RESEARCH ARTICLE

IL-17-triggered downregulation of miR-497 results in high HIF-1α expression and consequent IL-1β and IL-6 production by astrocytes in EAE mice

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Interleukin 17 (IL-17) is increasingly recognized as a key factor that contributes to the pathogenesis of multiple sclerosis (MS) and its experimental mouse autoimmune encephalomyelitis (EAE) model. However, the roles and regulatory mechanisms of IL-17-induced pro-inflammatory cytokine production in EAE mice remain largely unclear. In this study, the expression of IL-17, hypoxia inducible factor-1α (HIF-1α), IL-1β, IL-6 and microRNA-497 (miR-497), as well as their intrinsic associations, was investigated using EAE model mice and cultured astrocytes exposed to IL-17 *in vitro*. We observed markedly increased production of IL-17, HIF-1α, IL-1β and IL-6 in the brain tissues of EAE mice, while the expression and secretion of HIF-1α, IL-1β and IL-6 were also significantly increased when cultured primary astrocytes from mice were stimulated with IL-17. Meanwhile, the expression of miR-497 was downregulated both *in vivo* and *in vitro*. Subsequent *in vitro* experiments revealed that IL-17 induced the production of IL-1β and IL-6 in astrocytes through the upregulation of HIF-1α as a transcriptional factor, indicating that IL-17-mediated downregulation of miR-497 enhanced HIF-1α expression. Furthermore, astrocyte-specific knockdown of IL-17RA and HIF-1α or astrocyte-specific overexpression of miR-497 by infection with different lentiviral vectors containing an astrocyte-specific promotor markedly decreased IL-1β and IL-6 production in brain tissues and alleviated the pathological changes and score of EAE mice. Collectively, these findings indicate that decreased miR-497 expression is responsible for IL-17-triggered high HIF-1α expression and consequent IL-1β and IL-6 production by astrocytes in EAE mice.
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INTRODUCTION

Multiple sclerosis (MS), an organ-specific autoimmune disease, is characterized by chronic inflammatory demyelination within the central nervous system (CNS) of humans.[1–3] It is well known that autoreactive CD4^{+} T cells, especially Th17 cells and their secretion of interleukin-17 (IL-17), play important pathogenic roles in the inflammatory response and in demyelinating lesions within the CNS of MS patients.[4–9] In addition, interleukin-1β (IL-1β) and interleukin-6 (IL-6), two pro-inflammatory cytokines, are also related to the CNS damage that occurs in MS patients.[10,11] Reportedly, IL-17 not only

recruits immune cells but also increases the production of IL-lβ and IL-6 in astrocytes, macrophages and other cells, leading to aggravation of CNS injury. Neutralizing antibody against IL-17 or IL-1 receptor antagonist (IL-1Ra) partly decreases CNS damage in MS patients.[12–15] Nevertheless, the regulatory role and mechanism of IL-17 in IL-1β and IL-6 production in the CNS of patients with MS are still largely unclear.

Mouse experimental autoimmune encephalomyelitis (EAE) is a widely used animal model for studying human MS.[16–18] It has been revealed that, similar to human MS, Th17 cells are recruited into the CNS of EAE mice, and the levels of

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pro-inflammatory cytokines, such as IL-17, IL-1β and IL-6, are markedly elevated in the CNS and related to CNS damage in EAE mice.[19–25] Notably, IL-17 is a trigger that can also elevate the secretion of IL-1β and IL-6 from mouse astrocytes,[26–28] indicating that IL-17 is involved in the initiation and development of inflammatory injuries in EAE mice. However, the molecular mechanism by which the astrocytes of EAE mice contribute to the production of these cytokines in response to IL-17 stimulation needs to be investigated.

Increasing evidence shows that IL-17 is able to induce various biological responses in target cells through the induction of various transcriptional factors, such as Krüppel-like factor 2 (KLF2), CCAAT/enhancer-binding protein β (C/EBPβ), early growth response-1 (Egr-1) and hypoxia inducible factor-1α (HIF-1α).[29–31] HIF-1α is the alpha subunit of HIF-1, which is a transcription factor that responds to the changes in available oxygen.[32,33] As a master transcriptional regulator, HIF-1α can activate the transcription of over 40 genes, including cytokines or other factors, and it is also involved in inflammation.[34–37] Hence, whether the level of HIF-1α can be upregulated by IL-17 stimulation and whether the transcription of IL-lβ and IL-6 genes can be directly triggered by HIF-1α to increase EAE damage remains unknown and needs to be elucidated.

MicroRNAs (miRNAs) belong to a large family of endogenous small noncoding RNAs that regulate gene expression at the post-transcriptional level. Deregulated expression of specific miRNAs is also involved in many pathological processes, such as viral infection, cancer and autoimmune diseases, including EAE.[20,38–43] Our previous study reported that IL-17 stimulation induces upregulation or downregulation of specific miRNAs in mouse astrocytes, and among them, miR-873 can regulate the synthesis of inflammatory cytokines in mouse astrocytes by targeting A20 through NF-kB in EAE mice.[44] In the present study, because miR-497 was demonstrated to be downregulated both in the CNS of EAE mice (in vivo) and in the astrocytes exposed to IL-17 (in vitro), and miR-497 was also predicted to bind to the 3′UTR of HIF-1α mRNA, we selected miR-497 from the several downregulated miRNAs to explore its regulatory function.

In the current study, the induction of IL-17, HIF-1α, IL-1β, IL-6 and miR-497 in the CNS of EAE mice, as well as the production of these parameters in cultured primary mouse astrocytes exposed to IL-17, were examined. Subsequently, the role of HIF-1α in the synthesis of IL-1β and IL-6, including its upstream regulation by miR-497, was determined in IL-17-treated astrocytes. Finally, the effects of the IL-17-miR-497-HIF-1α axis on the production of IL-1β and IL-6, as well as the pathological changes and behavior of EAE mice, were observed using astrocyte-specific lentiviral vectors to overexpress or silence the above-mentioned genes.

MATERIALS AND METHODS

Reagents
Oligodendrocyte glycoprotein (MOG) 35–55 amino-acid peptide (MOG$_{35–55}$, MEVGWYRSPFSRVVHLYRNGK) and complete Freund's adjuvant (CFA) were purchased from CL Bio-scientific Co. Ltd (Xian, China) and Sigma-Aldrich (St Louis, MO, USA), respectively. Mouse IL-17 protein was from R&D Systems (Minneapolis, MN, USA). Dulbecco's modified Eagle's medium (DMEM) and DMEM/F12 were obtained from HyClone (San Angelo, TX, USA). Fetal bovine serum (FBS) was from Gibco (Carlsbad, CA, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). TaqMan microRNA reverse transcription kits, miR-497 TaqMan assay kits, TaqMan universal master mix II and SYBR select master mix were from Applied Biosystems (Foster City, CA, USA). A 2 × Taq Master Mix was purchased from Vazyme Biotech (Nanjing, China). PrimeScript RT reagent kits with gDNA eraser were from Takara Bio Inc. (Tokyo, Japan). RIPA lysis buffer and BCA protein assay kits were purchased from Beyotime (Nantong, China). Rabbit polyclonal antibodies against IL-17RA and HIF-1α were from Abcam (Cambridge, UK) and Bioworld Technology (St Louis, MN, USA), respectively. Mouse monoclonal antibodies against HIF-1α (H1alpha67, ChIP Grade) and β-actin were from Abcam and Boster (Wuhan, China), respectively. HRP-labeled goat anti-rabbit IgG was purchased from Cell Signaling Technology (Danvers, MA, USA). HRP-labeled goat anti-mouse IgG was obtained from Sigma-Aldrich. Enhanced chemiluminescence (ECL) western blotting substrate was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Mouse IL-17, IL-1β and IL-6 ELISA kits were from BioLegend (San Diego, CA, USA) and eBioscience (San Diego, CA, USA), respectively. A chromatin immunoprecipitation (ChIP) assay kit was obtained from Millipore (Bedford, MA, USA). The luciferase reporters pGL3-Basic, pGL3-Promoter and pRL-SV40 were purchased from Promega (Madison, WI, USA). A Neon transfection system was provided by Invitrogen. GenEscort III was from Wisegen (Nanjing, China). Polybrene was from Sigma-Aldrich.

Animals, EAE induction and score
C57BL/6 mice were purchased from Nanjing Medical University Laboratory Animal Center (Nanjing, China). All mice were maintained under specific pathogen-free conditions. All animal experiments were performed in compliance with the guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Ethics Committee of Nanjing Medical University. EAE was induced in female C57BL/6 mice at 8 weeks of age, and EAE scores were evaluated as described previously.[44]

Cell culture and IL-17 stimulation
Primary mouse astrocytes were obtained and cultured in DMEM/F12 with 10% FBS as described previously.[44] After different treatments, the astrocytes were stimulated with IL-17 (100 ng/ml) for different time points. In addition, HEK293T cells were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS (vol/vol), 100 μg/ml penicillin G and 100 μg/ml streptomycin.

Plasmid construction

The mouse HIF-1α expression plasmid pCMV/HIF-1α was a gift from Professor Fan Pan (Johns Hopkins University, USA). The mouse IL-17RA shRNA expression plasmid pSilencer2.1-U6/IL-17RA shRNA (shIL-17RA, 5′-GCACCTACGTTGTT TGCTA-3′) and scrambled control shRNA (shCTR) expression plasmid were a gift from Professor Dongqing Li (Wuhan University, China).

The luciferase reporters pGL3-Basic/IL-1β and pGL3-Basic/IL-6 were constructed by inserting the 1.169 kb mouse IL-1β promoter (−1142 to +27 nt) and the 0.736 kb mouse IL-6 promoter (−667 to +69 nt), respectively, into a pGL3-Basic vector at the *kpn* I restriction enzyme site. In addition, the luciferase reporter pGL3-Promoter/HIF-1α was constructed by inserting the 1.522 kb 3′UTR of mouse HIF-1α mRNA (NM_010431) into a pGL3-Promoter vector at the *Xba* I restriction enzyme site. The primer sequences for PCR are shown in Supplementary Table 1.

miRNA mimic and inhibitor synthesis

All of the miRNA mimics and a universal negative control miRNA mimic (CTR mimic) were purchased from Gene-Pharma (Shanghai, China). The sequences of different miRNA mimics are shown in Supplementary Table 2.

A miRCURY LNA microRNA inhibitor, namely LNA-anti-miR-497 (4101347-000, 5′-CAAACCACAGTGTGCTGCT-3′), and a universal negative control LAN, namely LNA-anti-miR-CTR (199006-000, 5′-TAACACGTCTATACGCCCA-3′), were purchased from Exiqon (Vedbæk, Denmark).

siRNA synthesis

To silence the mouse HIF-1α gene, an siRNA against mouse HIF-1α mRNA (siHIF-1α, 5′-GAACTAACTGGACACAGTG TGTT-3′) was synthesized by GenePharm (Shanghai, China). In addition, a universal scrambled control siRNA (siCTR, 5′-UUCUCCGAACGUGUCACGUTT-3′) was also produced by GenePharm.

Cellular transfection

Primary mouse astrocytes were transfected with corresponding miRNAs, siRNAs or plasmids using a Neon transfection system.[45,46] For miRNA or siRNA transfection, 4×10^5 cells were resuspended in 100 μl of resuspension buffer that included 100 nM miRNA or siRNA and then electroporated at 1100 V (30 ms, 1 time). For plasmid transfection, 4×10^5 cells were resuspended in 100 μl of resuspension buffer that included 100 nM plasmid and then electroporated at 1350 V (20 ms, 2 times). After transfection, the astrocytes (4×10^5 cells per well) were transferred to six-well cell culture plates pre-coated with 1 mg/ml poly-L-lysine and then incubated for 48 h. The transfection efficiency was examined by observing the fluorescence of GFP (Supplementary Figure 1).

HEK293T cells were transfected with corresponding miRNAs or plasmids using GenEscort III according to the manufacturer's instructions. Briefly, cells were seeded in 24-well cell culture plates 24 h before transfection. The medium was changed with new DMEM plus 10% FBS (500 μl per well) 30 min before transfection. Next, 1 μg of plasmid or 0.5 μg of plasmid plus 100 nmol of miRNA were mixed with 50 μl of serum-free DMEM, and then, 3 μl of GenEscort III mixed with 50 μl of serum-free DMEM was added and incubated for 10 min. Finally, 100 μl of the resultant mixture was added to each well.

Real-time PCR

Total RNA was extracted from the primary mouse astrocytes and the mouse brain tissues with TRIzol reagent. For miRNA detection, cDNA was synthesized with TaqMan microRNA reverse transcription kits. The expression level of miR-497 was assayed with miR-497 TaqMan assay kits and the TaqMan universal master mix II. The reaction program included an initial step for denaturation at 95 °C for 10 min and then 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The level of miR-497 was normalized to the level of the U6 gene. For mRNA detection, cDNA was synthesized using PrimeScript RT reagent kits with gDNA Eraser. The expression levels of mouse IL-17, IL-1β and IL-6 mRNA were quantified by real-time PCR with SYBR select master mix. Primer sequences are listed in Supplementary Table 3. The expression levels of mouse IL-17, IL-1β and IL-6 mRNA were normalized to the expression level of GAPDH mRNA. Amplification of cDNA was performed on an ABI Prism 7300 (Applied Biosystems) system with a reaction program that included an initial step at 50 °C for 2 min and 95 °C for 10 min, and then 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. Each sample was assayed in triplicate. Relative gene expression levels were obtained using the formula $2^{-\triangle\triangle Ct}$.

Western blotting

Extracted protein (50 μg per well) was loaded into a 12% SDS polyacrylamide gel for electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes with a PowerPacTM Basic system (Bio-Rad, Hercules, CA, USA). The PVDF membranes were incubated in blocking buffer (5% skim milk in TBS-T buffer) at room temperature (RT) for 1 h and then incubated with antibodies to HIF-1α and β-actin at 4 °C overnight. β-actin expression in each sample was identified as the internal standard. After being washed five times with TBST-T, the PVDF membranes were further incubated with HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG at RT for 1 h. The bands were visualized by regular X-ray film using an ECL detection system after washing the PVDF membranes five times. Finally, the density of the radiographic bands on the PVDF membranes was analyzed using Quantity One software (Bio-Rad).

ELISA

The protein levels of IL-17, IL-1β and IL-6 in the supernatant of cultured astrocytes and mouse sera and in the homogenate of mouse brain tissues (20 mg/ml in PBS) were measured with ELISAs according to the manufacturer's instructions.

Luciferase assays

For IL-1β and IL-6 promoter reporter assays, HEK293T cells in each well of 24-well cell culture plates were transfected with a mixture of 0.5 μg pGL3 luciferase vector (pGL3-Basic/IL-1β or pGL3-Basic/IL-6), 10 ng pRL-SV40 and 0.5 μg pCMV expression plasmid (pCMV/HIF-1α or pCMV) by using GenEscort III. For the HIF-1α 3′UTR reporter assay, HEK293T cells in each well of 24-well cell culture plates were transfected with a mixture of 0.5 μg pGL3-Promoter/HIF-1α, 2.5 ng pRL-SV40 and 100 nmol miRNA mimic (miR-195, miR-292-5p, miR-322, miR-497, miR-497 mutant or miR-CTR mimic) by using GenEscortIII. Cells were lysed 48 h after transfection, and then, the luciferase activity of IL-1β and IL-6 promoters as well as HIF-1α 3′UTR reporter was measured through a dual-luciferase reporter gene assay. The ratio of firefly luciferase to Renilla luciferase was calculated for each cellular sample.

ChIP assays

ChIP assays were performed with antibody against HIF-1α as mentioned previously.[44] The captured genomic DNA was further used for real-time PCR analysis with SYBR select master mix and for regular PCR analysis with 2× Taq Master Mix according to the manufacturer's instructions. The primers used for detection of the IL-1β promoter (−544 to −352 nt) were 5′-CTGTGTGTGCCCTGACCCA-3′ (forward) and 5′-GGATGTGCGGAACAAAGGTAG-3′ (reverse). The primers used for detection of the IL-6 promoter (−499 to −300 nt) were 5′-CAGACATACAAAAGAATCCTAGCCTC-3′ (forward) and 5′-CTTGAGCATGTCTTGATGGGAA-3′ (reverse).

Lentivirus construction and *in vivo* experimental design

Different lentiviral vectors encoding shIL-17RA, miR-497 mimic, miR-497 sponge, shHIF-1α and shCTR (namely, LV-shIL-17RA, LV-miR-497 mimic, LV-miR-497 sponge, LV-shHIF-1a and LV-shCTR, respectively) were purchased from GeneChem (Shanghai, China). The sequences for mouse IL-17RA and HIF-1α gene knockdown and for the miR-497 mimic were the same as those used *in vitro*. The sequence of the miR-497 sponge was 5′-TAGCAGCATTAGAGCAAGAAAGCATATGTAGCAGCAGACTGTGTTGCCCCAGATCT-TAGCAGCACATTAGCAACTTCT-3′. For all of these lentiviral vectors, an astrocyte-specific promoter of glial fibrillary acidic protein was used to confirm astrocyte-specific knockdown or overexpression *in vivo*.[47]

To explore the roles of IL-17, miR-497 and HIF-1α in IL-1β and IL-6 production and CNS damage in EAE, mice were divided into six groups as follows: (1) LV-CTR+CFA, (2) LV-CTR+EAE, (3) LV-shIL-17RA+EAE, (4) LV-miR-497 mimic +EAE, (5) LV-miR-497 sponge+EAE, (6) LV-shHIF-1a+EAE. For lentivirus infection *in vivo*, C57BL/6 mice were anesthetized before microinjection. Subsequently, a microsyringe was inserted 2.0 mm lateral and 1.0 mm caudal to the bregma and 2.0 mm below the skull surface, and then, 5 μl of lentivirus (0.5×10^7 IU) with 10 μg/ml polybrene was administered to each mouse at the speed of 1 μl/min. Transfection efficiency and organ specificity were determined by observing the GFP

expression in the main organs of the LV-CTR-infected mice (Supplementary Figure 2). The brain tissues of mice in different groups were obtained for detection of mRNA, miRNA and protein expression as well as for histopathology on day 20 after EAE induction.

Histopathology

To assess inflammation and demyelination, the 4-μm-thick paraffin-embedded sections of mouse brain tissues and spinal cords were stained with hematoxylin and eosin (H&E) and Luxol fast blue (LFB), and observed with light microscopy. For H&E staining, 30 randomly distributed fields (original magnification: ×200) within the brain tissues were captured from different sections of each of the mice. The inflammatory cells were counted using ImageJ software (https://imagej.nih.gov/ij). For LFB staining, 30 randomly distributed fields (original magnification: ×200) within the white matter of the spinal cords were captured from different sections of each of the mice. The area of the white matter and the area covered by the LFB stain were quantified using ImageJ software. The degree of demyelination was calculated with the following formula: (white matter area − LFB area)/white matter area×100%. In addition, ultrathin sections of spinal cords were stained with uranyl acetate and lead citrate and examined with electron microscopy. For demyelination quantification, the G-ratio, the value of the axon diameter divided by the entire fiber diameter of the axon plus myelin sheath was measured with ImageJ software (300 axons per mouse).[48]

Statistics

One-way analysis of variance was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the *t*-statistic and adjusting the critical value according to the Bonferroni method. In addition, a Mann–Whitney test for nonparametric data (EAE score) was used. Values of $P < 0.05$ were considered significant.

RESULTS

The production of IL-17, HIF-1α, IL-1β and IL-6 increases in the brain tissues of EAE mice

To investigate the potential roles of IL-17 in HIF-1α, IL-1β and IL-6 induction in the CNS of EAE mice, the expression of IL-17, HIF-1α, IL-1β and IL-6 at the mRNA and/or protein level was assessed in the brain tissues and/or in sera of EAE mice. Time course studies *in vivo* showed that IL-17 mRNA in brain tissues (Figure 1a) and IL-17 protein in the sera (Figure 1b) were noticeably elevated in mice with EAE, in which the time-dependent tendency was consistent with our previous study.[44] Meanwhile, the protein level of HIF-1α was also markedly increased in the mouse brain tissues from day 7 to day 20 after EAE induction (Figure 1c). In addition, the mRNA levels of IL-1β and IL-6 in the brain tissues (Figure 1d) as well as the protein levels of IL-1β and IL-6 in the sera of EAE mice (Figure 1e) were obviously elevated in a time-dependent manner. Notably, the expression time points of IL-17, HIF-1α,
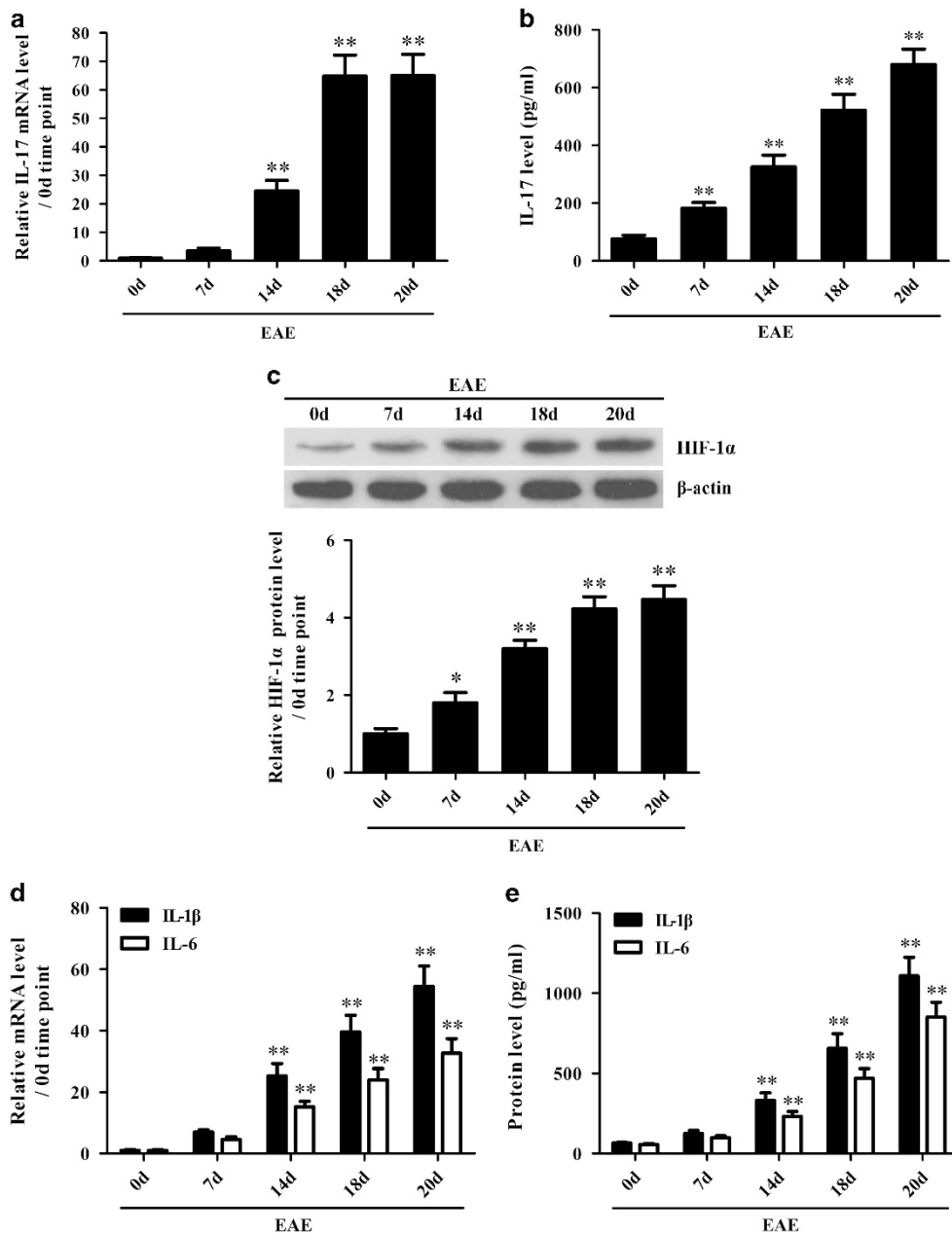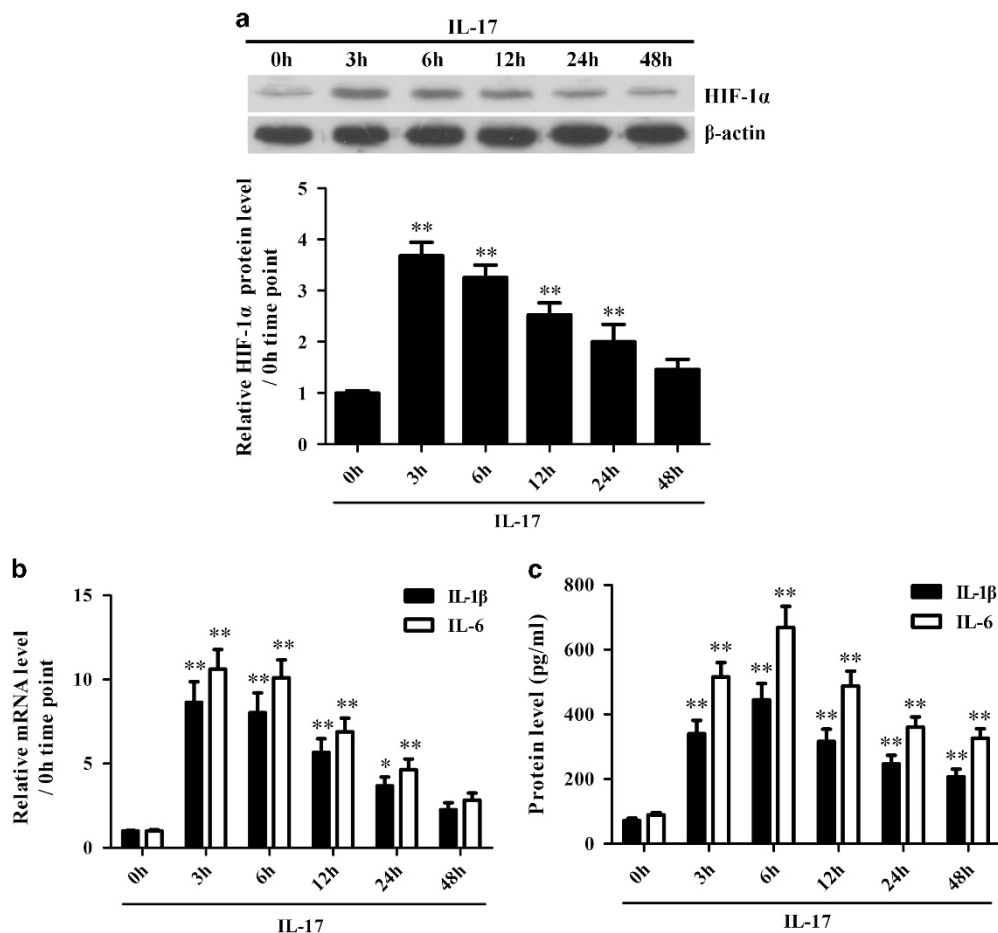
Figure 1 The expression of interleukin (IL)-17, hypoxia inducible factor-1α (HIF-1α), IL-1β and IL-6 both in the brain tissues and the sera of experimental autoimmune encephalomyelitis (EAE) mice. (**a**, **b**) The messenger RNA (mRNA) and protein levels of IL-17 in the brain tissues (**a**) and in the sera (**b**) of EAE mice were measured with real-time PCR and ELISAs, respectively. (**c**) The expression of HIF-1α protein in the brain tissues of EAE mice was analyzed with a western blot. (**d**, **e**) The expression of IL-1β and IL-6 at the mRNA level in the brain tissues (**d**) and at the protein level in the sera (**e**) of EAE mice was detected using real-time PCR and ELISAs, respectively. $*P<0.05$, $**P<0.01$ versus the day 0 (0 d) time point. The results from one representative experiment out of three are shown. Data are presented as the means ± s.d. ($n=6$ in each time point).

IL-1β and IL-6 were consistent with each other, suggesting that IL-17 might regulate HIF-1α, IL-1β and IL-6 production in the CNS of EAE mice.

IL-17 stimulates astrocytes to produce HIF-1α, IL-1β and IL-6

To explore the possible roles of IL-17 in HIF-1α, IL-1β and IL-6 production in astrocytes, cultured primary mouse astrocytes were stimulated with IL-17 *in vitro*, and then, the mRNA and/or protein levels of HIF-1α, IL-1β and IL-6 were detected in the cells or supernatant. Time course studies showed that the expression levels of HIF-1α, IL-1β and IL-6 were upregulated simultaneously in primary mouse astrocytes stimulated with IL-17 (Figure 2). Further experiments showed that IL-17RA knockdown obviously inhibited the expression of HIF-1α, IL-1β and IL-6 in primary mouse astrocytes exposed

Figure 2 The expression of hypoxia inducible factor-1α (HIF-1α), interleukin (IL)-1β and IL-6 in astrocytes induced by IL-17. Primary mouse astrocytes were incubated with IL-17 for different time points. (**a**) The protein expression of HIF-1α in the astrocytes was detected with a western blot. (**b**) The mRNA levels of IL-1β and IL-6 in the astrocytes were measured with real-time PCR. (**c**) The protein levels of IL-1β and IL-6 in the supernatant of astrocytes were determined by ELISA. *$P < 0.05$; **$P < 0.01$ versus the 0 h time point. The results from one representative experiment out of three are shown. Data are expressed as the means ± s.d. ($n = 3$ in each time point).

to IL-17 (Supplementary Figure 3), indicating that IL-17 induces the expression of these genes in astrocytes through its receptor IL-17RA.

HIF-1α mediates the production of IL-1β and IL-6 in astrocytes in response to IL-17

Since our present studies revealed that IL-17 stimulation *in vitro* could induce the expression of HIF-1α, IL-1β and IL-6 in primary mouse astrocytes, and HIF-1α was reported to mediate the production of pro-inflammatory cytokines,[34–36] overexpression and knockdown experiments were further performed to explore the effects of HIF-1α on IL-1β and IL-6 expression in primary mouse astrocytes exposed to IL-17. The results showed that HIF-1α overexpression obviously enhanced IL-1β and IL-6 production in astrocytes induced by IL-17, and HIF-1α gene silencing markedly decreased IL-1β and IL-6 production in the astrocytes stimulated with IL-17 (Figures 3a–d). These data indicate that IL-17-mediated upregulation of HIF-1α contributes to the induction of IL-1β and IL-6 in astrocytes.

HIF-1α directly promotes gene transcription of IL-1β and IL-6 in astrocytes exposed to IL-17

Reportedly, HIF-1α is able to trigger gene transcription by binding to the hypoxia response element (HRE) sequence (R'CGTG) within the promoter of target genes.[37,49] Therefore, to clarify the mechanism by which HIF-1α upregulates IL-1β and IL-6 expression, luciferase experiments were performed to assess the effects of HIF-1α on IL-1β and IL-6 gene promotion in HEK293T cells. The results showed that HIF-1α overexpression markedly enhanced the transcription of IL-1β and IL-6 genes (Figure 3e). Since TFsearch predicted that two HRE elements and one HRE element were, respectively, located in the promoters of IL-1β and IL-6 genes, a ChIP assay was further designed to discover whether HIF-1α protein can bind to the promoters of these two genes. As we expected, HIF-1α protein bound to the HRE element-containing regions within IL-1β and IL-6 gene promotors (Figure 3f and Supplementary Figure 4). These results indicate that IL-17-mediated upregulation of HIF-1α could activate both IL-1β and IL-6 genes through binding to the promotors of these two genes.
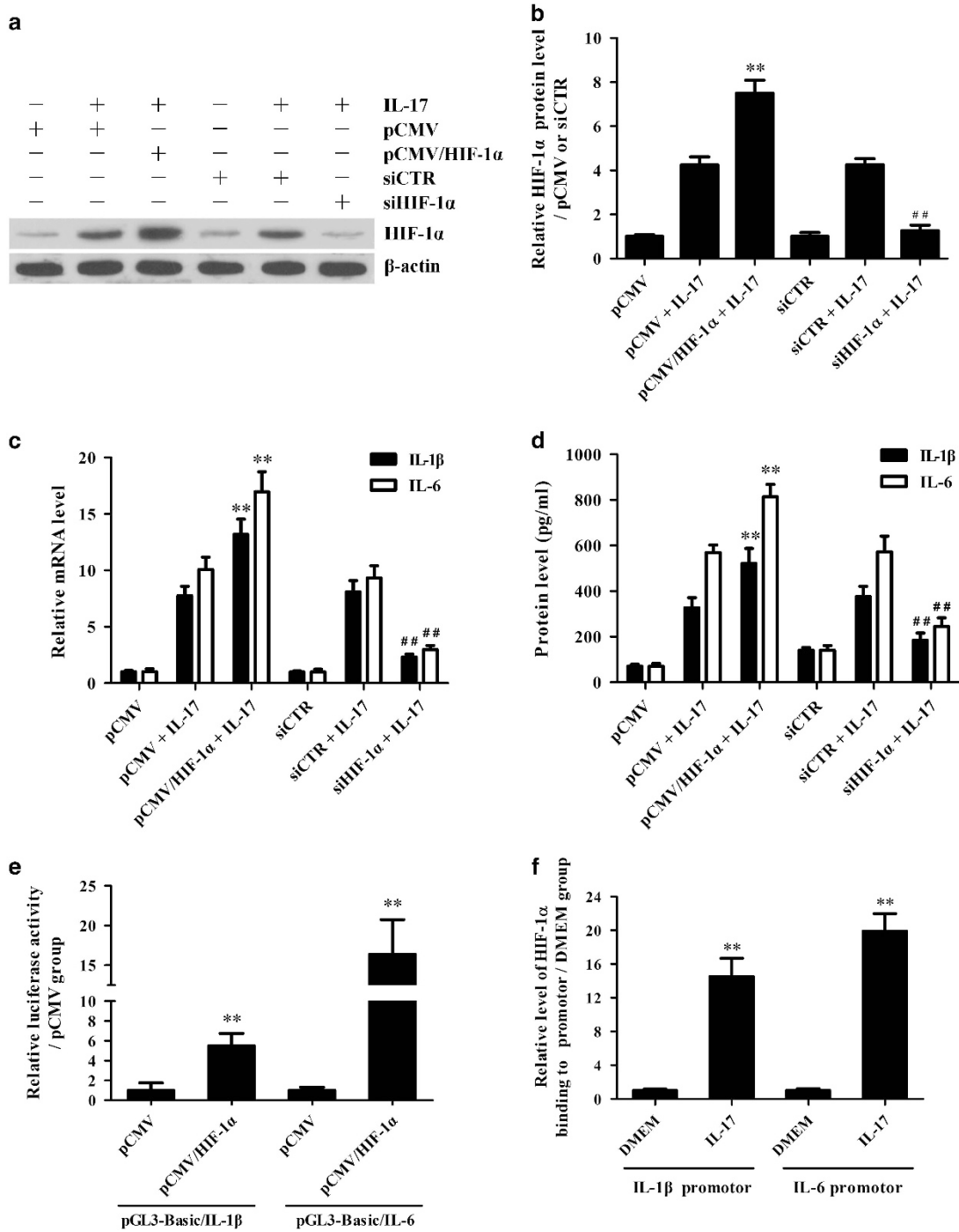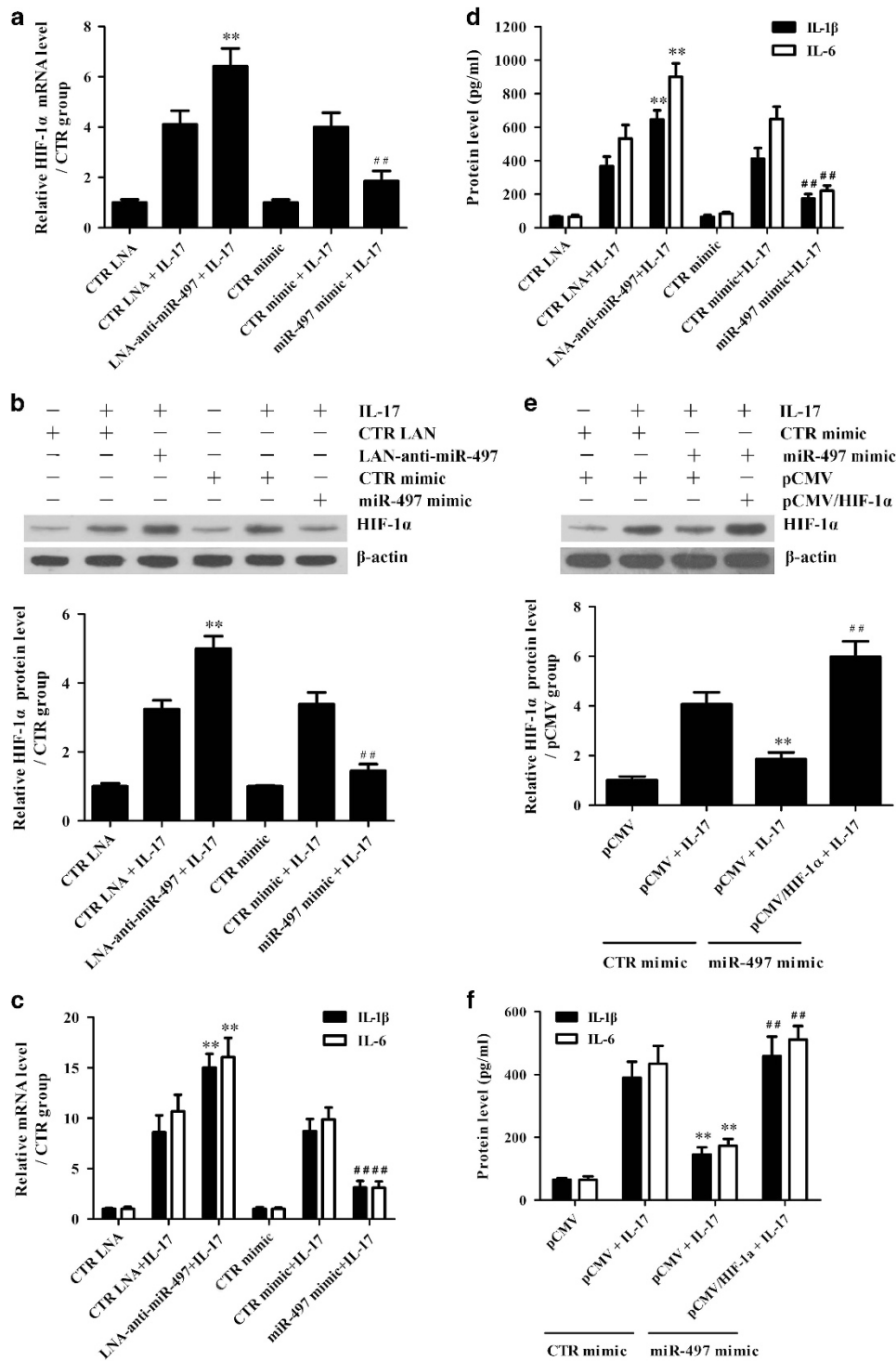
Figure 3 The effects of hypoxia inducible factor-1α (HIF-1α) on interleukin (IL)-1β and IL-6 production in astrocytes exposed to IL-17. (**a**–**d**) Primary mouse astrocytes were transfected with pCMV/HIF-1α, pCMV, siHIF-1α and siCTR, separately, for 48 h and then treated with or without IL-17 for 3 and 6 h. The protein level of HIF-1α (**a**, **b**) in the astrocytes 3 h after IL-17 stimulation was measured with western blotting. In addition, the mRNA levels of IL-1β and IL-6 in the astrocytes at 3 h (**c**) as well as the protein levels of IL-1β and IL-6 in the supernatant of astrocytes at 6 h (**d**) were analyzed separately with real-time PCR and ELISAs. **P<0.01 versus the pCMV+IL-17 group; ##P<0.01 versus the siCTR+IL-17 group. (**e**) HEK293T cells were transfected with pGL3-Basic/IL-1β or pGL3-Basic/IL-6 accompanied by pCMV/HIF-1α, pCMV, siHIF-1α or siCTR. The luciferase activity of IL-1β and IL-6 promoters was detected 48 h after transfection. **P<0.01 versus pCMV. (**f**) Primary mouse astrocytes were cultured with or without IL-17 for 3 h. A ChIP assay was performed with anti-HIF-1α antibody to pull down the DNA–protein complex, and then immunoprecipitated DNA was amplified using real-time PCR with two pairs of primers for the proximal promoters of the IL-1β or IL-6 gene. **P<0.01 versus the DMEM group. The results from one representative experiment out of three are shown. Data are presented as the means±s.d. (n=3 in each group).

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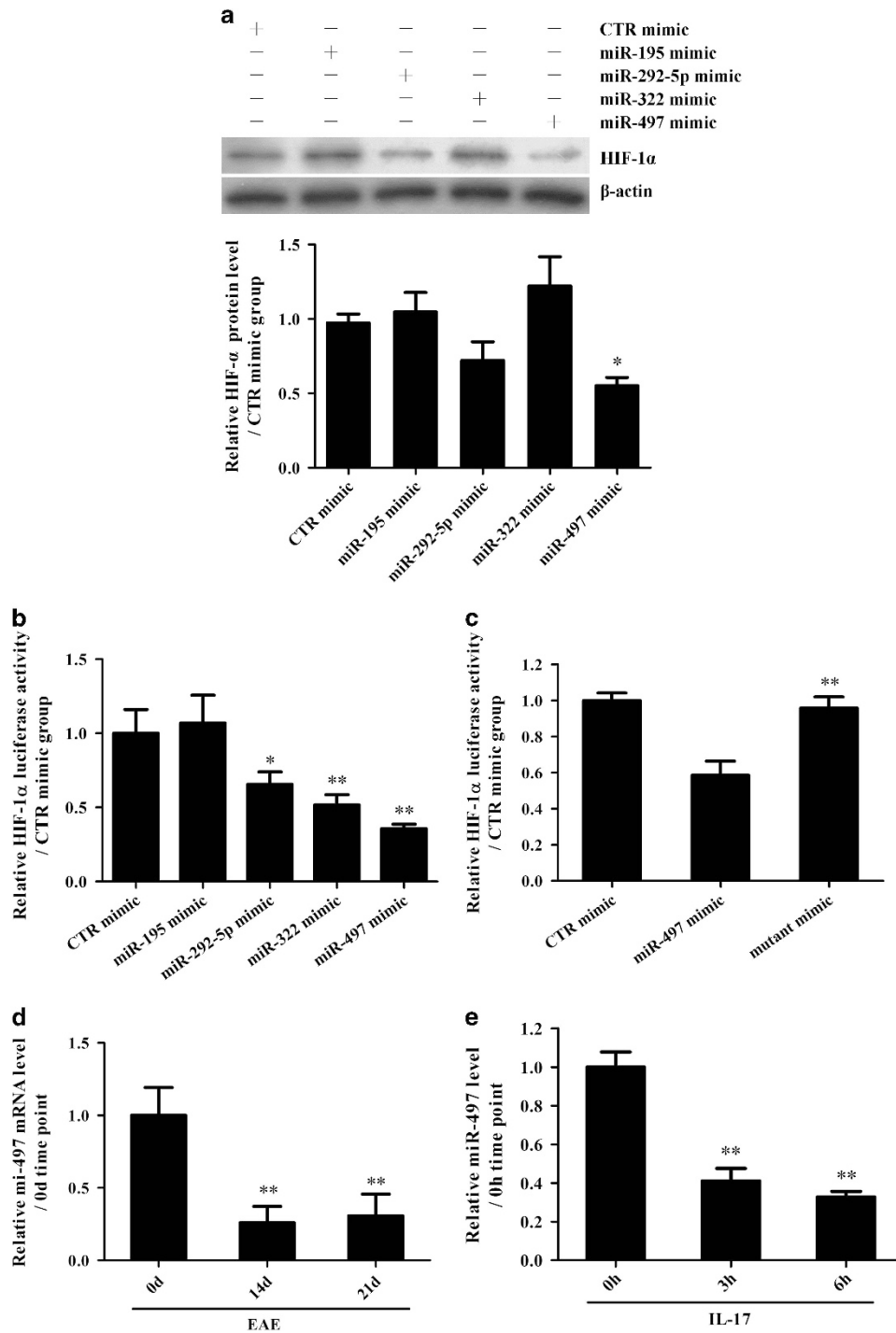
Figure 4 Effects of potential miRNAs on hypoxia inducible factor-1α (HIF-1α) in interleukin (IL)-17-stimulated astrocytes. (**a**) Primary mouse astrocytes were transfected with different mimics of miR-195, miR-292-5p, miR-322, miR-497 and CTR, and the protein level of HIF-1α in the astrocytes was detected with western blotting 48 h after transfection. *$P<0.05$ versus the CTR mimic group. (**b**) HEK293T cells were transfected with a mixture of pGL3-Promoter/HIF-1α and pRL-SV40 as well as one of the miRNA mimics (miR-195, miR-292-5p, miR-322, miR-497 or CTR). Subsequently, the luciferase activity of the HIF-1α 3′UTR reporter was measured 48 h after transfection. *$P<0.05$; **$P<0.01$ versus the CTR mimic group. (**c**) HEK293T cells were transfected with a mixture of pGL3-Promoter/HIF-1α and pRL-SV40 as well as a miR-497 mimic or miR-497 mutant mimic. Then, the luciferase activity of the HIF-1α 3′UTR reporter was examined 48 h after transfection. **$P<0.01$ versus the miR-497 mimic group. (**d**, **e**) Real-time PCR was used to measure the level of miR-497 in the brain tissues of mice on 0 d, 14 d and 21 d after experimental autoimmune encephalomyelitis (EAE) induction (**d**, **$P<0.01$ versus the 0 d time point) or in the astrocytes at 0, 3 and 6 h after IL-17 stimulation (**e**, **$P<0.01$ versus the 0 h time point). The results from one representative experiment out of three are shown. Data are shown as the means±s.d. ($n=3$ in each group).

Figure 5 The role of miR-497 in the expression of hypoxia inducible factor-1α (HIF-1α), interleukin (IL)-1β and IL-6 in astrocytes stimulated with IL-17. Primary mouse astrocytes were transfected with miR-497 mimic, CTR mimic, LNA-anti-miR-497 or CTR LNA for 48 h and then incubated with or without IL-17 for 3 and 6 h. (**a**, **b**) The mRNA and protein levels of HIF-1α were determined with real-time PCR (**a**) and western blotting (**b**) respectively, 3 h after IL-17 stimulation. **$P<0.01$ versus the CTR LNA+IL-17 group; ##$P<0.01$ versus the CTR mimic +IL-17 group. (**c**, **d**) The mRNA and protein levels of IL-1β and IL-6 were analyzed at 3 h in the astrocytes treated with IL-17 (**c**, real-time PCR) and at 6 h in the supernatant of astrocytes stimulated with IL-17 (**d**, ELISA). **$P<0.01$ versus the CTR LNA+IL-17 group; ##$P<0.01$ versus the CTR mimic+IL-17 group. (**e**, **f**) Primary mouse astrocytes were transfected with CTR mimic, miR-497 mimic, pCMV and pCMV/HIF-1α separately or in combination for 48 h and then cultured with or without IL-17 for 3 and 6 h. Finally, the protein levels of HIF-1α in the astrocytes (**e**, 3 h) as well as IL-1β and IL-6 in the supernatant of astrocytes (**f**, 6 h) were measured using western blotting and ELISAs, respectively. **$P<0.01$ versus the CTR mimic+pCMV+IL-17 group; ##$P<0.01$ versus the miR-497 mimic+pCMV+IL-17 group. The results from one representative experiment out of three are shown. Data are presented as the means±s.d. ($n=3$ in each group).

Identification of downregulated miRNAs targeting HIF-1α mRNA

Our previous study reported changes in miRNA expression both in the brain tissues of EAE mice (*in vivo*) and in IL-17-treated astrocytes (*in vitro*).[44] Since downregulation of miRNAs against HIF-1α mRNA is supposed to increase HIF-1α expression, a bioinformatics assay was used to find the potential downregulated miRNAs involved in HIF-1α upregulation in astrocytes stimulated with IL-17 in EAE mice, and, finally, four downregulated miRNAs (miR-195, miR-292-5p, miR-322 and miR--497) were predicted to bind to the 3′UTR of HIF-1α mRNA.

To further find which downregulated miRNA is possibly responsible for HIF-1α upregulation, overexpression experiments were performed through transfection of different miRNA mimics. We observed that although both miR-292-5p and miR-497 mimics decreased HIF-1α expression, the miR-497 mimic had a more significant inhibitory role (Figure 4a). A luciferase activity assay showed that the mimics of miR-292-5p, miR-322 and miR-497, especially miR-497, directly targeted the 3′UTR of HIF-1α mRNA (Figure 4b). Furthermore, a miR-497 mimic with a mutant seed-region did not target the 3′UTR of HIF-1α mRNA (Figure 4c), indicating that the inhibitory effect of the miR-497 mimic is sequence specific. Subsequent assays confirmed that miR-497 expression was markedly downregulated both *in vivo* (Figure 4d) and *in vitro* (Figure 4e), while IL-17RA knockdown obviously upregulated miR-497 expression in primary mouse astrocytes exposed to IL-17 (Supplementary Figure 5). Notably, Lindberg *et al.*[50] reported that miR-497 expression was reduced in the peripheral blood lymphocytes of MS patients, indicating that miR-497 downregulation might contribute to CNS damage in MS patients. However, the role of miR-497 in the pathological CNS changes during MS, including IL-17-inducing pro-inflammatory cytokine production and its regulatory mechanism, remain largely unclear. Therefore, further functional studies on miR-497 were performed both in astrocytes exposed to IL-17 and in the EAE mouse model.

Downregulation of miR-497 enhances production of IL-1β and IL-6 through upregulation of HIF-1α expression in astrocytes induced by IL-17

To study whether miR-497 downregulation promotes IL-1β and IL-6 production through HIF-1α upregulation in IL-17-stimulated astrocytes, LNA-anti-miR-497 (as a miR-497-specific inhibitor) and miR-497 mimic were transfected into primary mouse astrocytes, separately, and then, HIF-1α, IL-1β and IL-6 expression was detected after IL-17 stimulation. The expression of miR-497 was clearly downregulated by LNA-anti-miR-497 and upregulated by miR-497 mimic (Supplementary Figure 6). Furthermore, LNA-anti-miR-497 enhanced IL-17-induced HIF-1α, IL-1β and IL-6 expression in the astrocytes, while the miR-497 mimic exhibited the opposite effects (Figures 5a–d). More importantly, HIF-1α overexpression in the astrocytes reversed the IL-6 and IL-1β downregulation caused by the miR-497 mimic in IL-17-stimulated astrocytes (Figures 5e and f), confirming the upstream and downstream

relationship between miR-497 and HIF-1α. Taken together, our *in vitro* studies indicate that IL-17-mediated downregulation of miR-497 induces high HIF-1α expression and promotes IL-1β and IL-6 production via HIF-1α in astrocytes.
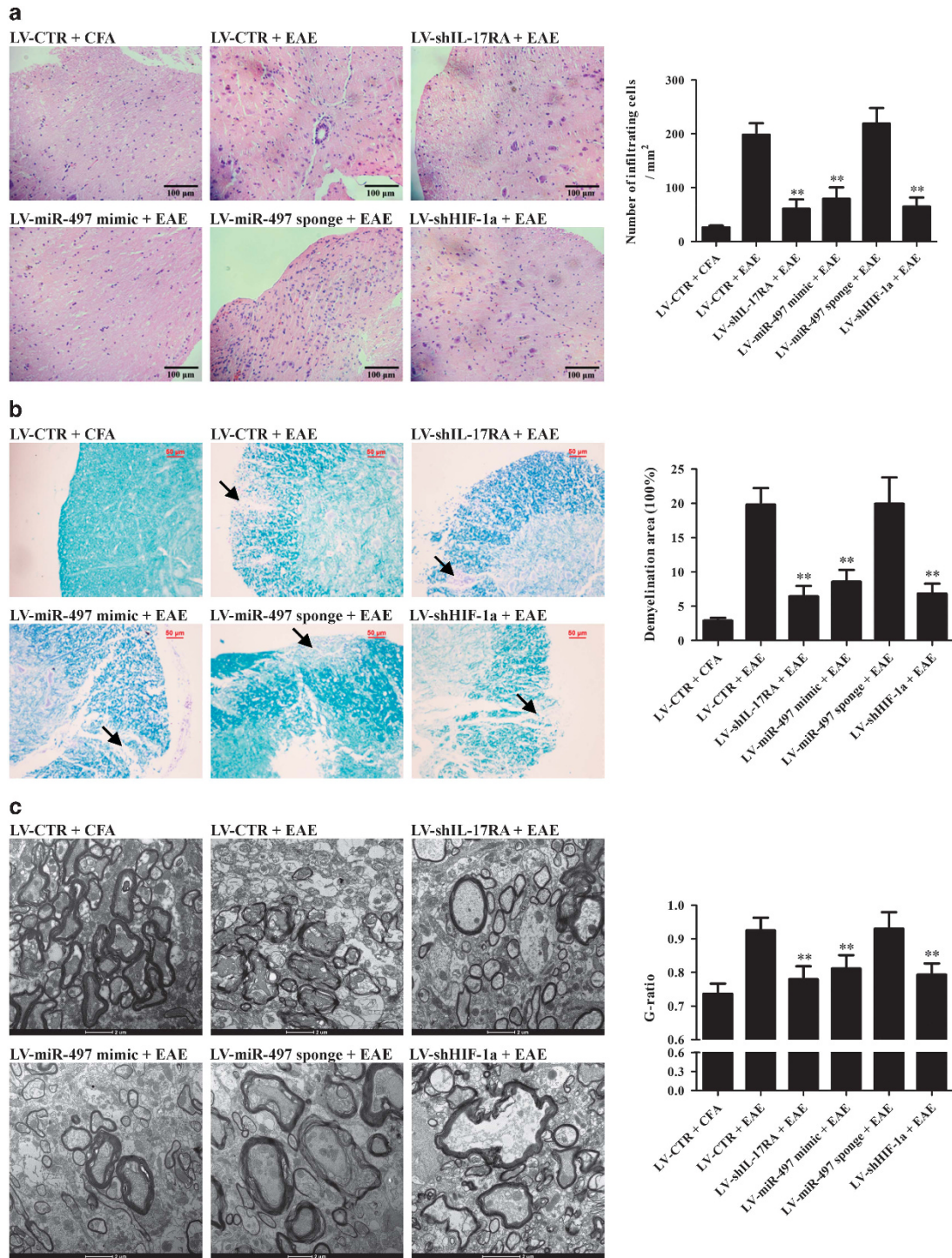
The IL-17-miR-497-HIF-1α axis contributes to the production of IL-1β and IL-6, as well as to pathological changes and behavior of EAE mice

To verify the roles of the IL-17-miR-497-HIF-1α axis in the production of IL-1β and IL-6 in astrocytes, as well as the pathological changes and behavior of EAE mice, corresponding *in vivo* experiments were subsequently performed. Different astrocyte-specific lentiviral vectors encoding shIL-17RA, miR-497 mimic, miR-497 sponge, shHIF-1α and shCTR were used to overexpress or knockdown corresponding target genes in mouse astrocytes *in vivo*. EAE was induced on day 7 after lentivirus injection, and the mice were killed on day 20 after EAE induction. Mice infected with LV-shIL-17RA, LV-miR-497 mimic and LV-shHIF-1α exhibited significantly decreased HIF-1α, IL-1β and IL-6 expression (Figures 6a–c) as well as lymphocyte infiltration and demyelination in the brain tissues and spinal cords (Figures 7a–c). LV-miR-497 mimic, LV-miR-497 sponge and LV-shHIF-1α had no effect on IL-17RA expression (Figure 6d). In addition, the mice were scored according to the degree of paralysis from day 7 to day 20 after EAE induction, and the mice infected with LV-shIL-17RA, LV-miR-497 mimic and LV-shHIF-1α exhibited much lower scores than the mice infected with LV-miR-497 sponge and LV-CTR (Figure 6e). Our *in vivo* experiments indicated that IL-17-mediated downregulation of miR-497 increases HIF-1α expression and IL-1β and IL-6 production in astrocytes and aggravates CNS inflammation in EAE mice.

DISCUSSION

As a critical pro-inflammatory cytokine, IL-17 is involved in various chronic autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis (RA) and MS.[7,51,52] In EAE mice, IL-17 can aggravate the damage of the CNS by promoting inflammatory responses in multiple types of cells.[53,54] Astrocytes, which can secrete various inflammatory cytokines, have been shown to play an indispensable role in the pathogenesis of EAE.[25,55,56] Our present studies demonstrated that the production of IL-17, HIF-1α, IL-1β and IL-6 was increased in the brain tissues of EAE mice in a time-dependent manner, with a relatively consistent time point tendency. Furthermore, IL-17 stimulation *in vitro* markedly upregulated IL-1β and IL-6 synthesis in primary mouse astrocytes, and IL-17RA knockdown clearly downregulated IL-1β and IL-6 production. In addition, our *in vivo* experiment showed that knockdown of IL-17RA in astrocytes with LV-shIL-17RA markedly decreased IL-1β and IL-6 expression and reduced lymphocyte infiltration and demyelination in the brain tissues or spinal cords of EAE mice. These data indicate that IL-17 can trigger the production of IL-1β and IL-6 in astrocytes and promote CNS damage in EAE mice, and blockade of the IL-17

Figure 6 The roles of the interleukin (IL)-17–miR-497–HIF-1α axis in the production of IL-1β and IL-6 as well as in the behavior of experimental autoimmune encephalomyelitis (EAE) mice. Mice were injected with different recombinant astrocyte-specific lentiviruses (LV-CTR, LV-shIL-17RA, LV-miR-497 mimic, LV-miR-497 sponge, LV-shHIF-1a) for 7 days and then immunized with MOG (35–55) for 20 days (*n*=6 each group). (**a**) The miR-497 level in the brain tissues of mice was detected with real-time PCR on day 20 after EAE induction. (**b**–**d**) The protein levels of IL-17RA, HIF-1a, IL-1β and IL-6 in the brain tissues of mice on day 20 after EAE establishment were measured using western blotting (**b**, HIF-1a; **d**, IL-17RA) and ELISAs (**c**, IL-1β and IL-6). (**e**) The EAE score was evaluated from day 7 to day 20. **P<0.01 versus the LV-CTR+EAE group; ^{NS}*P*>0.05 versus the LV-CTR+EAE group. The results from one representative experiment out of two are shown. Data are expressed as the means±s.d.

Figure 7 The roles of the interleukin (IL)-17–miR-497–HIF-1α axis in the pathological changes in experimental autoimmune encephalomyelitis (EAE) mice. Mice in different groups ($n = 6$ each group) were treated as mentioned in Figure 6. The histopathology of mouse brain tissues and spinal cords was observed using light microscopy (**a**, **b**) and electron microscopy (**c**) on day 20 after EAE induction. (**a**) H&E staining (original magnification: ×200) was performed to evaluate the inflammatory cell infiltration into brain tissues. The number of infiltrated inflammatory cells in the mice of the LV-shIL-17RA+EAE, LV-miR-497 mimic+EAE and LV-shHIF-1a+EAE groups was less than that of the LV-CTR+EAE and LV-miR-497 sponge+EAE groups. (**b**) LFB staining (original magnification: ×200) was used to observe pathological changes in spinal cords. The demyelination degree in the mice of the LV-shIL-17RA+EAE, LV-miR-497 mimic+EAE and LV-shHIF-1a+EAE groups was less than that in the mice of the LV-CTR+EAE and LV-miR-497 sponge+EAE groups. Arrows indicate areas of severe demyelination. (**c**) The spinal cords in the mice of the LV-shIL-17RA+EAE, LV-miR-497 mimic+EAE and LV-shHIF-1a+EAE groups viewed with EM exhibited reduced disruption or mild loosening of the medullary sheath and lower G-ratio compared with the mice of the LV-CTR+EAE and LV-miR-497 sponge+EAE groups. The results from one representative experiment out of two are shown. Data are expressed as the means ± s.d. **$P < 0.01$ versus the LV-CTR+EAE group.

signal by shIL-17RA can decrease the expression of IL-6 and IL-1β both *in vitro* and *in vivo*.

HIF-1α has been confirmed to play a regulatory role in the inflammatory response, and most investigations have focused on the involvement of HIF-1α in downstream cytokine signaling.[57,58] However, there is little research concerning the activity of HIF-1α in regulating pro-inflammatory cytokine production at the transcriptional level through binding to the core DNA sequence 5'-[A/G]CGTG-3' within the HRE of promoters.[34–36] Because upregulation of HIF-1α is involved in pathological CNS changes in both MS patients and EAE model mice,[59–61] studies were designed to explore the underlying molecular mechanism by which HIF-1α regulates IL-17-induced synthesis of IL-1β and IL-6 in astrocytes of EAE mice. Our present experiments revealed that the production of HIF-1α was increased in brain tissues of mice with EAE and in cultured astrocytes stimulated with IL-17. Overexpression and knockdown experiments *in vitro* demonstrated that IL-17-mediated upregulation of HIF-1α promoted the expression of IL-1β and IL-6 genes in the astrocytes. Meanwhile, we determined that HIF-1α could bind to the HRE element within the IL-1β and IL-6 gene promotor and then directly trigger the transcription of these two genes in astrocytes. In addition, astrocyte-specific knockdown of HIF-1α *in vivo* obviously inhibited the expression of IL-1β and IL-6 in the CNS of EAE mice. Collectively, our *in vivo* and *in vitro* studies indicate that IL-17-induced HIF-1α promotes the production of IL-1β and IL-6 in astrocytes of EAE mice.

Many miRNAs such as miR-23b, miR-124 and miR-326 have been demonstrated to play roles in EAE.[62–64] Our previous study reported changes in miRNA expression profiles both in brain tissues of EAE mice (*in vivo*) and in IL-17-treated astrocytes (*in vitro*), and we found that upregulation of miR-873 is involved in the pathogenesis of EAE by targeting A20 ubiquitin-editing enzyme.[44] However, in addition to miRNA upregulation, microRNA downregulation also plays pathogenic roles in several autoimmune diseases.[65] Given that HIF-1α expression was increased both *in vivo* and *in vitro*, a subsequent bioinformatics assay was used to predict which downregulated microRNA could target HIF-1α mRNA. The results showed that four miRNAs (miR-195, miR-292-5p, miR-322 and miR-497) among the downregulated miRNAs in the expression profile were predicted to bind to the 3′UTR of HIF-1α mRNA. Further function experiments confirmed that only a miR-497 mimic could target the 3′UTR of HIF-1α mRNA and markedly suppress the expression of HIF-1α, IL-1β and IL-6 in astrocytes. In contrast, LNA-anti-miR-497 enhanced the production of HIF-1α, IL-1β and IL-6 in astrocytes induced by IL-17. Moreover, HIF-1α overexpression was able to reverse the reduction in IL-6 and IL-1β caused by the miR-497 mimic in astrocytes upon IL-17 stimulation. Furthermore, our *in vivo* studies also revealed that astrocyte-specific miR-497 overexpression reduced the expression of HIF-1α, IL-1β and IL-6 as well as the infiltration of lymphocytes and demyelination in the brain tissues of EAE mice. It is worth mentioning that LV-miR-497 sponge treatment did not

aggravate EAE pathological changes *in vivo* as we expected, although LNA-anti-miR-497 did enhance IL-17-induced production of IL-1β and IL-6 in astrocytes *in vitro*. We think that this difference might be interpreted by considering that the downregulation of miR-497 *in vivo* was much more significant than that *in vitro*, and if miR-497 had been downregulated to a very low level *in vivo*, the miR-497 sponge could not reduce miR-497 expression to a lower level. Our present studies indicate that IL-17-triggered downregulation of miR-497 results in high HIF-1α expression and consequent IL-1β and IL-6 production by astrocytes in EAE mice. Notably, a recent study has revealed that miR-497 expression is decreased in the peripheral blood lymphocytes of MS patients.[50] Therefore, miR-497 downregulation could contribute to CNS damage in MS patients, and thus, miR-497 might be a new therapeutic target in clinical trials.

In summary, our current studies revealed that the production of IL-17, HIF-1α, IL-1β and IL-6 was markedly increased in the brain tissues of EAE mice (*in vivo*), and the production of HIF-1α, IL-1β and IL-6 in cultured primary mouse astrocytes was also greatly induced by IL-17 stimulation (*in vitro*). Meanwhile, the expression of miR-497 was decreased both *in vivo* and *in vitro*. In addition, *in vitro* experiments showed that IL-17 could induce the expression of IL-1β and IL-6 genes in astrocytes through upregulation of HIF-1α as a transcriptional factor. Furthermore, IL-17 promoted HIF-1α-mediated production of IL-1β and IL-6 via miR-497 downregulation in astrocytes, indicating that miR-497 downregulation could induce high HIF-1α expression. Moreover, our experiments *in vivo* demonstrated that astrocyte-specific knockdown of IL-17RA or HIF-1α or astrocyte-specific overexpression of miR-497 could reduce IL-1β and IL-6 production in brain tissues and alleviate the pathological changes and score of EAE mice. These results not only confirm the above-mentioned molecular mechanism discovered *in vitro* but also reveal the pathogenetic role of the IL-17-miR-497-HIF-1α axis in mouse EAE and provide new insight into the pathogenesis of human MS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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