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Modulating of ocular inflammation with macrophage migration inhibitory factor is associated with notch signalling in experimental autoimmune uveitis

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Summary

The aim of this study was to examine whether macrophage migration inhibitory factor (MIF) could exaggerate inflammatory response in a mouse model of experimental autoimmune uveitis (EAU) and to explore the underlying mechanism. Mutant serotype 8 adeno-associated virus (AAV8) (Y733F)-chicken β-actin (CBA)-MIF or AAV8 (Y733F)-CBA-enhanced green fluorescent protein (eGFP) vector was delivered subretinally into B10.RIII mice, respectively. Three weeks after vector delivery, EAU was induced with a subcutaneous injection of a mixture of *interphotoreceptor retinoid binding protein* (IRBP) peptide with CFA. The levels of proinflammatory cytokines were detected by real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). Retinal function was evaluated with electroretinography (ERG). We found that the expression of MIF and its two receptors CD74 and CD44 was increased in the EAU mouse retina. Compared to AAV8.CBA.eGFP-injected and untreated EAU mice, the level of proinflammatory cytokines, the expression of Notch1, Notch4, delta-like ligand 4 (Dll4), *Notch receptor intracellular domain* (NICD) and hairy enhancer of split-1 (Hes-1) increased, but the ERG a- and b-wave amplitudes decreased in AAV8.CBA.MIF-injected EAU mice. The Notch inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) reduced the expression of NICD, Hes-1 and proinflammatory cytokines. Further, a MIF antagonist ISO-1 attenuated intraocular inflammation, and inhibited the differentiation of T helper type 1 (Th1) and Th17 in EAU mice. We demonstrated that over-expression of MIF exaggerated ocular inflammation, which was associated with the activation of the Notch signalling. The expression of both MIF and its receptors are elevated in EAU mice. Over-expression of MIF exaggerates ocular inflammation, and this exaggerated inflammation is associated with the activation of the Notch signalling and Notch pathway. Our data suggest that the MIF–Notch axis may play an important role in the pathogenesis of EAU. Both the MIF signalling pathways may be promising targets for developing novel therapeutic interventions for uveitis.

Keywords: EAU, inflammation, MIF, Notch, retina

Introduction

Uveitis, a group of intraocular inflammatory diseases, causes retinal damage and in severe cases leads to blindness [1]. Experimental autoimmune uveitis (EAU) induced with human interphotoreceptor retinoid-binding protein $(IRBP)_{161–180}$ peptide in B10.RIII mice is an established rodent model of human autoimmune uveitis. EAU is mediated by $CD4^+$ T cells [2]. Interferon (IFN)-γ-producing T helper type 1 (Th1) and IL-17-producing Th17 cells drive ocular autoimmunity in EAU [3].

Macrophage migration inhibitory factor (MIF) is an upstream activator of innate immunity that regulates subsequent adaptive responses [4]. MIF is a proinflammatory cytokine that plays an important role in regulating macrophage

function and T cell activation [4], and also up-regulates Toll-like receptor 4 (TLR-4) expression [5]. MIF has a reciprocal relationship with glucocorticoids, which are used commonly in the treatment of uveitis to relieve the intraocular inflammation. MIF expression is induced by glucocorticoids [6], but it exerts glucocorticoid antagonistic proinflammatory effects [7] and regulates glucocorticoid sensitivity [8]. MIF is a crucial mediator of several immunoinflammatory disorders, including experimental autoimmune encephalomyelitis (EAE) [9], experimental autoimmune myocarditis [10], rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and atherosclerosis [11]. It has been reported that patients with uveitis exhibit prominent increases in MIF within their sera and ocular fluids [12–14]. Moreover, we demonstrated recently that the MIF gene has a strong association with Behçet's disease (BD) [15] and Vogt–Koyanagi–Harada (VKH) syndrome [16], two common forms of autoimmune uveitis.

Accumulating evidence suggests that Notch plays an important role in the pathogenesis of various autoimmune disorders [17,18], and it is a vital modulator of T cell-mediated immune diseases [17]. Numerous studies have demonstrated that Notch signalling is involved in the regulation of the immune response and is critical for the development of EAU [19], EAE [20] and RA [21]. Notch signalling is initiated by ligand engagement of Notch receptors. To date, four Notch receptors (Notch 1–4) and five of their ligands (delta-like 1, 3, 4 and Jagged 1, 2) have been identified in mammals. Upon ligand binding, the intracellular domain (ICD) of the receptor is cleaved proteolytically by γ-secretase and translocates into the nucleus, where it associates with the RBP–Jk transcription factor and regulates the expression of various target genes, including members of the hairy enhancer of split (Hes) and hairy-related (Hey and Hrt) gene families.

However, although increasing evidence indicates a pivotal role of MIF in autoimmune diseases [22], its role and underlying mechanism in the pathogenesis of EAU remains unclear. To gain a better understanding of the molecular mechanisms of MIF in EAU, we analysed the status of the Notch signalling pathway during the intervention of MIF. We hypothesized that MIF was critical in modulating intraocular inflammation in EAU, and the MIF–Notch axis may be involved in the pathogenesis of this disease.

Recent studies have shown that the serotype 8 adeno-associated virus (AAV8) vector-mediated gene therapy was effective in restoring retinal structure and function in animal models of retinal degeneration, which were shown previously to be refractory to AAV2 or AAV5 vectors [23,24]. Investigators attributed the high efficacy to rapid onset of transgene expression mediated by AAV8 relative to other serotypes [25]. AAV8, containing a single point mutation (Y733F) in a surface-exposed tyrosine residue, confers earlier onset and stronger transgene expression in photoreceptor cells than the standard AAV8 [26]. We have applied the AAV8-Y733F vector to over-express the MIF gene in this study.

Materials and methods

Ethics statement

This study was carried out according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. Every effort was made to minimize animal discomfort and stress.

Animals and reagents

Male and female B10.RIII mice at 6–8 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed under specific pathogen-free conditions. Human interphotoreceptor retinoid binding protein peptide spanning amino acid residues 161–180 (IRBP161–180, SGIPYII-SYLHPGNTILHVD) was synthesized by Shanghai Sangon Biological Engineering Technology and Services Co Ltd. The MIF antagonist, ISO-1, was purchased from Calbiochem (Billerica, MA, USA). ISO-1 was administered in sterile 1% dimethylsulphoxide (DMSO)/phosphate-buffered saline (PBS) at a dose of 20 mg/kg daily by intraperitoneal (i.p.) injection. Control mice received vehicle alone. N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Selleck Chemicals, Houston, TX), an inhibitor of Notch signalling, was diluted from stock solutions in DMSO to yield a final concentration to 1% DMSO in PBS, and 5 mg/kg DAPT or vehicle was administered daily via the intraperitoneal route.

Recombinant adeno-associated virus construction and packaging

Two adeno-associated viral vectors (AAVs), AAV8-chicken β-actin promoter (CBA)-MIF and AAV8-CBA-enhanced green fluorescent protein (eGFP), were constructed exactly as detailed previously [27]. These constructs contained expression cassettes flanked by the rAAV8 terminal repeats. Expression of eGFP and MIF was driven by a CBA with a human cytomegalovirus enhancer. Site-directed mutagenesis of surface-exposed tyrosine residues on the capsids to generate AAV serotype 8 Y733F mutation has been described recently [26]. Vector plasmid was packaged in AAV serotype 8 Y733F by transfection of H293 cells according to previously published methods [28]. Vector doses were expressed as genome copies.

Subretinal injections

One μl of AAV8 (Y733F).CBA.eGFP or AAV8 (Y733F). CBA.MIF (1×10^{11} vector genome copies/ml) was injected

subretinally into one eye according to a method described previously [29]. All procedures were performed under sterile conditions. Under a dissecting microscope, an aperture within the dilated pupil area was made through the cornea with a 30-gauge needle, and a blunt 33-gauge needle was inserted through the corneal opening, avoiding damage to the lens and penetrating the neuroretina. The injected retinal area was visualized by fluorescein-positive subretinal blebs demarcating the retinal detachment. Such detachments usually resolved within 1 to 2 days. All animals received antibiotic ointment to the cornea and were observed daily after operation.

Induction and histopathology of EAU

EAU was made following a previous protocol [30,31]. Briefly, mice were immunized subcutaneously at the base of the tail and both thighs with 50 μg human $IRBP_{161–180}$ peptide in 100 ml PBS, emulsified 1 : 1 v/v in complete Freund's adjuvant (CFA) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 1·0 mg/ml *Mycobacterium tuberculosis* strain (MTB). A total of 200 μl emulsion was administered in one mouse.

Eyeballs were enucleated at day 14 after a mixture of IRBP peptide with CFA immunization and were fixed in 4% buffered paraformaldehyde for 1 h at room temperature. Serial 4–6 μm sections were collected through the pupillary–optic nerve axis and stained with haematoxylin and eosin (H&E). At least four sections of each eye were evaluated histologically. The severity of EAU was graded in a masked fashion on a scale of 0–4, as described earlier [31].

Western blotting analysis

Western blotting was performed as described previously [31]. Retinas were dissected from enucleated eyeballs at day 14 after immunization. Protein was extracted in radio immunoprecipitation assay buffer (RIPA; Beyotime, Shanghai, China) including 1% protease inhibitor (Beyotime, Shanghai, China). Equal amounts of protein (50 μg) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, using 10% polyacrylamide gels. The resolved proteins were electroblotted onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk and incubated with specific primary antibodies against MIF (1 : 1500, Abcam, Cambridge, UK), Notch receptor intracellular domain (NICD) (1 : 500; Abcam) and hairy enhancer of split (Hes) (1 : 1000; Abcam) overnight at 4°C. Blots were washed and incubated with a secondary antibody for 1 h at 37°C. Bands were analysed using Image J software version 1·43 (Broken Symmetry Software, Bethesda, MD, USA). Analysis was normalized against a housekeeping β-actin (1 : 100, Abcam) protein. The measurements were repeated three times in each experiment.

Real-time PCR analysis

Total RNA was isolated from mouse retinas using the Trizol Reagent (Ambion, Carlsbad, CA, USA) [31]. For quantitative polymerase chain reaction (PCR), total RNA was reverse-transcribed using RT Primer Mix and oligo dT primers (Takara, Dalian, China). The cDNA was quantified by the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using primers specific for mouse. The sequences and accession numbers of the primers are shown in Table 1. PCR amplification was performed in a volume of 20 μl, using all-in-one quantitative PCR (qPCR) Mix (ABI, Carlsbad, CA, USA). The conditions were 95°C for 10 min, followed by 40 cycles of 10 s at 95°C, 20 s at

Table 1. Sequences and accession numbers of primers for real-time polymerase chain reaction (PCR)

Gene	Forward	Reverse	Accession number
MIF	5′-CCATGCCTATGTTCATCGTG-3′	5′-GAACAGCGGTGCAGGTAAGTG-3′	NM_010798.2
CD74	5′-ATGACCCAGGACCATGTGAT-3′	5′-CCAGGGAGTTCTTGCTCATC-3′	NM_010545.3
CD44	5′-TTACCCACCATTGACCAAAT-3′	5′-GGTCACTCCACTGTCCTGGT-3′	NM_001039150.1
TNF-α	5′-GCCTCTTCTCATTCCTGCTT-3′	5′-CTCCTCCACTTGGTGGTTTG-3′	NM_013693.2
IL-1β	5′-TCCTTGTGCAAGTGTCTGAAGC-3′	5′-ATGAGTGATACTGCCTGCCTGA-3′	NM_008361.3
IL-6	5′-TCGGCAAACCTAGTGCGTTA-3′	5′-CCAAGAAACCATCTGGCTAGG-3′	NM_031168.1
IL-17	5′-ACCGCAATGAAGACCCTGAT-3′	5′-TCCCTCCGCATTGACACA-3′	NM_002190.2
IFN-γ	5′-AGAGGATGGTTTGCATCTGGGTCA-3′	5′-ACAACGCTATGCAGCTTGTTCGTG-3′	NM_008337.3
Notch1	5′-TGCCTGTGCACACCATTCTGC-3′	5′-CAATCAGAGATGTTGGAATGC-3′	NM_008714.3
Notch2	5′-ATGCACCATGACATCGTTCG-3′	5′-GATAGAGTCACTGAGCTCTCG-3′	NM_010928.2
Notch3	5′-TTGGTCTGCTCAATCCTGTAGC-3′	5′-TGGCATTGGTAGCAGTTGCTG-3′	NM_008716.2
Notch4	5′-AAGCGACACGTACGAGTCTGG-3′	5′-ATAGTTGCCAGCTACTTGTGG-3′	NM_010929.2
Dll1	5′-GACACCAAGTACCAGTCGGTGTATG-3′	5′-AACCTGGTTCTCAGCAGCAGTC-3′	NM_007865.3
Dll4	5′-AGCTGGAAGTGGACTGTGGT-3′	5′-TAGAGTCCCTGGGAGAGCAA-3′	NM_019454.3
GAPDH	5′-TGAACGGGAAGCTCACTGG-3′	5′-TCCACCACCCTGTTGCTGTA-3′	NM_008084

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IFN = interferon; IL = interleukin; TNF = tumour necrosis factor; MIF = macrophage migration inhibitory factor.

60°C and 15 s at 72°C. Fluorescence data were acquired at 72–95°C to decrease the non-specific signal and amplification of specific transcripts was confirmed by melting curve profiles at the end of each PCR. Measurements were masked to group assignment.

Flow cytometry analysis

The spleens and draining lymph nodes were removed from immunized mice on day 14. Cell suspension was prepared by mechanical disruption and followed by passage through a sterile stainless steel screen. For intracellular cytokine evaluation [31], the lymphocytes (2×10^5 in 100 μl) were pretreated with 100 ng/ml phorbol-12-myristate-13-acetate (PMA) and 1 μg/ml ionomycin for 1 h at 37°C, 10 μg/ml brefeldin A (Sigma-Aldrich, St Louis, MO) for another 4 h, then washed, fixed and permeabilized using the Cytofix/Cytoperm kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. The cells were stained intracellularly with fluorescent antibodies including anti-mouse CD4-allophycocyanin (APC), anti-mouse interferon (IFN)-γ-phycoerythrin (PE)-cyanin 7 and anti-mouse IL-17A-PE (eBioscience) for 30 min.

Immunohistochemistry

At day 14 after immunization, eyeballs were collected from EAU mice and fixed in 4% paraformaldehyde and embedded in paraffin. Sections were blocked with 5% bovine serum albumin (BSA) for 30 min and incubated with rabbit anti-mouse MIF antibody (1 : 250; Abcam) overnight at 4°C. Sections were incubated with a secondary horseradish peroxidase-labelled anti-rabbit immunoglobulin (Ig)G antibody (Sigma-Aldrich). Negative controls were obtained by replacing the primary antibody with serum or PBS. Intraocular macrophages were counted for the vitreous cavity or the retina of each mouse section from microscopic fields of ×100 or ×200, respectively.

Enzyme-linked immunosorbent assay (ELISA)

Retinas from EAU mice were harvested and protein was extracted by RIPA (Beyotime, Shanghai, China) buffer including 1% proteases inhibitor (Beyotime). Tumour necrosis factor (TNF)-α, interleukin (IL)-6 or IL-1β concentration in the supernatants were measured using a commercial ELISA kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) with a detection threshold of 3 or 7 pg/ml.

Electroretinography (ERG)

Retinal function was evaluated by recording of dark- and light-adapted ERG (RetiMINER System; AiErXi Medical Equipment Co. Ltd, Chongqing). Mice were dark-adapted overnight, anaesthetized and the pupils were dilated as described [32]. Active electrodes were placed on the cornea centre. Reference and ground electrodes were attached, respectively, to the back neck and near the tail subcutaneously. The a-wave amplitude was measured from the baseline to the trough of a-wave and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Each experimental group consisted of five to six mice.

Statistical analysis

Data were presented as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). A Mann–Whitney *U*-test was used to compare the EAU score. Continuous variables of band intensity and relative mRNA expression experiments were analysed with the unpaired Student's *t*-test. One-way analysis of variance (ANOVA) followed by a Bonferroni correction were applied for multiple comparisons. A *P*-value less than 0·05 was considered statistically significant.

Results

The expression of MIF was increased in EAU

It has been reported that MIF levels are increased significantly in the sera and ocular fluids of uveitis patients [12,13]. We examined MIF expression by immunohistochemistry and found that MIF was expressed abundantly in the inner nuclear and ganglion cell layers in the retina of EAU (Fig. 1a). We quantitated the expression of MIF in the retina by real-time PCR. MIF expression was significantly higher at day 14 in the EAU than in the naive mice ($P < 0{\cdot}001$) (Fig. 1b). Because the MIF signal transduction pathway is mediated by its two receptors, CD74 and CD44 [33], we detected the gene expression of CD74 and CD44 in EAU mice by real-time PCR. The levels of CD74 ($P < 0{\cdot}01$) and CD44 ($P < 0{\cdot}01$) were increased significantly in EAU mice compared with the naive mice at day 14 (Fig. 1c,d).

AAV8 (Y733F) vector-mediated eGFP reporter gene expression in the retina

An eGFP reporter gene was constructed and was packaged into mutant AAV8. AAV8.CBA.eGFP vector was injected subretinally into the eyes of naive mice [29]. An image of the injection area (white arrow) at 1 week after injection was shown in Fig. 2a. The eGFP gene was expressed at the injection site at 1 week after injection (data not shown). Three weeks after injection, the whole fundus of the injected eye showed a wide range of GFP expression (Fig. 2b). Further, the GFP expression was detected mainly in the photoreceptor cell layer and in the RPE layer in frozen retinal sections (Fig. 2d). No GFP expression was seen in the control slides (Fig. 2c). These data confirmed that the AAV8 (Y733F)-mediated gene delivery was effective.

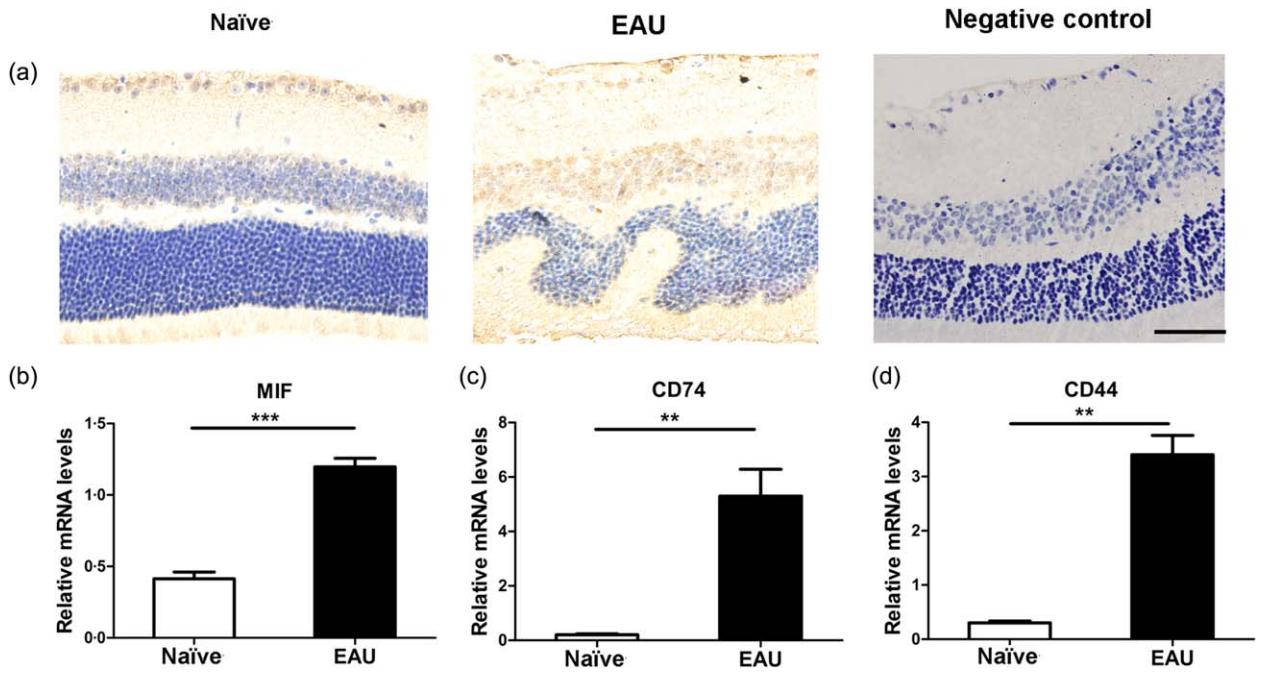

Fig. 1. Expression of macrophage migration inhibitory factor (MIF) and its two receptors in the retina of experimental autoimmune uveitis (EAU) mice at day 14 post-immunization. EAU was induced in B10.RIII mice by active immunization with a mixture of interphotoreceptor retinoid binding protein (IRBP) peptide with complete Freund's adjuvant (CFA). The eyeballs were collected 14 days later to evaluate the expression of MIF, CD74 and CD44. (a) Representative images of immunostaining showed MIF-positive cells (brown) in naive mice (left panel), EAU mice (middle panel) and negative control (right panel). Real-time polymerase chain reaction (PCR) showed that the levels of MIF (b), CD74 (c) and CD44 (d) mRNAs increased in EAU than in naive mice. The relative gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are mean ± standard error of the mean (s.e.m.) and were representative of four independent experiments. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. The scale bar represents 50 μm. Six mice were used in each group.

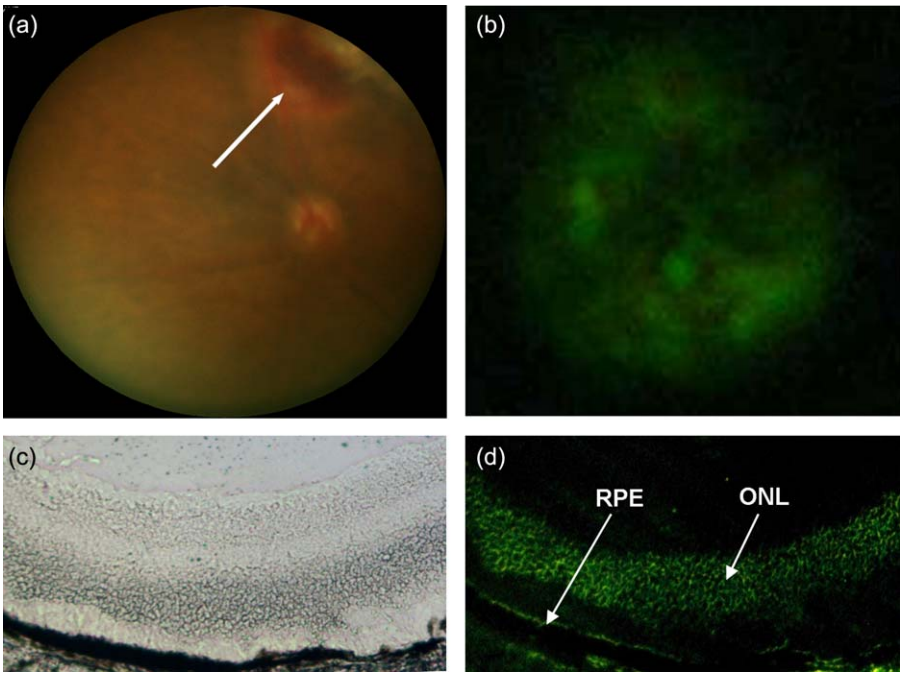

Fig. 2. Validation of serotype 8 adeno-associated virus [AAV8(Y733F)]-mediated enhanced green fluorescent protein (eGFP) reporter gene expression in mouse retina. (a) One week after subretinal injection of AAV8.chicken β-actin (CBA).eGFP, the white arrow indicates the injection area. (b) Three weeks after subretinal injection of AAV8.CBA.eGFP, GFP expression was detected by fundus examination. Delivery of the vector (1 μl) led to a wide range of transduction. (d) Higher transduction of GFP was seen in the photoreceptor and in the RPE layers. (c) The control image of GFP expression was examined under white light by fluorescence microscopy. Green = GFP; RPE = retinal pigment epithelium; ONL = outer nuclear layer.

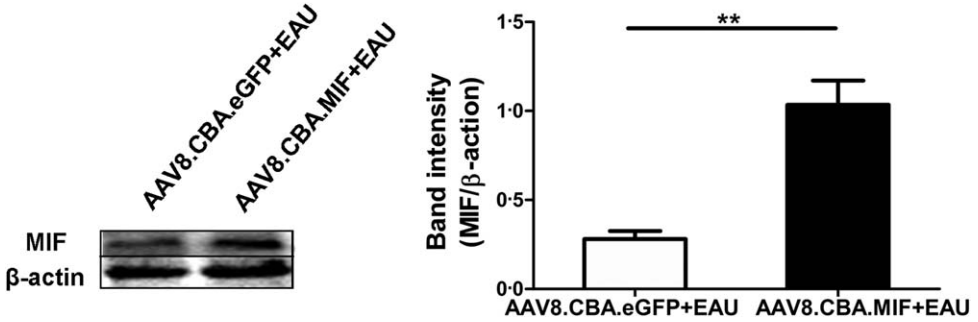

Fig. 3. Retinal protein expression of macrophage migration inhibitory factor (MIF) in serotype 8 adeno-associated virus (AAV8).chicken β-actin (CBA).MIF-injected experimental autoimmune uveitis (EAU) mice. MIF protein level was higher in AAV8.CBA.MIF than in the AAV8.CBA.enhanced green fluorescent protein (eGFP)-injected eyes. The relative expression was normalized to β-actin. Data are mean ± standard error of the mean (s.e.m.) and were representative of three independent experiments. $^*P < 0{\cdot}05$. Six mice were used in each group.

AAV8 (Y733F).CBA.MIF increased MIF protein level in EAU retina

We examined whether subretinal injection of AAV8.CBA.-MIF led to over-expression of MIF in EAU mice. Three weeks after the vector injection, EAU was induced. Retinal MIF expression was assayed by Western blotting analysis. The result demonstrated that the expression of MIF protein in AAV8.CBA.MIF-injected retinas was higher than the AAV8.CBA.eGFP-injected controls ($P < 0{\cdot}05$) (Fig. 3).

MIF promoted the production of proinflammatory cytokines in EAU

Proinflammatory cytokines contribute to the pathogenesis of autoimmune inflammatory disorders. Among these, TNF-α, IL-1β and IL-6 play active roles in uveitis [34]. Three weeks after subretinal injection of AAV8.CBA.MIF, we detected the levels of these proinflammatory cytokines in the retina. The mRNA levels of TNF-α ($P < 0{\cdot}05$), IL-1β ($P < 0{\cdot}05$) and IL-6 ($P < 0{\cdot}01$) were higher in the retinas of AAV8.CBA.MIF-injected than the AAV8.CBA.eGFP-injected EAU mice (Fig. 4a). The expression of TNF-α ($P < 0{\cdot}05$), IL-1β ($P < 0{\cdot}05$) and IL-6 ($P < 0{\cdot}01$) proteins was also higher in the AAV8.CBA.MIF-injected EAU mice (Fig. 4b). The expression of proinflammatory cytokines in the eGFP-injected EAU mice was not significantly different from those in the EAU mice ($P > 0{\cdot}05$, Fig. 4a,b).

MIF exaggerated retinal function damage in EAU

To examine the effect of over-expression of MIF on retinal function, we employed ERG in mice. At day 14 post-

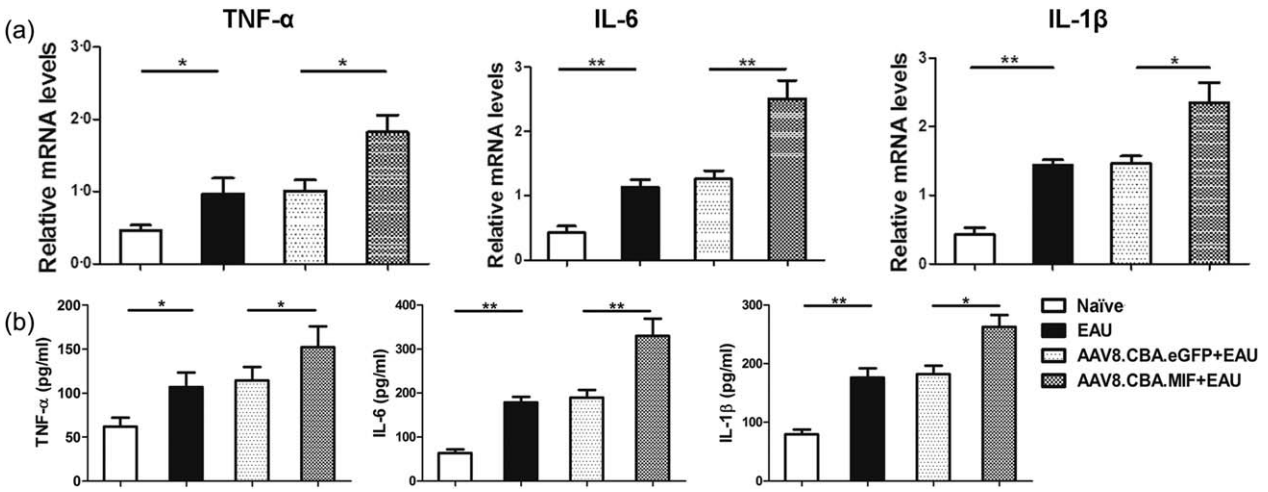

Fig. 4. Expression of proinflammatory cytokines in macrophage migration inhibitory factor (MIF) transduced experimental autoimmune uveitis (EAU) mice. Retinal mRNA and protein were measured at day 14 after immunization with a mixture of interphotoreceptor retinoid binding protein (IRBP) peptide with complete Freund's adjuvant (CFA). (a) Real-time polymerase chain reaction (PCR) showed increase of proinflammatory cytokines [tumour necrosis factor (TNF)-α, interleukin (IL)−1β and IL-6] in AAV8.CBA.MIF-injected EAU mice. (b) TNF-α, IL-1β and IL-6 levels increased in AAV8.chicken β-actin (CBA).MIF-injected EAU mice than in AAV8.CBA.enhanced green fluorescent protein (eGFP)-injected EAU mice. The relative gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are mean ± standard error of the mean (s.e.m.) and were representative of three to four independent experiments. $^*P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$. Six mice were used in each group.

immunization, the dark-adapted ERG amplitudes of the a-wave and b-wave and the light-adapted amplitudes of the b-wave decreased in EAU mice. In the AAV8.CBA.MIF-injected mice, no noticeable ERG response could be elicited (Fig. 5a,b). MIF exaggerated the retinal function damage in EAU mice.

MIF-regulated Notch signal pathway in EAU

Ishida *et al.* have reported the kinetic changes of Notch receptors and their ligands in the eyes during the development of EAU. They showed that the expression of Notch1, 2, 4 and Dll4 were up-regulated significantly in EAU [19]. Also, both Dll1 and Dll4 induced differentiation along the

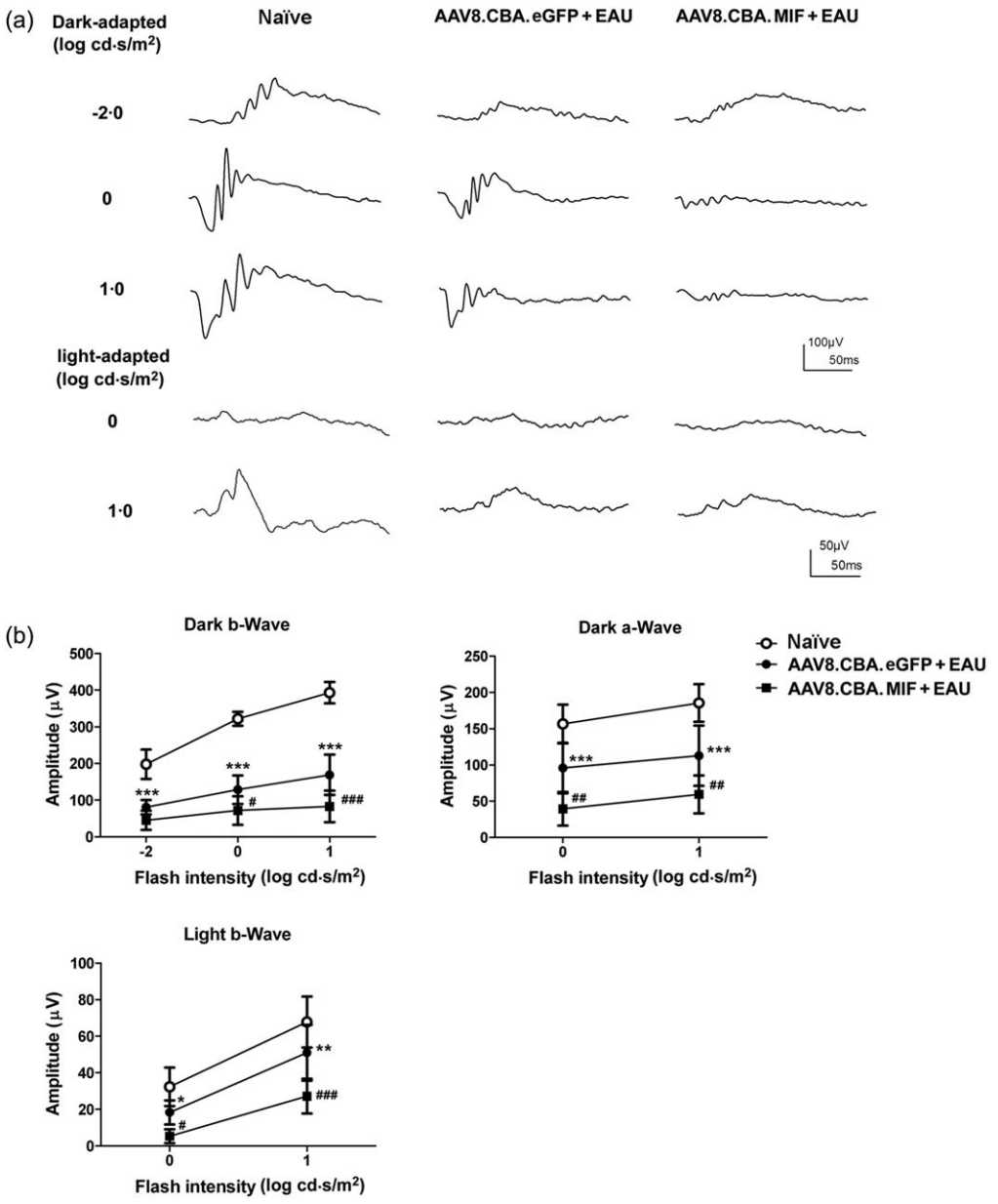

Fig. 5. The effect of macrophage migration inhibitory factor (MIF) on retinal function in experimental autoimmune uveitis (EAU) mice. Retinal functions were evaluated by electroretinography (ERG) at day 14 after immunization with a mixture of interphotoreceptor retinoid binding protein (IRBP) peptide with complete Freund's adjuvant (CFA). (a) Representative ERG responses in the naive mice, AAV8.chicken β-actin (CBA).enhanced green fluorescent protein (eGFP)-injected EAU mice and AAV8.CBA.MIF-injected EAU mice. (b) ERG amplitudes *versus* flash intensity for dark-adapted a-wave, b-wave and light-adapted b-wave. Dark-adapted amplitudes of ERG a-wave and b-wave, light-adapted amplitudes of ERG b-wave decreased in the EAU mice. MIF exaggerated the functional damage in EAU mice. Data are presented as mean ± standard error of the mean (s.e.m.) of 12 mice. $^{*}P < 0{\cdot}05$ *versus* naive group; $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, $^{\#}P < 0{\cdot}05$ *versus* EAU group; $^{\#\#}P < 0{\cdot}01$; $^{\#\#\#}P < 0{\cdot}001$.

Th1 pathway [35,36]. The mRNA expression of Notch receptors and their ligands was evaluated by real-time PCR. Expression of Notch1 ($P < 0{\cdot}01$) and Dll4 ($P < 0{\cdot}01$) was up-regulated significantly at day 14 after EAU induction (Fig. 6a), which was in agreement with a former report [19].

To test whether MIF was interrelated with Notch signalling, the EAU model was induced 3 weeks after subretinal injection of AAV8.CBA.MIF. The expression of Notch signalling components in retina was analysed 14 days of immunization. MIF increased the expression of Notch1 ($P < 0{\cdot}05$) and Notch4 ($P < 0{\cdot}05$) (Fig. 6b), but not Notch2 and Notch3 mRNA (data not shown). The mRNA expression of a Notch ligand, Dll4 ($P < 0{\cdot}05$), was also induced in EAU mouse retina (Fig. 6b), whereas no noticeable change was seen for Dll1 (data not shown).

To examine further whether Notch signalling was up-regulated in EAU mice after AAV8.CBA.MIF injection, NICD (the intracellular domain of Notch receptors) and Hes-1 protein expression were evaluated by Western blotting. Densitometric analysis demonstrated that the levels of NICD ($P < 0{\cdot}05$) and Hes-1 ($P < 0{\cdot}05$) were increased in the AAV8.CBA.MIF-injected compared with the AAV8.CBA.eGFP-injected EAU eyes (Fig. 6c,d).

DAPT inhibited the exaggerated intraocular inflammation induced by MIF

We tested whether suppressing of the Notch signalling pathway could inhibit the exaggerated intraocular inflammation mediated by over-expression of MIF in EAU. DAPT, a Notch pathway inhibitor (γ-secretase inhibitor), effectively blocks the signalling by preventing cleavage of the intracellular fragment from the receptor. The dosage of DAPT was adopted from previous studies [21,37]. DAPT or vehicle was, respectively, administered intraperitoneally to the AAV8.CBA.MIF-injected EAU mice and the mRNA expression of proinflammatory cytokines was detected by real-time PCR. The expression of proinflammatory cytokines, including IL-17 ($P < 0{\cdot}05$), IFN-γ ($P < 0{\cdot}05$), TNF-α ($P < 0{\cdot}05$) and IL-1β ($P < 0{\cdot}05$), decreased in the DAPT-treated AAV8.CBA.MIF-injected EAU mice (Fig. 7).

A MIF inhibitor ISO-1 attenuated the severity of EAU

Next, we evaluated the effect of ISO-1, a small molecule MIF antagonist which binds to the MIF tautomerase site [38], on the severity of EAU. ISO-1 was administered intraperitoneally for 14 consecutive days at a dose of 20 mg/kg/day, starting from 2 days before to 14 days after immunization as a prophylactic regimen. The dosage was referred to the former study [39]. Retinal damage was ameliorated in the ISO-1-treated EAU mice ($P < 0{\cdot}05$) (Fig. 8b). Representative histological images of vehicle-treated and ISO-1-treated EAU mice at day 14 after immunization are shown in Fig. 8a. We also detected the mRNA levels of proinflammatory cytokines by real-time PCR after administration of ISO-1. The mRNA levels of IL-17 ($P < 0{\cdot}05$), IFN-γ ($P < 0{\cdot}05$), TNF-α ($P < 0{\cdot}05$) and IL-1β ($P < 0{\cdot}05$) were decreased significantly in ISO-1-treated EAU mice compared with the vehicle-treated EAU mice (Fig. 8c).

ISO-1 reduced the differentiation of Th1 and Th17 in EAU

The effect of ISO-1 on the differentiation of Th1 and Th17 was examined in the lymphocytes from the spleen and lymph nodes of the EAU mice. Lymphocytes were collected from the vehicle- and the ISO-1-treated EAU mice, and intracellular expression of IFN-γ and IL-17 were examined. The $CD4^+$ IFN-γ^+ ($P < 0{\cdot}05$) and $CD4^+$ IL-17^+ ($P < 0{\cdot}05$) Th populations were reduced significantly in the ISO-1-treated EAU mice (Fig. 9a–d). Meanwhile, the number of the IFN-γ/IL-17 double-positive cells decreased after ISO-1 treatment (data not shown).

Discussion

Cytokines are important contributors to many acute and chronic inflammatory diseases. MIF is one of the important proinflammatory cytokines, and acts as an immune regulator, an anterior pituitary hormone, a high-activity enzyme and is well known for its role in inflammatory and autoimmune diseases [40–42]. However, although MIF is associated with some autoimmune uveitis [12–16], its role in uveitis is largely unclear. The optimized capsid-mutant AAV vector is becoming a useful gene-transfer tool to study the mechanism as well as to develop therapeutic applications in a variety of ocular diseases [26]. We applied a mutant AAV8 vector to explore the proinflammatory effect and underlying mechanism of MIF in intraocular inflammation.

Our results suggested a critical role of MIF in EAU. We found that: (i) MIF exaggerated the intraocular inflammation and exacerbated retinal function damage in EAU; (ii) the Notch signalling pathway was involved in the exaggerated inflammation induced with over-expression of MIF, as demonstrated by increased expression of NICD and Hes-1, and by decreased intraocular inflammation with a Notch signalling pathway antagonist DAPT; (iii) suppression of MIF with ISO-1 reduced intraocular inflammation and inhibited the differentiation of Th1 and Th17 in EAU; and (iv) the expression of the Notch signalling pathway components NICD and Hes-1 were reduced significantly in MIF inhibitor-treated EAU mice. To our knowledge, this is the first study to show that MIF exaggerates intraocular inflammation via regulating the Notch signal pathway *in vivo*.

T cells are the main cellular source of MIF in the immune system, while monocytes, macrophages, blood dendritic cells and B cells also express MIF [43]. Under normal physiological conditions, MIF is expressed in the

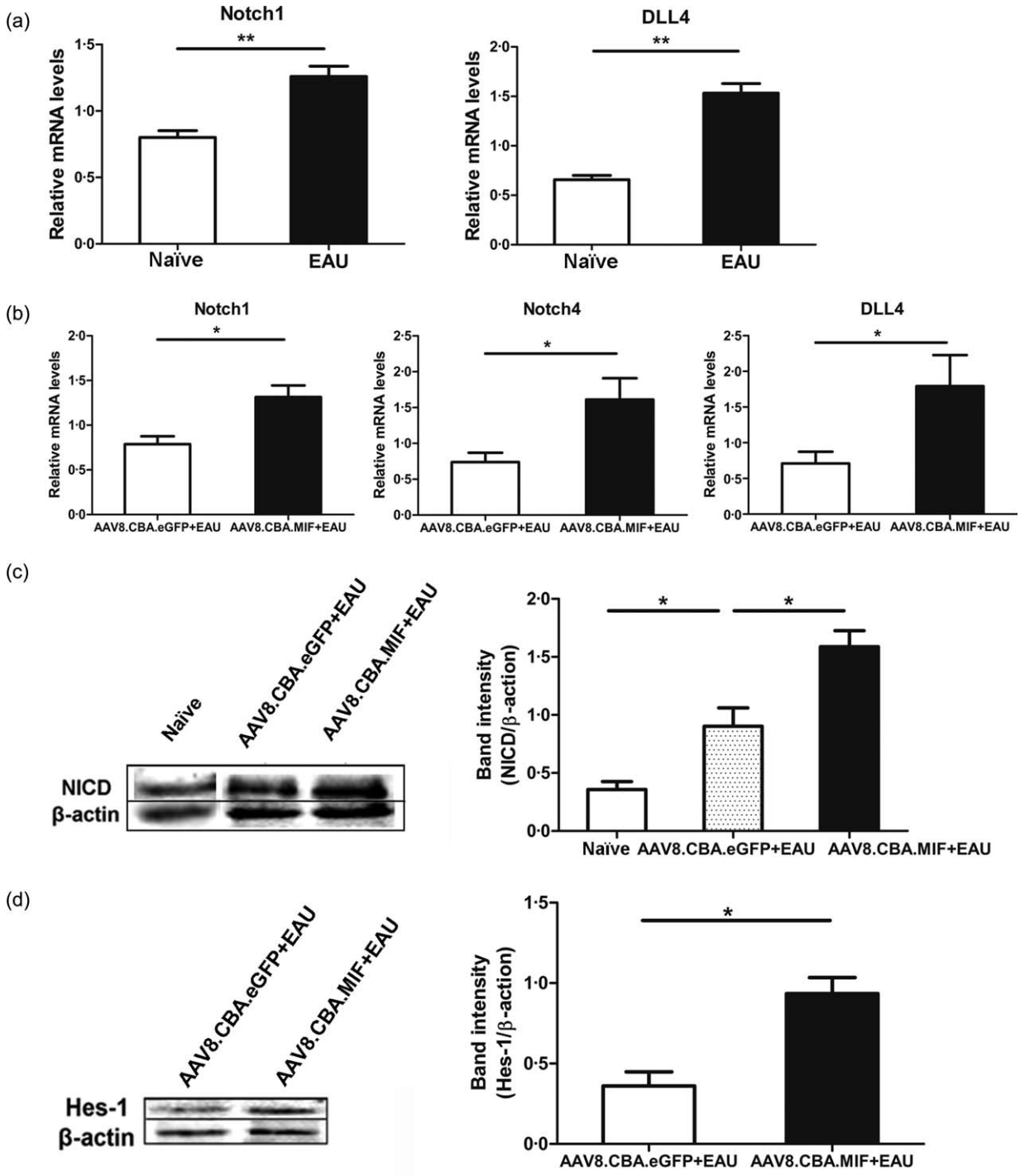

Fig. 6. Expression of Notch signal pathway in macrophage migration inhibitory factor (MIF) over-expressed experimental autoimmune uveitis (EAU) mice. (a) Expression of Notch receptors and their ligands in the retina of EAU. The levels of Notch1 and delta-like ligand 4 (Dll4) increased in EAU mice at day 14 after immunization with a mixture of interphotoreceptor retinoid binding protein (IRBP) peptide with complete Freund's adjuvant (CFA). (b) Expression of Notch receptors and their ligands in the AAV8.chicken β-actin (CBA).MIF-injected EAU mice. The expression of Notch1, Notch4 and Dll4 was higher in the AAV8.CBA.MIF-injected EAU mice than in the AAV8.CBA.enhanced green fluorescent protein (eGFP)-injected EAU mice. (c) Protein expression of Notch receptor intracellular domain (NICD) in the AAV8.CBA.MIF-injected EAU mice. NICD protein level was higher in the AAV8.CBA.MIF-injected eyes than in the AAV8.CBA.eGFP-injected eyes. (d) Protein expression of hairy enhancer of split-1 (Hes-1) in the AAV8.CBA.MIF-injected EAU mice. Hes-1 protein level increased in the AAV8.CBA.MIF-treated eyes compared with the AAV8.CBA.eGFP-injected eyes. The relative expression was normalized to β-actin. Data are mean ± standard error of the mean (s.e.m.) and were representative of three independent experiments. $^*P < 0{\cdot}05$. Six mice were used in each group.

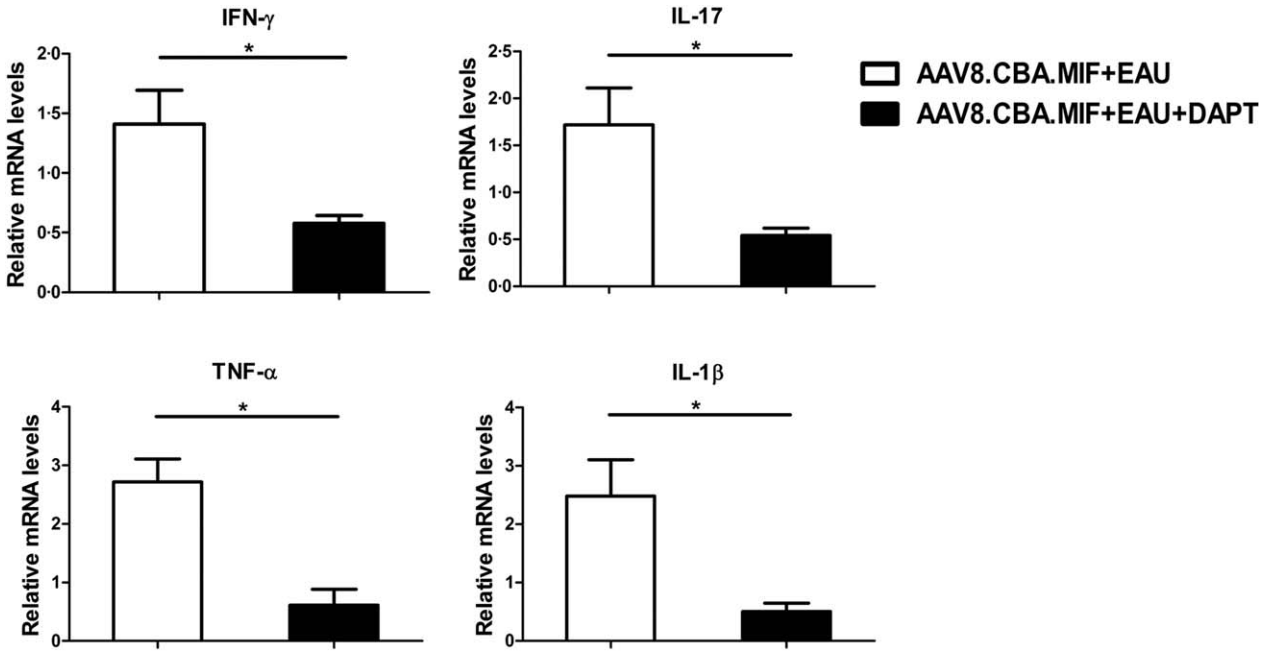

Fig. 7. Expression of proinflammatory cytokines in the N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT)-treated AAV8.CBA.macrophage migration inhibitory factor (MIF)-injected experimental autoimmune uveitis (EAU) mice. Retinal mRNA and protein were examined at day 14 after a mixture of interphotoreceptor retinoid binding protein (IRBP) peptide with complete Freund's adjuvant (CFA) immunization. Real-time polymerase chain reaction (PCR) showed increased level of proinflammatory cytokines [tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-17 and interferon (IFN)-γ] in the AAV8.CBA.MIF-injected EAU eyes. The relative gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are mean ± standard error of the mean (s.e.m.) and were representative of three to four independent experiments. $^*P < 0{\cdot}05$. Six mice were used in each group.

rat retina [44]. In uveitis, the expression of MIF in the sera and ocular fluid was increased significantly [12,13]. MIF signalling through CD74 requires CD44 in a kidney inflammatory disease, in which the levels of CD74, CD44 and CXCR4 are up-regulated, and MIF activation through CD74 promotes a kidney inflammatory response [45]. We found that the expression of MIF as well as its two receptors in the retina was significantly higher at day 14 in EAU. Together with the previous reports, our findings indicated that the MIF pathway may be operational in both normal physiological conditions and in uveitis.

One of the major characteristics of inflammatory disease is abnormal regulation of cytokines in the target tissues or organs. We proved that MIF stimulated the secretion of proinflammatory cytokines, including TNF-α, IL-1β and IL-6 in the retina of EAU, which is consistent with its role in EAE and rheumatoid arthritis (RA) [46,47]. In agreement with a recent report [48], we showed that dark- and light-adapted ERG responses decreased remarkably in EAU, and the dark- and light-adapted ERG responses in AAV8.CBA.MIF-injected EAU mice were reduced further. Thus, the functional impairment of the retina was exaggerated by MIF. To examine whether MIF was a key player in regulating the intraocular inflammation in EAU, we administered an inhibitor of MIF ISO-1 to EAU mice via intraperitoneal injection. ISO-1 attenuated the severity of EAU and reduced intraocular macrophage recruitment in the retina (data not shown).

It was demonstrated that MIF triggers several cell signalling pathways in various cell types. Protein kinase B (AKT), extracellular regulated kinase (ERK), 5′ AMP-activated protein kinase (AMPK) and signal transducer and activator of transcription 3 (STAT-3) pathways were activated by MIF in the proliferation and survival of neural stem/progenitor cells (NSPC) [49,50]. Although the Notch signal pathway plays a critical role in T cell differentiation and autoimmune diseases [20,40], the interaction between MIF and Notch signalling pathways, as well as the functions of their interaction in uveitis, remains unknown. After subretinal injection of AAV8.CBA.MIF, the levels of Notch1, Notch4 and the ligand Dll4 increased in the retina. The activated Notch NICD and a classic target gene Hes-1 were also up-regulated. By using DAPT, a Notch signalling pathway inhibitor, the proinflammatory effect of MIF was inhibited. Our data show that the Notch signalling pathway was associated with the MIF-mediated amplification of intraocular inflammation, suggesting an interaction between the MIF and Notch signalling pathways. We demonstrate that the Notch signalling pathway and MIF are involved in EAU. However, the detailed mechanism concerning the association between Notch signalling and MIF remains to be investigated.

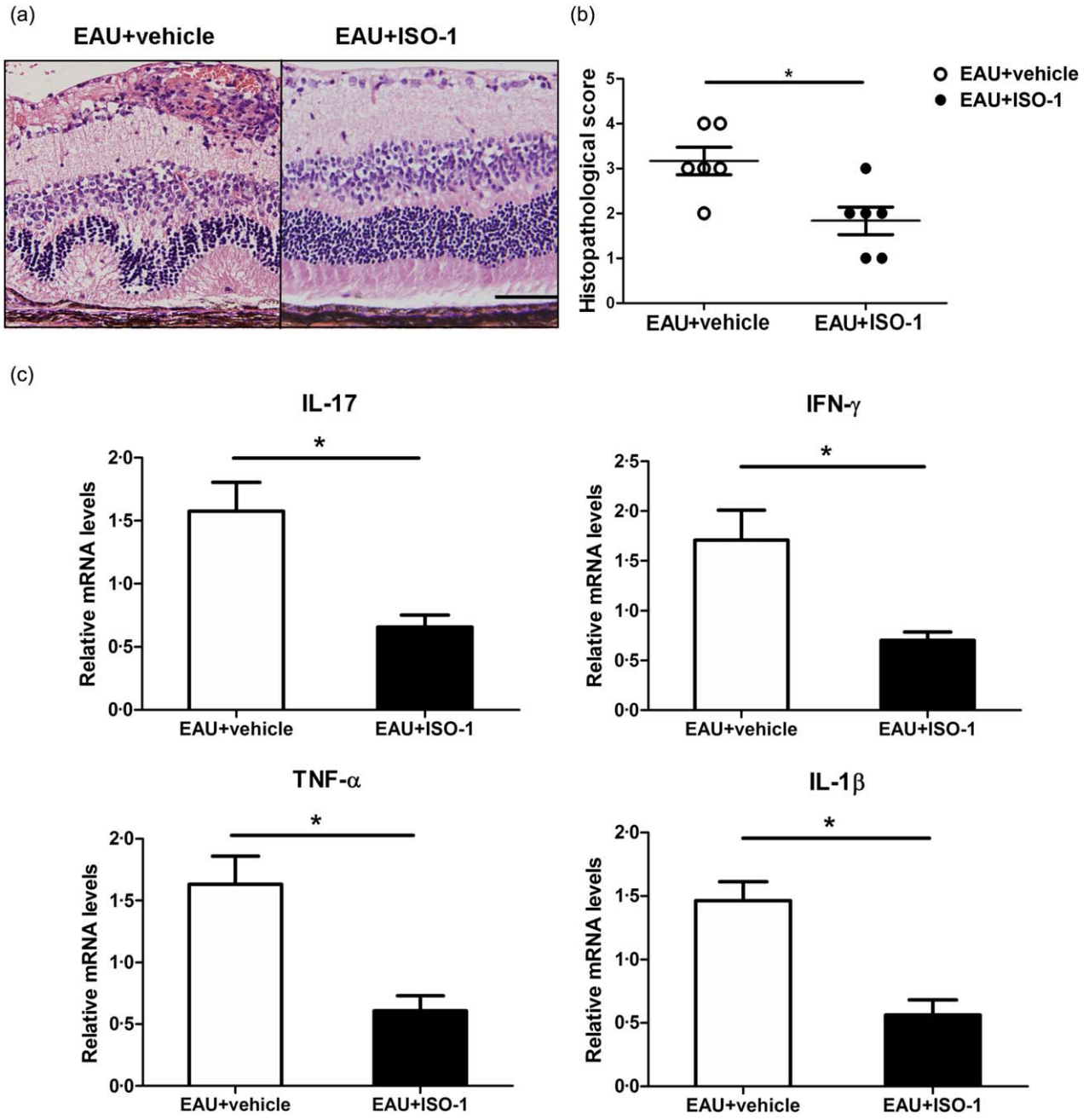

Fig. 8. The severity of histological score and the expression of proinflammatory cytokines in the ISO-1-treated experimental autoimmune uveitis (EAU) mice. Histological scores were assessed with haematoxylin and eosin (H&E) staining at day 14 after immunization. Retinal damage was ameliorated in the ISO-1-treated EAU mice (b). Representative retinal histological images of the vehicle-treated and the ISO-1-treated EAU mice (a). Inflammatory cellular infiltration and retinal folds were evident in the vehicle-treated EAU mice, but was not seen in the ISO-1-treated groups. The levels of proinflammatory cytokines [tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-17 and interferon (IFN)-γ] in the ISO-1-treated EAU mice were reduced (c). The scale bar represents 50 μm. Data are shown as mean ± standard error of the mean (s.e.m.). $^*P < 0{\cdot}05$.

Th1 and Th17 cell responses mediated the pathogenesis of EAU, and Notch is essential in the differentiation of Th1 and Th17 cells. Amsen *et al.* reported that the Notch ligands Dll1 and/or Dll4 were up-regulated in the increased Th1 cell response [51]. Further, Dll4 could promote the generation of Th17 cells through activation of the Th17 cell-specific transcription factor RAR-related orphan receptor (ROR)γt [52]. In our study, Notch 1, Notch 4 and Dll4 were up-regulated when MIF exaggerated the retinal functional damage and the production of proinflammatory cytokines in EAU. Thus, we speculate that MIF, Notch signalling and Th1 and Th17 may form a cascade in the

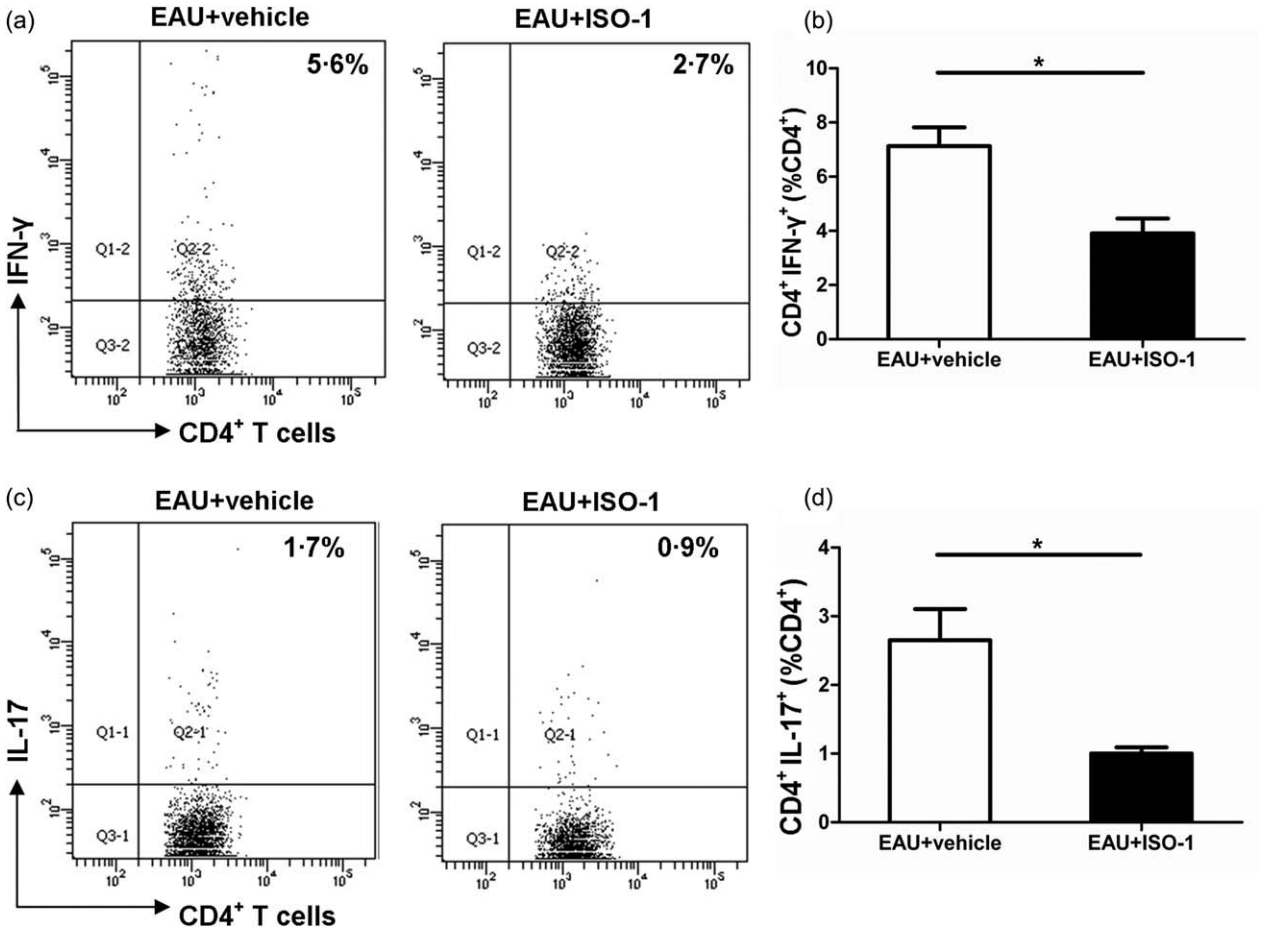

Fig. 9. The differentiation of T helper type 1 (Th1) and Th17 in the ISO-1-treated experimental autoimmune uveitis (EAU) mice. ISO-1 reduced the proportions of Th1 and Th17 cells expressing interferon (IFN)-γ, interleukin (IL)-17 in EAU at day 14. Lymphocytes from the vehicle and the ISO-1-treated EAU mice were collected at day 14 post-immunization and the frequency of IFN-γ^+CD4$^+$ and IL-17$^+$CD4$^+$ T cells were tested by flow cytometry. (a,c) Representative intracellular expression of IFN-γ and IL-17, respectively. (b,d) The averages of four independent experiments presented as the percentages of IFN-γ- and IL-17-positive cells in the lymphocytes. Numbers represent percentage of positive cells. Data are mean ± standard error of the mean (s.e.m.) . *$P < 0{\cdot}05$. Six mice were used in each group.

development of ocular inflammation. Also, Notch induces the Th1 cell response by interacting with the nuclear factor kappa β (NF-κB) pathway [53]. It has been shown that an increase of plasma MIF was associated with higher translocation of NF-κB p65 in patients with severe injury [54]. However, the detailed mechanism concerning MIF, Notch signalling and NF-κB in EAU deserves further study.

In conclusion, we have demonstrated that MIF is a key cytokine in the process of EAU. MIF plays an important role in exaggerating intraocular inflammation. It increases proinflammatory cytokine release and exacerbates retinal function damage. Suppression of MIF reduces the differentiation of Th1 and Th17 in EAU. Furthermore, the amplification of inflammation by MIF is associated with Notch signalling. Our data suggest that the MIF-Notch axis may play an important role in the pathogenesis of EAU. Both the MIF and Notch signalling pathways may be promising targets for developing novel therapeutic interventions for uveitis.

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Disclosure

The authors have no financial disclosures.

Author contributions

H. Y. and B. L. conceived and designed the project. H. Y. and S. Z. performed most of the experiments. C. S. provided MIF plasmid; Y. M. and H. L. packed recombinant adeno-associated virus; Z. C. performed the

electroretinography examination. C. Z. helped with Western blotting; H. Y. and B. L. analysed the data and wrote the manuscript. P. Y., Q. L. and B. L. reviewed and revised the manuscript.

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