., Huang, S., Li, Z., Wu, Z., and Pei, G. (2009). MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. Nat. Immunol. *10*, 1252–1259.

Eijkelenboom, A., and Burgering, B.M. (2013). FOXOs: Signalling integrators for homeostasis maintenance. Nat. Rev. Mol. Cell Biol. *14*, 83–97.

El-Behi, M., Ciric, B., Dai, H., Yan, Y., Cullimore, M., Safavi, F., Zhang, G.X., Dittel, B.N., and Rostami, A. (2011). The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. Nat. Immunol. *12*, 568–575.

Harada, Y., Harada, Y., Elly, C., Ying, G., Paik, J.H., DePinho, R.A., and Liu, Y.C. (2010). Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells. J. Exp. Med. *207*, 1381–1391.

Hedrick, S.M., Hess Michelini, R., Doedens, A.L., Goldrath, A.W., and Stone, E.L. (2012). FOXO transcription factors throughout T cell biology. Nat. Rev. Immunol. *12*, 649–661.

Hirota, K., Duarte, J.H., Veldhoen, M., Hornsby, E., Li, Y., Cua, D.J., Ahlfors, H., Wilhelm, C., Tolaini, M., Menzel, U., et al. (2011). Fate mapping of IL-17-producing T cells in inflammatory responses. Nat. Immunol. *12*, 255–263.

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121–1133.

Korn, T., Bettelli, E., Oukka, M., and Kuchroo, V.K. (2009). IL-17 and Th17 Cells. Annu. Rev. Immunol. *27*, 485–517.

Kurebayashi, Y., Nagai, S., Ikejiri, A., and Koyasu, S. (2013). Recent advances in understanding the molecular mechanisms of the development and function of Th17 cells. Genes Cells *18*, 247–265.

Lee, Y., Awasthi, A., Yosef, N., Quintana, F.J., Xiao, S., Peters, A., Wu, C., Kleinewietfeld, M., Kunder, S., Hafler, D.A., et al. (2012). Induction and molecular signature of pathogenic TH17 cells. Nat. Immunol. *13*, 991–999.

Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W., Bartel, D.P., and Burge, C.B. (2003). Prediction of mammalian microRNA targets. Cell *115*, 787–798.

Lukens, J.R., Barr, M.J., Chaplin, D.D., Chi, H., and Kanneganti, T.D. (2012). Inflammasome-derived IL-1β regulates the production of GM-CSF by CD4(+) T cells and γδ T cells. J. Immunol. *188*, 3107–3115.

Lumayag, S., Haldin, C.E., Corbett, N.J., Wahlin, K.J., Cowan, C., Turturro, S., Larsen, P.E., Kovacs, B., Witmer, P.D., Valle, D., et al. (2013). Inactivation of the microRNA-183/96/182 cluster results in syndromic retinal degeneration. Proc. Natl. Acad. Sci. USA *110*, E507–E516.

McGeachy, M.J., Bak-Jensen, K.S., Chen, Y., Tato, C.M., Blumenschein, W., McClanahan, T., and Cua, D.J. (2007). TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. Nat. Immunol. *8*, 1390–1397.

McGeachy, M.J., Chen, Y., Tato, C.M., Laurence, A., Joyce-Shaikh, B., Blumenschein, W.M., McClanahan, T.K., O'Shea, J.J., and Cua, D.J. (2009). The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. Nat. Immunol. *10*, 314–324.

Miossec, P., Korn, T., and Kuchroo, V.K. (2009). Interleukin-17 and type 17 helper T cells. N. Engl. J. Med. *361*, 888–898.

Nurieva, R., Yang, X.O., Martinez, G., Zhang, Y., Panopoulos, A.D., Ma, L., Schluns, K., Tian, Q., Watowich, S.S., Jetten, A.M., and Dong, C. (2007). Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature *448*, 480–483.

O'Connell, R.M., Rao, D.S., Chaudhuri, A.A., and Baltimore, D. (2010). Physiological and pathological roles for microRNAs in the immune system. Nat. Rev. Immunol. *10*, 111–122.

Ouyang, W., Beckett, O., Ma, Q., Paik, J.H., DePinho, R.A., and Li, M.O. (2010). Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. Nat. Immunol. *11*, 618–627.

Paik, J.H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., et al. (2007). FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell *128*, 309–323.

Peters, A., Lee, Y., and Kuchroo, V.K. (2011). The many faces of Th17 cells. Curr. Opin. Immunol. *23*, 702–706.

Shuai, K., and Liu, B. (2003). Regulation of JAK-STAT signalling in the immune system. Nat. Rev. Immunol. *3*, 900–911.

Stetson, D.B., Mohrs, M., Reinhardt, R.L., Baron, J.L., Wang, Z.E., Gapin, L., Kronenberg, M., and Locksley, R.M. (2003). Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. J. Exp. Med. *198*, 1069–1076.

Stittrich, A.B., Haftmann, C., Sgouroudis, E., Kühl, A.A., Hegazy, A.N., Panse, I., Riedel, R., Flossdorf, M., Dong, J., Fuhrmann, F., et al. (2010). The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. Nat. Immunol. *11*, 1057–1062.

Sutton, C., Brereton, C., Keogh, B., Mills, K.H., and Lavelle, E.C. (2006). A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J. Exp. Med. *203*, 1685–1691.

Symons, A., Budelsky, A.L., and Towne, J.E. (2012). Are Th17 cells in the gut pathogenic or protective? Mucosal Immunol. *5*, 4–6.

Travis, M.A., and Sheppard, D. (2014). TGF-β activation and function in immunity. Annu. Rev. Immunol. *32*, 51–82.

Wan, Q., Kozhaya, L., ElHed, A., Ramesh, R., Carlson, T.J., Djuretic, I.M., Sundrud, M.S., and Unutmaz, D. (2011). Cytokine signals through PI-3 kinase pathway modulate Th17 cytokine production by $CCR6^{+}$ human memory T cells. J. Exp. Med. *208*, 1875–1887.

Wang, X. (2008). miRDB: A microRNA target prediction and functional annotation database with a wiki interface. RNA *14*, 1012–1017.

Wang, X., Zhang, Y., Yang, X.O., Nurieva, R.I., Chang, S.H., Ojeda, S.S., Kang, H.S., Schluns, K.S., Gui, J., Jetten, A.M., and Dong, C. (2012). Transcription of Il17 and Il17f is controlled by conserved noncoding sequence 2. Immunity *36*, 23–31.

Wu, C., Yosef, N., Thalhamer, T., Zhu, C., Xiao, S., Kishi, Y., Regev, A., and Kuchroo, V.K. (2013). Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. Nature *496*, 513–517.

Xiao, N., Eto, D., Elly, C., Peng, G., Crotty, S., and Liu, Y.C. (2014). The E3 ubiquitin ligase Itch is required for the differentiation of follicular helper T cells. Nat. Immunol. *15*, 657–666.

Xu, S., Witmer, P.D., Lumayag, S., Kovacs, B., and Valle, D. (2007). MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. J. Biol. Chem. *282*, 25053–25066.

Yang, X.O., Panopoulos, A.D., Nurieva, R., Chang, S.H., Wang, D., Watowich, S.S., and Dong, C. (2007). STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J. Biol. Chem. *282*, 9358–9363.

Yang, X.O., Pappu, B.P., Nurieva, R., Akimzhanov, A., Kang, H.S., Chung, Y., Ma, L., Shah, B., Panopoulos, A.D., Schluns, K.S., et al. (2008a). T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity *28*, 29–39.

Yang, X.O., Chang, S.H., Park, H., Nurieva, R., Shah, B., Acero, L., Wang, Y.H., Schluns, K.S., Broaddus, R.R., Zhu, Z., and Dong, C. (2008b). Regulation of inflammatory responses by IL-17F. J. Exp. Med. *205*, 1063–1075.