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Full-length Article

MicroRNA-181c promotes Th17 cell differentiation and mediates experimental autoimmune encephalomyelitis

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ABSTRACT

Among T helper (Th) cell subsets differentiated from naive CD4$^+$ T cells, IL-17-producing Th17 cells are closely associated with the pathogenesis of autoimmune diseases, including multiple sclerosis (MS) and the MS animal model, experimental autoimmune encephalomyelitis (EAE). The modulation of Th17 differentiation offers a potential avenue for treatment. Although a series of microRNAs (miRNAs) that modulate autoimmune disease development have been reported, further studies on miRNA roles in Th17 differentiation and MS pathogenesis are still warranted. Here, we demonstrated that mice with miR-181c knockdown presented with delayed EAE and slowed disease progression, along with a decreased Th17 cell population. We also found that miR-181c was a Th17 cell-associated miRNA and that Smad7, a negative regulator of TGF-β signaling, was a potential target of miR-181c. miR-181c knockdown rendered T cells less sensitive to TGF-β-induced Smad2/3, enhancing the expression of IL-2 which has been reported to inhibit Th17 cell differentiation. Moreover, through the analysis of published miRNA expression profiles from the Gene Expression Omnibus database, increased miR-181c levels were found in peripheral blood from MS patients. Our results identified a novel miRNA that promotes Th17 cell differentiation and autoimmunity, thus miR-181c may serve as a potential treatment target in patients with MS.

1. Introduction

MicroRNAs (miRNAs) are single-stranded non-coding RNAs that are approximately 21 nucleotides in length and that regulate gene expression in a posttranscriptional manner. The miRNA-induced silencing complex (miRISC) leads to translational repression, mRNA degradation and destabilization by binding to complementary sequences on target mRNAs. It has been revealed that miRNA expression can control dynamic aspects of autoimmunity that are important for maintaining homeostasis (Baumjohann and Ansel, 2013).

Multiple sclerosis (MS) is a typical autoimmune neurodegenerative disease in which proinflammatory cells invade the CNS, resulting in multifocal demyelination. Millions of people worldwide are affected by paralytic symptoms caused by MS (Nylander and Hafler, 2012). Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS characterized by demyelination, impaired nerve conduction, and paralysis (Wekerle, 2008). Although the exact pathogenesis of MS remains unidentified, it is generally believed that autoimmunity-related CD4$^+$ T cells within the central nervous system (CNS) contribute to the most important components. T helper (Th) cell subsets are differentiated from naive CD4$^+$ T cells and IL-17-producing Th17 cells are closely associated with the development of autoimmunity (Goverman, 2009).

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Transforming growth factor-β (TGF-β) signaling activation has been shown to play an important role in Th17 cell differentiation and is transmitted via Smad-dependent or non-Smad pathways (Mangan et al., 2006; Moustakas and Heldin, 2005). Alterations in TGF-β or TGF-β receptor modulate Th17-mediated autoimmunity. It has been reported that mice deficient in TGF-β receptor II exhibit a defect in Th17 generation, conferring resistance to EAE (Veldhoen et al., 2006). Moreover, enhanced TGF-β signaling is activated by the transcription factor IRF8 and triggers increased Th17 cell differentiation in EAE mice (Yoshida et al., 2014). In the Smad-dependent pathway, TGF-β-induced of Smad2/3 phosphorylation can be inhibited by Smad7 (Akhurst and Hata, 2012) and the TGF-β signaling can inhibit Il-2 expression which serves as negative regulator of Th17 differentiation (Cejas et al., 2010).

It has been revealed that a series of miRNAs play important roles in T cell responses and MS. Du et al. first linked miR-326 to Th17 differentiation and MS pathogenesis (Du et al., 2009). Guerau-de-Arellano et al. reported that miRNA dysregulation in MS inhibited Th2 cell development and favored proinflammatory Th1 responses (Guerau-de-Arellano et al., 2011). Moreover, subsequent studies stated that miR-27b, miR-103a, miR-128, and miR-628-3p inhibit inducible regulatory T cell development, and miR-21 promotes Th17 differentiation (Murugaiyan et al., 2015; Severin et al., 2016). The miR-181 family is another regulator of the Th cell subset population and consists of four unique mature miRNAs (miR-181a, miR-181b, miR-181c and miR-181d) encoded in three miRNA clusters (*miR-181a/b-1*, *miR-181a/b-2* and *miR-181c/d*) from independent paralog precursor transcripts on separate chromosomes(Baumjohann and Ansel, 2013; Zietara et al., 2013). The miR-181 family has been widely studied in several fields such as cancer (Ji et al., 2014; Parikh et al., 2014), myocardial infarction (Li et al., 2009) and ischemic stroke (Ma et al., 2016). Notably, members in the miR-181 family, especially those encoded in separate clusters, function distinctly within the same disease. For instance, miR-181a and miR-181b negatively regulate tumor suppressor genes and promote gastric cancer (Zhang et al., 2012; Zhou et al., 2016), while miR-181c reportedly is a tumor suppressor in gastric cancer (Yang et al., 2016). In MS/EAE, miR-181a and miR-181b inhibited Th1 generation, while miR-181a-1/b-1-knockout mice exhibited a delay in EAE induction due to defective miR-181a-mediated migration (Ghorbani et al., 2017; Schaffert et al., 2015). It has been reported that miR-181c serves as a negative regulator of *in vitro* T cell activation and proliferation (Xue et al., 2011); however, whether and how miR-181c modulates Th cell differentiation and influences EAE progression remains unknown.

In this study, we showed that miR-181c is overexpressed in Th17 cells specifically and that miR-181c-knockdown mice with EAE presented with defective Th17 differentiation and relief of disease progression. miR-181c downregulated Smad7 expression and thereby rendered T cells more sensitive to TGF-β-induced Smad2/3, limiting the inhibitory effects of autocrine IL-2 and thus contributing to Th17 cell differentiation and EAE induction. Moreover, through the analysis of published miRNA expression profiles in the Gene Expression Omnibus database, increased miR-181c levels were found in the peripheral blood of MS patients. Thus, our results identified a novel miRNA promoting Th17 cell differentiation and autoimmunity, suggesting that miR-181c may serve as a valuable target for clinical applications in patients with MS.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice (wild-type, WT) were purchased from the Academy of Military Medical Science (Beijing, China). The animals were housed and fed in a specific pathogen-free animal facility at the Experimental Animal Center of Tianjin Medical University (Tianjin, China). Experiments were performed in accordance with the guidelines for animal care and were approved by the Animal Ethics Committee of Tianjin Medical University (Tianjin, China).

2.2. Active EAE induction, treatment and evaluation

For EAE induction, C57BL/6 mice (aged 6–8 weeks) were immunized (s.c.) with 150 mg of myelin oligodendrocyte glycoprotein (MOG residues 35–55). The peptide sequence was Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys, and the purity was >95% (CL. Bio-Scientific Co., Ltd., Xi'an, China). Immunization was performed by mixing the MOG_{35-55} peptide with complete Freund's adjuvant (Difco, Detroit, MI) containing 5 mg/ml heat-killed H37Ra, a Mycobacterium tuberculosis strain (Difco, Detroit, MI). Pertussis toxin (200 ng) (List Biological Laboratories, Campbell, CA) in PBS and 50 mM NaCl were administered i.p. on the day of immunization and again 48 h later. After induction, neurological deficits were assessed with clinical scores according to the clinical syndromes in mice, which were assessed using the following standard scoring system: 0, no obvious changes in motor functions; 1.0, limp tail; 2.0, limp tail and wobbly gait; 3.0, bilateral hind limb paralysis; 4.0, complete hind limb and partial fore limb paralysis; and 5.0, death. The stages of disease were defined as follows: disease onset, around day 14 after immunization; peak, around day 20 after immunization; rest (remitting), around day 28 after immunization.

2.3. Lentiviral constructs and infection

For RNA interference, we used a validated shRNA, which was cloned into the shRNA expression plasmid miRZip™ (System Biosciences). The shRNA sequences for mmu-mir-181c-5p were as follows: forward: 5′-GATCCAAGTATCAACCTGTCGGTGAGTCTT CCTGTCAGAA CTCACCGACAGGTTGAATGTTTTTTTAAGCTTGAAGA CG-3′; and reverse: 5′-AATTCGT CTTCAAGCTTAAAAAAACATT CAACCTGTCGGTGAGTTCTGACAGGAAGACTCACCGACAGGTTGATAC TTG-3′. shRNA was transfected into HEK293T cells with the packaging vectors pSpax (Addgene plasmid 12260) and pMD2 (Addgene plasmid 12259) using PEI (Polyscience). After 48 and 72 h, the culture medium was collected and centrifuged at 2000×*g* for 5 min to remove the cell debris via sedimentation, and the supernatant was filtered through a membrane. The samples were subsequently placed in 40-ml ultracentrifugation tubes, 1/4 volume PEG 8000 was added to the supernatant, and media were incubated overnight at 4 °C. The next day, the samples were centrifuged at 4000 rpm/min for 30 min at 4 °C and resuspended for virus precipitation with ice-cold sterile PBS to collect LV-shRNA-181c-GFP-Puro virus. The negative control viruses LV-shRNA-control-GFP-Puro viruses were obtained similarly. Mice were infected with freshly purified virus (titer $\geq 1 \times 10^8$ IU/ml) by tail vein injection. For 7- to 8-week-old mice, 200 μl of concentrated recombinant lentivirus LV-shRNA-control or LV-shRNA-181c (containing 8 μg/ml polybrene) was administered through intravenous injection 7 days before immunization.

2.4. Histology

Spinal cords from mice transcardially perfused with 4% paraformaldehyde were dissected and post-fixed overnight. Following standard protocols, paraffin-embedded 6-μm spinal cord sections were stained with hematoxylin-eosin (H&E) for routine histological analysis of inflammatory infiltration and with Luxol fast blue (Alfa Aesar, Ward Hill, USA) to evaluate demyelination.

Inflammatory infiltration and demyelination were quantified using ImageJ software.

2.5. Preparation of CNS cells

Animals were perfused with cold PBS. The brains and spinal cords were dissected and incubated in 2.5 mg/ml collagenase D for 30 min at 37 °C. Single-cell suspensions were prepared by passing them through a 70-μm strainer. The cells were washed in RPMI 1640 medium, and mononuclear cells were isolated using a discontinuous Percoll gradient (Pharmacia). Cells were washed twice and stimulated with PMA plus ionomycin for 4 h for intracellular cytokine analysis by flow cytometry.

2.6. Flow cytometry

The cells isolated from mice were cultured in complete RPMI 1640 medium (containing 100 mM sodium pyruvate, 200 mM L-glutamine, 1 mg/ml penicillin/streptomycin, and 10% fetal bovine serum) and were restimulated with cell stimulation cocktail (plus protein transport inhibitors) (eBioscience, CA, USA) for 5 h before being collected and washed with PBS. Anti-CD4, anti-CD25, anti-IL-17, anti-IFN-γ, and anti-FOXP3 antibodies conjugated to fluorochromes were stained according to the instructions. Nonspecific staining was monitored with isotype antibody controls. (All antibodies were purchased from BioLegend). Data were acquired on a FACSCalibur instrument (BD Biosciences) and analyzed with FlowJo software (Tree star, Ashland, OR). The criteria for establishing phenotype of T cell subsets as follows was described in previous study (Bettelli et al., 2006): Th1 cells, $CD4^+IFN\gamma^+$; Th17 cells, $CD4^+IL17^+$; Treg cells, $CD4^+CD25^+Foxp3^+$.

2.7. 3′ UTR luciferase assays

The 3′ UTR of WT or mutant *smad7* was amplified by PCR and cloned into a p-mirGLO Dual-luciferase miRNA Target Expression Vector (Promega). The miR-181c precursor expression vector and pmirGLO dual-luciferase 3′ UTR vector were cotransfected into 293T cells using PEI transfection reagents. Cells were harvested and lysed at 48 h posttransfection. The interaction between miR-181c and the target 3′ UTR was measured by a dual-luciferase assay system (Promega).

2.8. Small interfering RNAs and transfection

For gene silencing, small interfering RNA (siRNA) duplexes targeting the mouse gene were synthesized by Integrated DNA Technologies. The siRNA duplexes for mouse miR-181c-5p and an siRNA control, as well as miR-181c-5p mimics and respective controls were purchased from RiboBio (Guangzhou, China). The cells were treated with siRNAs (final concentration, 25 nM) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and were harvested 24 h after siRNA treatment.

2.9. Th cell differentiation

Naive $CD4^+CD62L^+$ helper T cells were isolated from the spleens of female C57BL/6 mice by magnetic separation using microbeads (Miltenyi Biotec, Auburn, CA), followed by activation with plate-bound anti-CD3 (10 μg/ml; BD Pharmingen) and anti-CD28 (2 μg/ml; BD Pharmingen). For *in vitro* Th17 cell differentiation, the isolated cells were treated with siRNA or controls, and stimulated in the presence of TGF-β (10 ng/ml; R&D), IL-1β (10 ng/ml; R&D), IL-23 (10 ng/ml; R&D), IL-6 (50 ng/ml; R&D), anti-IFN-γ (10 μg/ml; BD Pharmingen), anti-IL-4 (10 μg/ml; R&D) and anti-IL-2 (1 μg/ml; R&D) antibodies. For Th1 polarization, the isolated cells were stimulated with IL-12 (20 ng/ml; R&D) and anti-IL-4 (20 μg/ml) antibodies. For Th2 polarization, the isolated cells were stimulated with IL-4 (20 ng/ml; R&D) and anti-IFN-γ (20 μg/ml) antibodies. To induce Treg differentiation, isolated cells were stimulated with TGF-β (5 ng/ml) and IL-2 (2.5 ng/ml; R&D). After 24 h of culture, transcription factor expression was analyzed by RT-PCR. Four days after activation, cells were restimulated with Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience, CA, USA) for 5 h for intracellular cytokine analysis by flow cytometry. The mRNA levels of specific transcription factors or cytokines validating the characterization of differentiated cells are shown in Supplementary Fig. 4A–D.

2.10. Quantitative real-time PCR

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) in accordance with the manufacturer's instructions. After RNA purification, the samples were treated with DNase to remove the contaminating genomic DNA. Reverse transcription was performed using random hexamers and M-MLV reverse transcriptase (Promega, Madison, USA). All other reverse transcription reagents were supplied by Takara (Takara, Japan). Gene-specific primers were synthesized at Genewiz (Suzhou, China). For relative quantitative real-time PCR, SYBR Green master mix (Takara, Japan) was used in accordance with the manufacturer's instructions. The reactions were performed in triplicate on an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, California, USA), and the generated products were analyzed using ABI 7500 software (Version 2.0.5). The primers used are listed in the Supplementary Material.

2.11. Western blot analysis

Mouse naive $CD4^+$ T cells were stimulated for 24 h with plate-bound anti-CD3 and anti-CD28. After a 16-hour resting period, the cells were stimulated with TGF-β (2 ng/ml) for specific time points (Murugaiyan et al., 2015). The cells were lysed with RIPA buffer containing 1 mM PMSF and 1% phosphatase inhibitor cocktail. Equal amounts of protein (20 μg) were separated on SDS-PAGE. Proteins were transferred onto PVDF membranes for western blot analysis using mouse anti-Gapdh, rabbit anti-Smad7 (Proteintech), p-Smad2, p-Smad2, Smad-2/3, p-p38, p-JNK, p-ERK, p38, JNK, and ERK antibodies (Cell Signaling Technology). Chemiluminescent HRP substrate (Millipore, MA, USA) was used to detect the antibody-antigen complexes.

2.12. Statistic

Statistical analysis was performed using unpaired two-tailed Student's *t*-tests for comparisons of two groups. Two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was applied for multiple comparisons. For EAE scoring, groups were compared using the Mann-Whitney *U* test. A *P* value of less than 0.05 was considered statistically significant and is represented as $^*P < 0.05$ and $^{**}P < 0.01$. All tests were performed with GraphPad Prism 6 software (GraphPad Software, San Diego, USA). For microarray analysis, gene expression microarray data were downloaded from the GEO database. The .CEL files were analyzed using the affy package within R/Bioconductor (Gentleman et al., 2004). Robust multichip averaging (RMA) was used to correct for the background and normalize and generate expression data (Irizarry et al., 2003).

3. Results

3.1. miR-181c-knockdown mice exhibit attenuated EAE clinical symptoms and decreased spinal cord inflammation and demyelination

$CD4^+$ T cells play a major role in the development of EAE (Goverman, 2009). By using $CD4^+$ T cells isolated from draining lymph nodes of mice, we found that miR-181c expression was significantly increased in EAE mice at the onset and peak stage of the disease compared with the expression level at these time points in wild-type mice, which exhibited a normal level when the disease remitted (Fig. 1A). To determine the potential role of miR-181c in EAE pathogenesis, we constructed lentiviral vectors and delivered recombinant lentivirus (LV) to the mice to knockdown miR-181c expression. On day 7 after the systemic administration injection of the LV through the tail vein, it was observed that the LV induced significant miR-181c knockdown in various organs without affecting the expression of other members in the miR-181 family, especially miR-181a and miR-181b (Supplementary Fig. 1A–C). After the immunization to induce EAE on day 7 after the lentivirus injection, compared with the LV-Control-infected mice, the LV-shRNA-miR-181c- infected mice exhibited attenuated EAE clinical symptoms including clinical scores as well as the area under the curve (AUC) values and peak clinical scores (Fig. 1B, C). Histological analysis of spinal cord sections revealed that significant inflammatory infiltration and demyelination were developed in the LV-Control-infected mice, whereas the LV-sh-miR-181c-infected mice presented with alleviated CNS pathology which was associated with relatively mild paralytic symptoms (Fig. 1D, E).

3.2. miR-181c affects the in vivo Th17 cell population in EAE mice

Alterations in the infiltrating effector helper T cells (mainly Th17 and Th1 cells) and regulatory T cells (Treg cells) in the CNS and peripheral lymphoid organs (spleen and lymph nodes) are considered hallmarks of inflammation in EAE (Goverman, 2009). We then investigated whether the distinct EAE pathology between the two groups of LV-infected mice was associated with the altered infiltration of effector helper T cells and Treg cells. As measured by CD4 surface staining, we found that decreased $CD4^+$ T cell accumulation in the peripheral lymphoid organs of the LV-sh-miR-181c-infected mice (Fig. 2A). In addition, the LV-sh-miR-181c-infected mice presented with fewer $CD4^+$ T cells in the CNS than the LV-Control-infected mice (Fig. 2B). Intracellular cytokine staining demonstrated that the percentage of $CD4^+IL\text{-}17^+$ cells was markedly reduced in the peripheral lymphoid organs and in the CNS of the LV-sh-miR-181c-infected mice at both the onset and peak stages of EAE development (Fig. 2C–H). In contrast, the percentage of $CD4^+IFN\text{-}\gamma^+$ cells and $CD4^+CD25^+FOXP3^+$ T cells primed in the peripheral lymphoid organs were respectively similar between the two groups of LV-infected EAE mice (Supplementary Fig. 2). The infiltration of $CD4^+IFN\text{-}\gamma^+$ cells and $CD4^+CD25^+FOXP3^+$ T cells in the CNS were not obviously altered by miR-181c expression (Supplementary Fig. 2). Furthermore, the percentage of the $CD4^+$ T cells and T cell subsets was comparable between the LV-Control-infected and LV-sh-miR-181c-infected mice that did not suffer from EAE (Supplementary Fig. 3A, B). These data collectively indicated that miR-181c greatly affected the Th17 cell population during EAE development.

3.3. miR-181c affects in vitro Th17 cell differentiation

To investigate whether miR-181c affected Th17 differentiation, an *in vitro* T cell differentiation assay was performed. We first activated naive $CD4^+CD62L^+$ T cells under *in vitro* Th1-, Th2-, Th17- and Treg-polarizing conditions and observed that miR-181c was highly expressed specifically in Th17 cells and that relatively fewer and similar amounts were expressed in Th1 and Th2 cells and

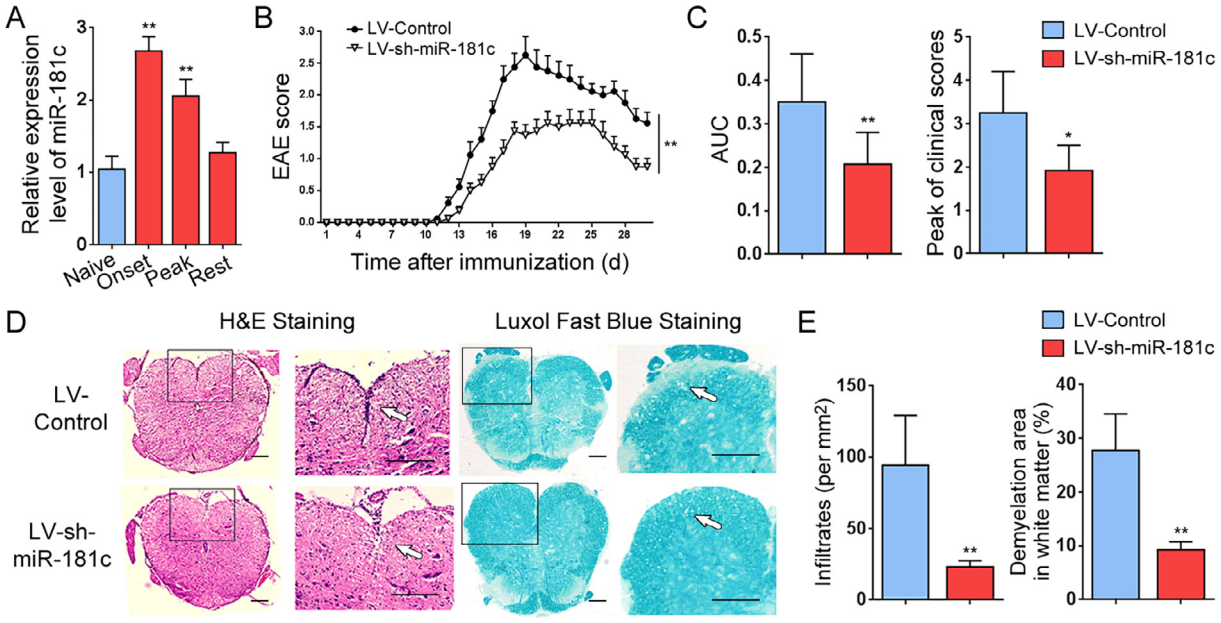


Fig. 1. miR-181c-knockdown mice exhibit attenuated EAE clinical symptoms and decreased spinal cord inflammation and demyelination. LV-Control-infected mice (LV-Control) or LV-shRNA-miR-181c-infected mice (LV-sh-miR-181c) were immunized to suffer EAE (n = 8–10 per group). (A) miR-181c expression in $CD4^+$ T cells isolated from the draining lymph nodes of naive mice and EAE mice at various stages of disease, as measured by quantitative PCR. (B) Clinical scores for EAE in the LV-Control-infected or LV-sh-miR-181c-infected mice. (C) The area under the curve (AUC) values of daily clinical scores and the peak clinical scores calculated from the EAE mice observed from days 1 to 30 after immunization. (D) Spinal cord sections were harvested from EAE mice on day 20 after immunization and were stained with hematoxylin-eosin (H&E) to assess inflammation or Luxol fast blue to examine demyelination. Arrows indicate inflammatory cell infiltration and demyelination, respectively. Scale bars, 100 μm. (E) Quantification of the infiltration and demyelination in spinal cord sections shown in D. Data shown are representative of 3 independent experiments. Error bars shown are the mean ± SEM. $^*P < 0.05$ and $^{**}P < 0.01$ using unpaired Student's *t*-test. Two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, was applied for multiple comparisons. For EAE scoring, groups were compared using Mann-Whitney *U* test.

inducible Treg cells (Fig. 3A). The isolated naive $CD4^+CD62L^+$ T cells were then transfected with siRNA to knockdown miR-181c expression followed by culturing under Th17-polarizing conditions. miR-181c knockdown significantly decreased the percentage of IL-17-producing T cells compared with that in the control group (Fig. 3B). Furthermore, miR-181c knockdown reduced the expression of Th17-associated cytokines, transcription factors and surface receptors (Fig. 3C, D). After treatment with miR-181c mimics, the percentage of IL-17-producing T cells and the expression of genes specific to Th17 cells were significantly upregulated (Fig. 3E–G). These results demonstrated that miR-181c positively regulates Th17 cell differentiation.

3.4. miR-181c affects the TGF-β-induced Smad-dependent signaling pathway

We next investigated the mechanism by which miR-181c regulates the differentiation of Th17 cells. To select and identify the potential downstream targets of miR-181c based on the presence of binding sites in the 3′UTR, we used three online target prediction software programs, including TargetScan (www.targetscan.org), DIANA (diana.imis.athena-innovation.gr) and miRDB (www.mirdb.org). Among these three predicted target gene sets, 397 genes overlapped and then were functionally annotated via KEGG pathway analysis by the DAVID tool (Fig. 3H, I). Among the top pathway terms, the TGF-β signaling pathway has been reported to play a critical role in Th17 cell differentiation. The TGF-β cytokine functions via the Smad-dependent signaling pathway and several non-Smad signaling pathways (Mangan et al., 2006; Moustakas and Heldin, 2005). We first observed the influence of miR-181c on the Smad-dependent TGF-β signaling pathway and found that the miR-181c knockdown resulted in the decreased phosphorylation of both Smad2 and Smad3 in the $CD4^+$ T cells compared with the phosphorylation level in the control group (Fig. 3J). For Smad-independent TGF-β signaling pathways, such as p38, Jun-amino-terminal kinase (JNK) and ERK signaling, no obvious alteration was found between the two groups (Fig. 3K). Collectively these data suggested that miR-181c affected the TGF-β-induced Smad-dependent signaling pathway and that miR-181c knockdown led to decreased sensitivity to TGF-β signaling in T cells.

3.5. Smad7 is a functional target of miR-181c

To further identify the functional target of miR-181c, we searched the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) database for raw data from mRNA expression profiles of MS patients and healthy control subjects and analyzed the differential expression of eight TGF-β signaling pathway-associated candidate target genes (Figs. 3H; 4A). Among these eight candidate miR-181c-targeted genes, *SMAD7* was markedly downregulated in the clinical datasets GSE43592 (Jernas et al., 2013) and GSE38010 (Han et al., 2012); meanwhile, the Smad7 protein was a negative regulator of Smad-dependent TGF-β signaling. In mice with EAE, Smad7 expression was decreased (Fig. 4B). Therefore, Smad7 might

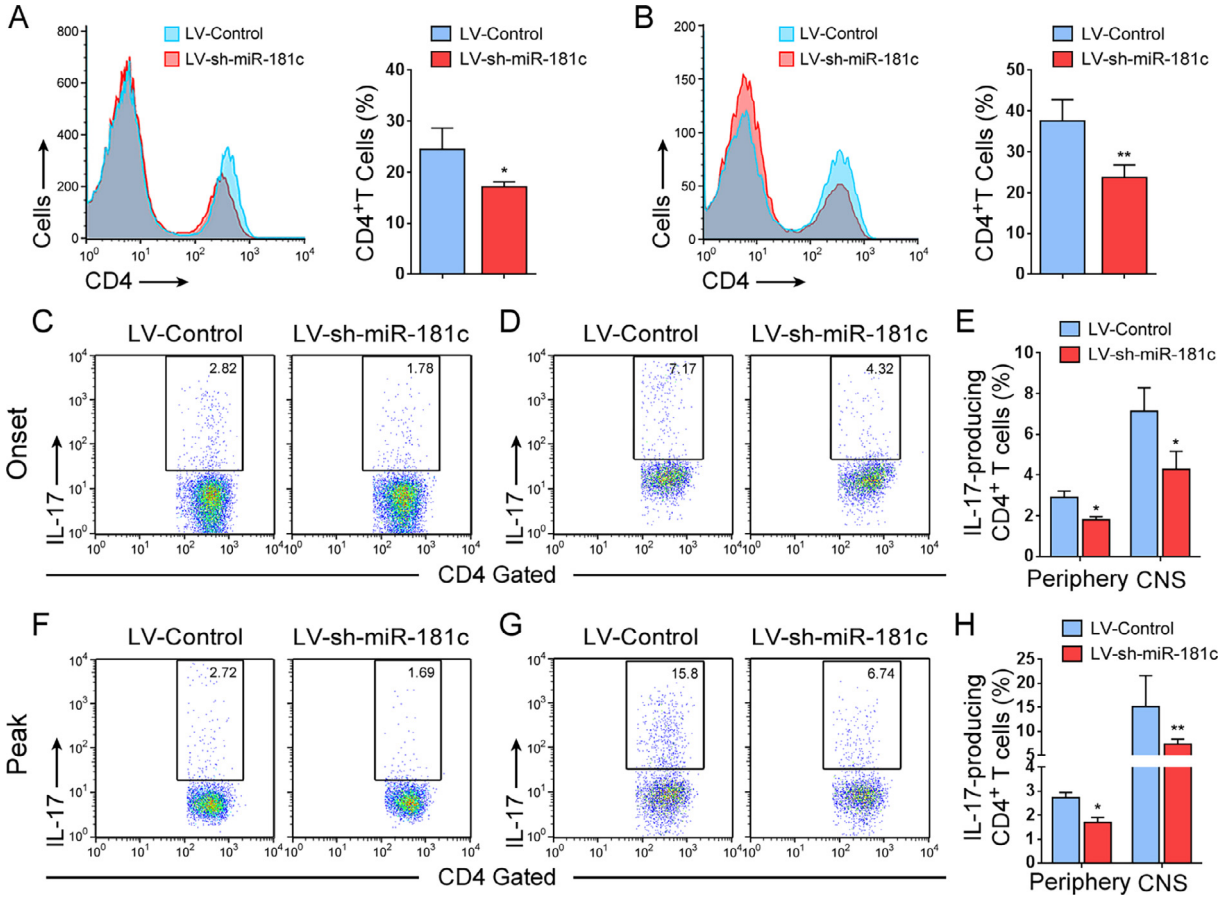
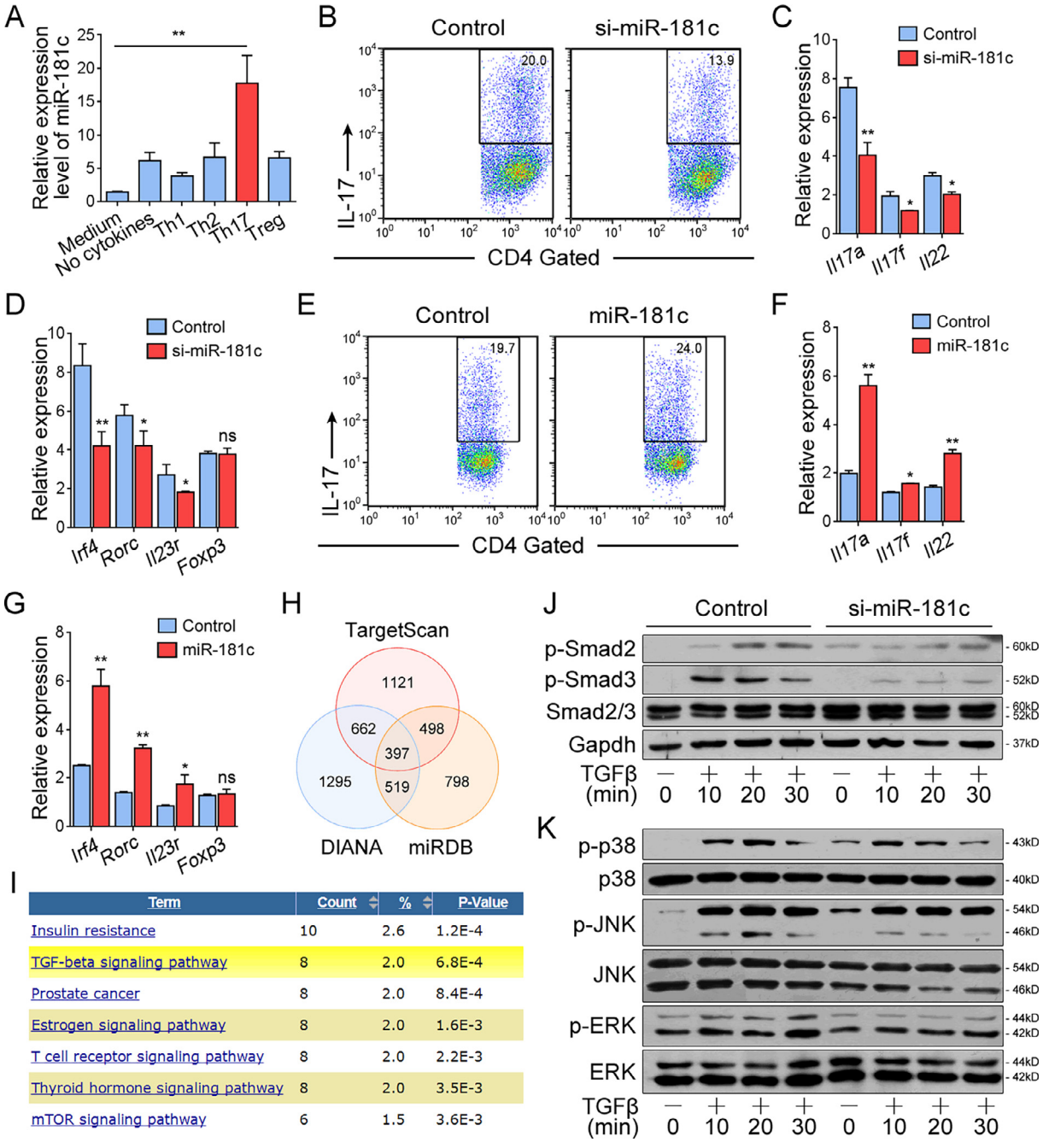


Fig. 2. miR-181c affects the *in vivo* Th17 cell population in EAE mice. Cells were isolated from lentivirus-infected mice at the onset (A, C, D, E) or peak stage (B, F, G, H) of EAE. (A, B) Quantification of $CD4^+$ T cells in the peripheral lymphoid organs (A) and mononuclear cell infiltrates isolated from the CNS (B). (C, D, E, F, G, H) Intracellular IL-17 staining in $CD4^+$ T cells from peripheral lymphoid organs (C, F) and mononuclear cell infiltrates isolated from the CNS (D, G). Data shown are representative of 3 independent experiments. Error bars shown are the mean ± SEM. $^*P < 0.05$ and $^{**}P < 0.01$ using unpaired Student's *t* test.

be a potential mediator that is targeted by miR-181c and induces decreased TGF-β signaling activation.

For functional validation, we applied the prediction program TargetScan to identify the putative miR-181c-binding element in the 3′ untranslated region (UTR) of *Smad7* and then performed the luciferase assays (Fig. 4C, D). miR-181c suppressed the 3′ UTR of WT *Smad7* but did not affect the mutated 3′ UTR. In $CD4^+$ T cells, the treatment with miR-181c mimics decreased Smad7 expression and enhanced Smad2 and Smad3 phosphorylation (Fig. 4E), while miR-181c inhibition by siRNA exhibited the inverse effects (Fig. 4F). In addition, naive $CD4^+CD62L^+$ T cells were transfected with si-miR-181c and cultured in a Th17-polarizing condition. miR-181c knockdown in the naive T cells led to downregulated *Il17a* expression with a concomitant increase in *Il2* expression, which serves as negative regulator of Th17 differentiation (Fig. 4G). Together, these data



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Term	Count	%	P-Value
Insulin resistance	10	2.6	1.2E-4
TGF-beta signaling pathway	8	2.0	6.8E-4
Prostate cancer	8	2.0	8.4E-4
Estrogen signaling pathway	8	2.0	1.6E-3
T cell receptor signaling pathway	8	2.0	2.2E-3
Thyroid hormone signaling pathway	8	2.0	3.5E-3
mTOR signaling pathway	6	1.5	3.6E-3

Fig. 3. miR-181c affects the *in vitro* Th17 cell differentiation and the TGF-β-induced Smad-dependent signaling pathway. (A) Quantitative PCR analysis of miR-181c expression *in vitro*- in differentiated Th1, Th2, Th17 and inducible Treg cells. "No cytokines" indicates activation with plate-bound anti-CD3/CD28 in the absence of supplementary polarizing conditions. (B–G) Intracellular IL-17 staining (B, E) and quantitative PCR analysis of the levels of representative cytokines (C, F), transcription factors and surface receptor (D, G) of naive $CD4^+CD62L^+$ T cells transfected by siRNA-anti-miR-181c (si-miR-181c) or miR-181c mimics or respective scrambled control and cultured in Th17-polarizing conditions. (H) Potential target genes of miR-181c as predicted by TargetScan, DIANA and miRDB. (I) Functional annotation of the 397 genes that overlapped, via KEGG pathway analysis by the DAVID tool, are shown in H. Count, indicates the number of corresponding pathway-associated candidate target genes. (J, K) Representative immunoblots of Smad-dependent signaling-associated and non-Smad signaling-associated proteins in $CD4^+$ T cells transfected by siRNA followed by TGF-β stimulation (2 ng/ml). Data shown are representative of 3 independent experiments. Error bars shown are the mean ± SEM. $^*P < 0.05$ and $^{**}P < 0.01$ using unpaired Student's *t*-test.

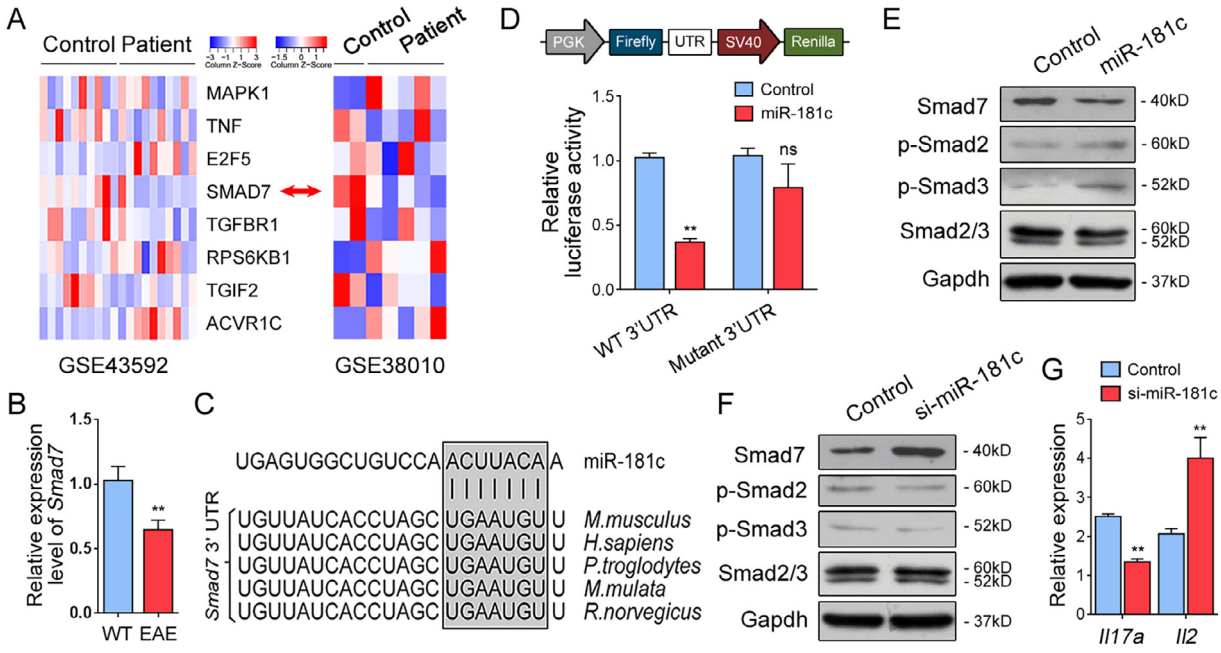

Fig. 4. Smad7 is a functional target of miR-181c. (A) Analysis of two published clinical datasets, GSE43592 and GSE38010, revealed that Smad7 expression is downregulated in patients with MS. (B) miR-181c expression in $CD4^+$ T cells isolated from draining lymph nodes of WT mice and EAE mice at the peak stage of disease, as measured by quantitative PCR. (C) Sequence alignment of the *Smad7* 3′ UTR with miR-181c among multiple species. (D) Activity of luciferase reporters and *Renilla* plasmids containing WT or mutant *Smad7* 3′ UTRs that were co-transfected with miR-181c mimics or respective controls into HEK-293T cells. (E) Immunoblot analysis of Smad7, p-Smad2, p-Smad3 and Smad2/3 expression in mouse naive $CD4^+$ T cells transfected with miR-181 mimics or the respective control. (F) Immunoblot analysis of Smad7, p-Smad2, p-Smad3 and Smad2/3 expression in mouse naive $CD4^+$ T cells transfected with siRNA-anti-miR-181 (si-miR-181c) or the respective control. (G) *Il17* and *Il2* expression in naive $CD4^+CD62L^+$ T cells transfected with si-miR-181c or the respective control and cultured under Th17 conditions. Data shown are representative for 3 independent experiments. Error bars shown are the mean ± SEM. $^*P < 0.05$ and $^{**}P < 0.01$ using unpaired Student's *t*-test.

indicated that Smad7 serves as a functional target of miR-181c during Th17 cell differentiation.

3.6. Expression of miR-181c is upregulated in patients with MS

To determine how miR-181c expression is altered in MS patients, we further searched the Gene Expression Omnibus database for raw data from the miRNA expression profiles. Clinical dataset GSE31568 (Keller et al., 2011) contained the expression profiles of 863 miRNAs by array analysis of 454 peripheral blood samples from healthy human individuals and patients of 14 different diseases. Group 1 (containing 22 healthy controls) and Group 201 (containing 23 patients with MS) contributed by the same institution were selected and analyzed. Clinical dataset GSE43592 (Jernas et al., 2013) contained the miRNA profiles of T cells isolated from the peripheral blood of 11 relapsing-remitting MS patients and 9 healthy individuals. We found that miR-181c was upregulated in MS patients in these two clinical datasets (Fig. 5A–D), which was consistent with the alteration in miR-181c expression in the EAE mice (Fig. 1A).

4. Discussion

T helper (Th) cell subsets are differentiated from naive $CD4^+$ T cells and serve as indispensable components of the adaptive immune system. Among these subsets, Th17 cells have huge effects on the modulation of inflammation and autoimmunity (Baumjohann and Ansel, 2013; Yang et al., 2014). MS is a typical autoimmune neurodegenerative disease in which proinflammatory cells invade the CNS, resulting in multifocal demyelination (Nylander and Hafler, 2012). Dysregulated IL-17 levels and Th17 cells are considered to be closely associated with the pathogenesis of MS, and the regulation of Th17 differentiation is considered an important avenue for clinical applications (Zepp et al., 2011). IL-$17^{-/-}$ EAE mice exhibit delayed onset, reduced maximum severity scores, ameliorated histological changes, and early recovery (Komiyama et al., 2006). miRNA pathways control dynamic aspects of autoimmunity and miR-181c is reported to inhibit the *in vitro* proliferation of T cells (Xue et al., 2011); however, whether and how miR-181c modulates Th cell differentiation and influences EAE progression remains unknown. Here, we have reported that miR-181c expression is increased in $CD4^+$ T cells of EAE mice. miR-181c-knockdown mice with EAE presented with defective Th17 differentiation in peripheral lymphoid organs and in the CNS *in vivo* as well as relief of disease progression. After *in vitro* culture under polarizing conditions, we found that miR-181c was specifically overexpressed in differentiated Th17 cells (Fig. 6).

Through Smad-dependent and non-Smad pathways, TGF-β signaling activation play a vital role in Th17 cell differentiation (Mangan et al., 2006; Moustakas and Heldin, 2005). Our results indicated that miR-181c knockdown inhibited the differentiation of naive $CD4^+CD62L^+$ T cells into Th17 cells via disrupting the TGF-β-induced Smad-dependent signaling pathway, while non-Smad signaling pathways, including p38, JNK and ERK signaling, were scarcely affected. By analyzing two published MS clinical datasets and conducting a luciferase assay, we selected and identified Smad7, a negative regulator of TGF-β signaling, to be a potential functional target of miR-181c. Activated TGF-β receptors phosphorylate Smad2/3 and then form trimers with Smad4 that translocate into the nucleus. Smad7 inhibits Smad2/3 activation by competitively binding to the receptors to block TGF-β-induced transcriptional responses (Akhurst and Hata, 2012). In Th17 cells, IL-2 was reportedly suppressed by Smad-dependent TGF-β signaling, inhibiting Th17 cell differentiation (Cejas et al., 2010; Meisel et al., 2013). A recent study

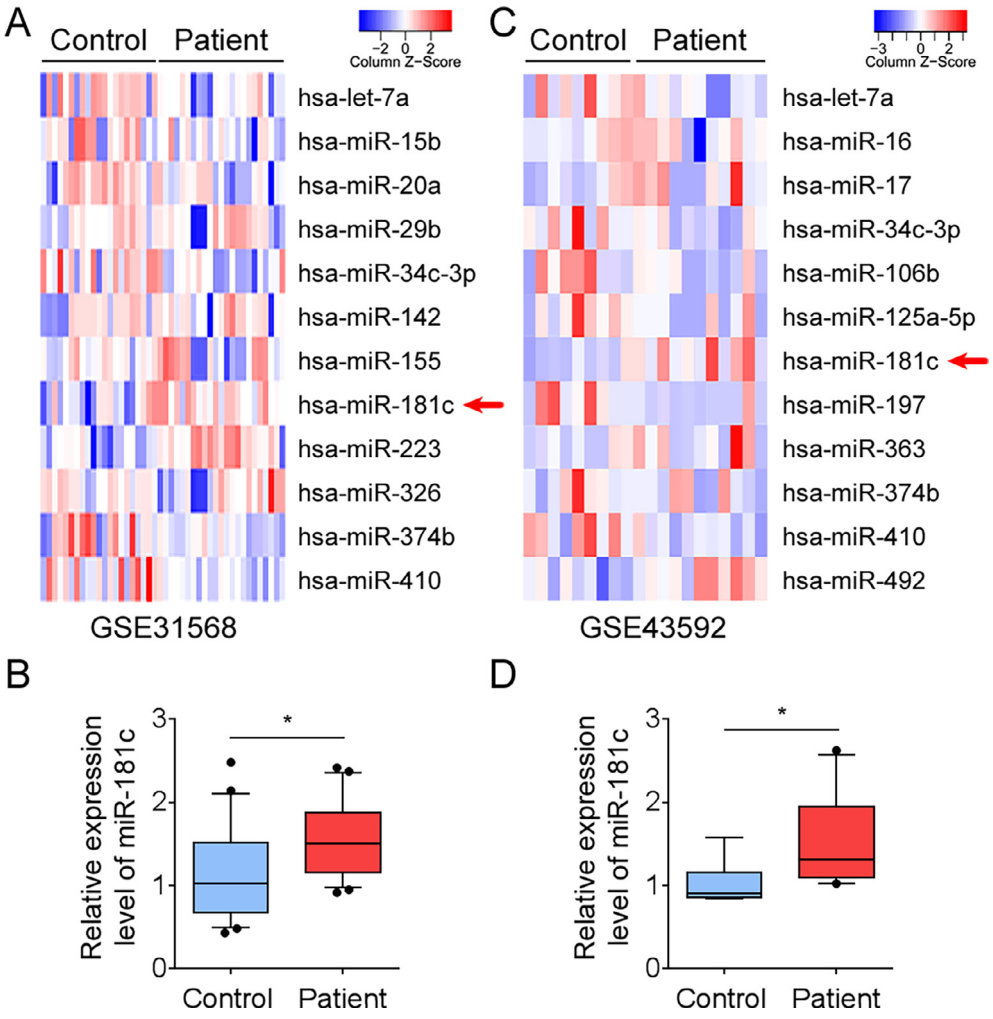

Fig. 5. Expression of miR-181c is upregulated in patients with MS. (A–D) Analysis of two published MS clinical datasets GSE31568 and GSE43592 and quantification of miR-181c expression from corresponding datasets. $^{*}P < 0.05$. The statistical significance of the differences was estimated by using non-parametric two-sided Mann-Whitney tests.

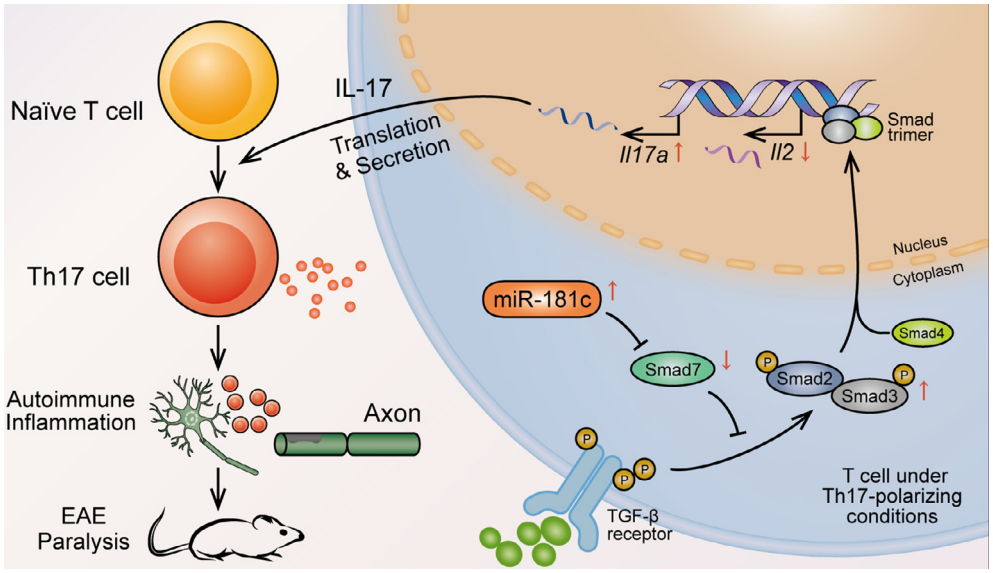

Fig. 6. Schematic for the molecular mechanism by which miR-181c regulates Th17 cell differentiation. In Th17-polarizing conditions, miR-181c was increased and thereby downregulated Smad7 expression, rendering T cells more sensitive to TGF-β-induced Smad2/3, limiting the inhibitory effects of autocrine IL-2 and thus contributing to Th17 cell differentiation and EAE induction.

demonstrated that silencing Smad7 resulted in enhanced IL-17 production, which correlated with increased Smad2/3 activation in T cells (Murugaiyan et al., 2015).

In addition, TGF-β signaling activation has an effect on Treg differentiation (Zheng et al., 2002). Smad2 and Smad3 were shown to play a partial role in the development of induced Treg cells *in vitro*; however, Smad2- or Smad3-knockout mice exhibited an insignificant alteration in Foxp3 induction *in vivo*, and it was suggested that non-Smad pathways, especially p38 signaling, were the primarily required for Treg differentiation and function (Lu et al., 2010). Another study demonstrated that Smad2 and Smad3 double-deficient mice maintained a normal phenotype regarding development, homeostasis, and function of Treg cells (Gu et al., 2012). Moreover, consistent with our data, a previous study demonstrated that increased TGF-β-mediated Smad2/3 activation induced the downregulation of *Il2* transcription but generated normal numbers of Foxp3-expressing induced Treg cells, which might result from complicated transcriptional mechanisms (Cejas et al., 2010). Collectively, these studies might explain our result that miR-181c knockdown failed to influence the differentiation of Tregs *in vivo* and *in vitro* (Supplementary Figs. 2, 3).

Through the analysis of two published miRNA expression profiles in the Gene Expression Omnibus database, increased miR-181c levels were found in peripheral blood and peripheral T cells collected from relapsing-remitting MS patients. The association between miR-181c and MS has also been investigated in several previous clinical studies. In the cerebro-spinal fluid (CSF) of patients with MS, miR-181c was found to be significantly upregulated, compared with other neurological diseases, which provided rationale for a confirmation study in larger MS cohorts(Haghikia et al., 2012). In the brain of MS patients, miR-181c was found to be negatively correlated with magnetic resonance Imaging (MRI) measures of disease severity (Regev et al., 2017). In peripheral blood mononuclear cells (PBMCs), miR-181c was downregulated in MS (Ma et al., 2014). Indeed, the present findings of miR-181c dysregulation in MS are partially consistent with these previous reports, and some conflicts in miR-181c expression are demonstrated. Apart from the difference in tissue types, different type of MS and disease stages may contribute to the diversity in miRNA level. It should be noted that miR-181c level changed dynamically at different stages during EAE development. miR-181c was upregulated at the onset and peak stage but was decreased when the disease remitted (Fig. 1A). We speculated that selected patients at various stages of disease might also exhibit different miR-181c levels. This might partly explain the inconsistency among different datasets, and thus further clinical studies need to be conducted. In addition, although the development of miRNA-targeted therapeutics still encounters great challenges, such as the broad-spectrum miRNA suppression and lentiviral-based genotoxicity, our study offers clues for understanding MS pathogenesis and might impact on the future diagnosis and treatment of patients with MS.

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Conflict of interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbi.2018.03.011.

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