Decreased microRNA-155 Expression in Ocular Behcet's Disease but Not in Vogt Koyanagi Harada Syndrome

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Purpose. MicroRNAs (miRNAs) have emerged as a class of gene expression regulators involved in immune regulation. In the present study, we investigated the role of miRNA in two uveitis entities: Behcet's disease (BD) and Vogt Koyanagi Harada syndrome (VKH).

Methods. The expression of five miRNAs was studied in PBMCs, DCs, and $CD4^+$ T cells from BD patients with active and inactive uveitis, VKH patients with active uveitis, and healthy controls using real-time PCR. MiR-155 mimics and inhibitor were transfected to DCs to evaluate the effect on DC maturation and cytokine production by these cells and $CD4^+$ T cells. Luciferase reporter assays and Western blotting were performed to identify the target gene of miR-155.

Results. Only miR-155 expression was significantly decreased in PBMCs and DCs from BD patients with active uveitis and no differences were observed in the miRNA expression in cells from patients with VKH as compared with controls. Overexpression of miR-155 in DCs was shown to inhibit the production of IL-6 and IL-1β, and to promote the expression of IL-10 by these cells. MiR-155 transfected DCs significantly inhibited intracellular IL-17 expression in allogeneic $CD4^+$ T cells; however, it did not influence the expression of cell surface markers CD80, CD40, CD83, CD86, and HLA-DR. Luciferase reporter assays revealed that TAB2 was a target gene of miR-155, which was confirmed by Western blotting.

Conclusions. The present results suggest that miR-155 expression is decreased in active BD but not in VKH patients. Downregulated miR-155 may be involved in BD pathogenesis by targeting TAB2. (*Invest Ophthalmol Vis Sci.* 2012;53:5665–5674) DOI:10.1167/iovs.12-9832

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Uveitis is one of common causes of blindness in the world. Behcet's disease (BD) and Vogt-Koyanagi-Harada (VKH) are the two major uveitis entities in China. BD is a chronic, multisystem inflammatory disease characterized by recurrent uveitis, oral aphthae, genital ulcers, and skin lesions.[1,2] It is more prevalent in the region that extends from China and Japan in the Far East to the Mediterranean Sea.[2,3] VKH disease is an autoimmune disorder characterized by bilateral granulomatous panuveitis frequently associated with systemic involvement and frequently affects Asians and Native Americans.[4,5] Although the etiology and pathogenesis of these two diseases remain unclear, several reports have suggested that an autoinflammatory or autoimmune response may play a crucial role.[6,7]

Mammalian microRNAs (miRNAs) are small (18–25 nucleotides long), endogenous, noncoding RNA oligonucleotides. They are highly conserved during evolution and have recently emerged as potent regulators of gene expression linked to many biological functions.[8] miRNAs posttranscriptionally regulate gene expression by binding to the sequences in the 3′ untranslated region (3′-UTR) of target mRNAs.[9] More than 900 miRNAs have been identified in mammals. miRNAs have long been known for their role in cellular proliferation, organ development, homeostasis and function.[10] Several miRNAs exhibit a tissue-specific or developmental stage-specific expression pattern and are associated with human diseases, such as leukemia, cancer, and viral infection.[11,12] More recently, studies have demonstrated that miRNAs are crucial in both innate and adaptive immunity, including the control of differentiation and function of various immune cell subsets.[13,14] The role of miRNAs in the innate immune response was previously confirmed in a study that identified miR-146a as a negative feedback regulator in Toll-like receptor (TLR) signaling by targeting TRAF6 and IL-1R–associated kinase (IRAK) 1.[14] MiR-155 is one of the first miRNAs linked to inflammation by virtue of its potent upregulation of multiple immune cell lineages via TLR ligands and expression of proinflammatory cytokines.[14–17] Silencing miR-155 has been shown to ameliorate the inflammatory response during experimental autoimmune disease.[18] Furthermore, aberrant expression of miR-155 has been reported in human autoimmune disorders such as rheumatoid arthritis (RA), and systemic lupus erythematosus.[19,20]

Whether miR-155 plays a role in the pathogenesis of intraocular inflammation is not yet known and was therefore the subject of this study. Our results showed a significantly decreased expression of miR-155 in peripheral blood mononuclear cells (PBMCs) and monocyte-derived dendritic cells (mo-DCs) of BD patients with active uveitis but not in patients with VKH syndrome. Furthermore, the miR-155 was shown to negatively regulate the inflammatory cytokine production by DCs in the TLR/IL-1 signaling cascade by targeting TAB2 (TGF-beta activated kinase 1 binding protein 2).

Materials and Methods

Clinical Specimens

Twenty-three BD patients with active uveitis and 19 BD patients without active uveitis were included in the study. The diagnosis of BD was made according to the International Study Group.[21] Twenty-one VKH syndrome patients with active uveitis who did not receive any systemic immunosuppressives were also included. These patients were diagnosed using the diagnostic criteria revised for VKH by an international committee on nomenclature.[22] Twenty healthy individuals served as controls. All investigated subjects provided informed consent before collection of blood and the Ethical Committee of Chongqing Medical University approved the study. The tenets of the Declaration of Helsinki were conducted during all procedures of the present study.

Cell Isolation and Culture

We included BD patients with active intraocular inflammation and active VKH patients who had not received any systemic corticosteroids or other immunosuppressive agents for at least 1 week before being referred to our hospital. BD patients without active intraocular inflammation were included if they had not received any systemic corticosteroids or other immunosuppressive drugs for at least 2 weeks before blood sampling. A total amount of 15 mL of blood was collected from each subject. PBMCs were isolated from heparinized blood samples by Ficoll-Hypaque density-gradient centrifugation. Peripheral $CD4^+$ T cells were isolated from PBMCs by human CD4 microbeads (Miltenyi Biotec, Palo Alto, CA) according to the manufacturer's instructions. CD14- positive monocytes were isolated using human CD14 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Isolated $CD14^+$ cells (1.5×10^6 cells per well) were seeded in 24-well plates and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Greiner, Wemmel, Belgium), 100 U/mL penicillin, 100 μg/mL streptomycin, recombinant human granulocyte-macrophage colony-stimulating hormone (GM-CSF) 100 ng/mL (PeproTech, London, UK) and recombinant human IL-4 50 ng/mL (PeproTech) for 6 days to promote differentiation into immature DCs (imDCs). On day 3, half of the volume of the medium was replaced with fresh medium. On day 6 following culture, a pure population of DCs was identified by flow cytometry using a FC500 cytometer and CXP analysis software (Beckman Coulter, Pasadena, CA) and more than 90% of purified CD1a+ cells were obtained. For DC maturation, 100 ng/mL LPS (100 ng/mL; Sigma, St. Louis, MO) was added to the cells at day 6 and cultured for 24 hours.

DC-$CD4^+$ T Coculture

The imDCs were transfected with controls (negative control mimics or inhibitors with irrelevant sequence), miR-155 mimics (double-stranded RNA [dsRNA] oligonucleotides), and miR-155 inhibitors (single-stranded chemically modified oligonucleotides) at a final concentration of 100 nM. After 48 hours, DCs were stimulated with 100 ng/mL LPS for 24 hours and then washed and cocultured with $CD4^+$ T cells obtained from healthy controls. The mixed lymphocyte reaction was set up by culturing the purified $CD4^+$ T cells from different tested groups (1×10^5 cells/0.2 mL of RPMI 1640 complete medium per well in triplicate) with allogeneic monocyte-derived DCs obtained 24 hours after maturation. Coculture was performed at a DC/T ratio of 1:4 in 96-well plates for 7 days, and the $CD4^+$ T cells were collected for intracellular IL-17 assay by flow cytometry.

RNA Preparation and Real-Time Quantitative PCR Analysis

Total RNA, containing miRNA, was extracted with TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA concentrations were determined with a Nano instrument (NanoDrop Technologies, Wilmington, DE). For miRNA analysis, miRNA TaqMan assays for the five miRNAs and U6 small nuclear RNA (Applied Biosystems, Foster City, CA) as internal control were used according to the manufacturers' instructions. The sequence of the primers used is not provided by the manufacturer and is covered by a patent. The relative expression level of miRNAs was normalized to that of the internal control U6 by using the $2^{-\Delta\Delta Ct}$ cycle threshold method.[23]

Transfection of Human Monocyte-Derived DCs with miRNA Mimics and Inhibitors

We used miR-155 mimics (dsRNA oligonucleotides) and miR-155 inhibitors (single-stranded chemically modified oligonucleotides) from GenePharma (Shanghai, China) for the overexpression and inhibition of miR-155 activity in human monocyte-derived DCs (mo-DCs), respectively. Negative control mimics or inhibitors (GenePharma) were used as matched controls. For transfection of mo-DCs, 1.5×10^6 cells were seeded in 24-well plates and cultured in RPMI 1640 medium. To overexpress or silence miR-155, imDCs were transfected with miR-155 mimics or inhibitors at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen) at day 4 and cultured for 48 hours.

Cell Cytotoxicity Assay

To investigate the effects of transfection on the viability of DCs, the Cell Counting Kit-8 (CCK-8, Sigma) assay was used, which is based on the conversion of water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium, monosodium salt] to a water-soluble formazan dye upon reduction in the presence of an electron carrier by dehydrogenases.[24] In brief, isolated $CD14^+$ cells (1×10^5 cells per well) were seeded in 96-well plates and cultured in 100 ng/mL GM-CSF and 50 ng/mL IL-4 to promote differentiation into imDCs. The miR-155 mimics or inhibitors were transfected into cells at different final concentrations (0, 20, 50, 100, 200 nM) on day 4 and cultured for 48 hours. To detect cytotoxicity, 10 μL of CCK8 was added to each well. The optical density was read at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Cells cultured without transfection were used as the control. All groups were tested five times.

ELISA for IL-6, IL-10, IFN-γ, and IL-1β

Serum levels of IL-1β and IL-17 were measured with a human Duoset ELISA development kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols. IL-6, IL-10, IFN-γ and IL-1β levels in the supernatants were also detected using the same method.

Flow Cytometry

To evaluate the transfection rate of miR-155 mimics or inhibitors in mo-DCs, cells were gently washed after transfection for 48 hours and directly detected by flow cytometry. For cell surface marker analysis, DCs were incubated for 30 minutes at 4°C with fluorescein-conjugated and isotype-matched antibodies in the labeling solution. To detect intracellular expression of IL-17 in $CD4^+$ T cells cocultured with mo-DCs pretreated with mimics or inhibitors, cells were recovered and stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin (Sigma) for 5 hours. During the final 4 hours, 10 μg/mL Brefeldin A (Sigma) was added to the cultured $CD4^+$ T cells. The stimulated cells were washed, fixed, permeabilized, and subsequently stained with anti-CD3, anti-CD8, and anti-IL-17 antibodies or appropriate isotypes (eBioscience, San Diego, CA). Flow cytometry was conducted on FACS Aria, (BD Bioscience, San Diego, CA) and the data were analyzed with FACSDiva Software (BD Bioscience).

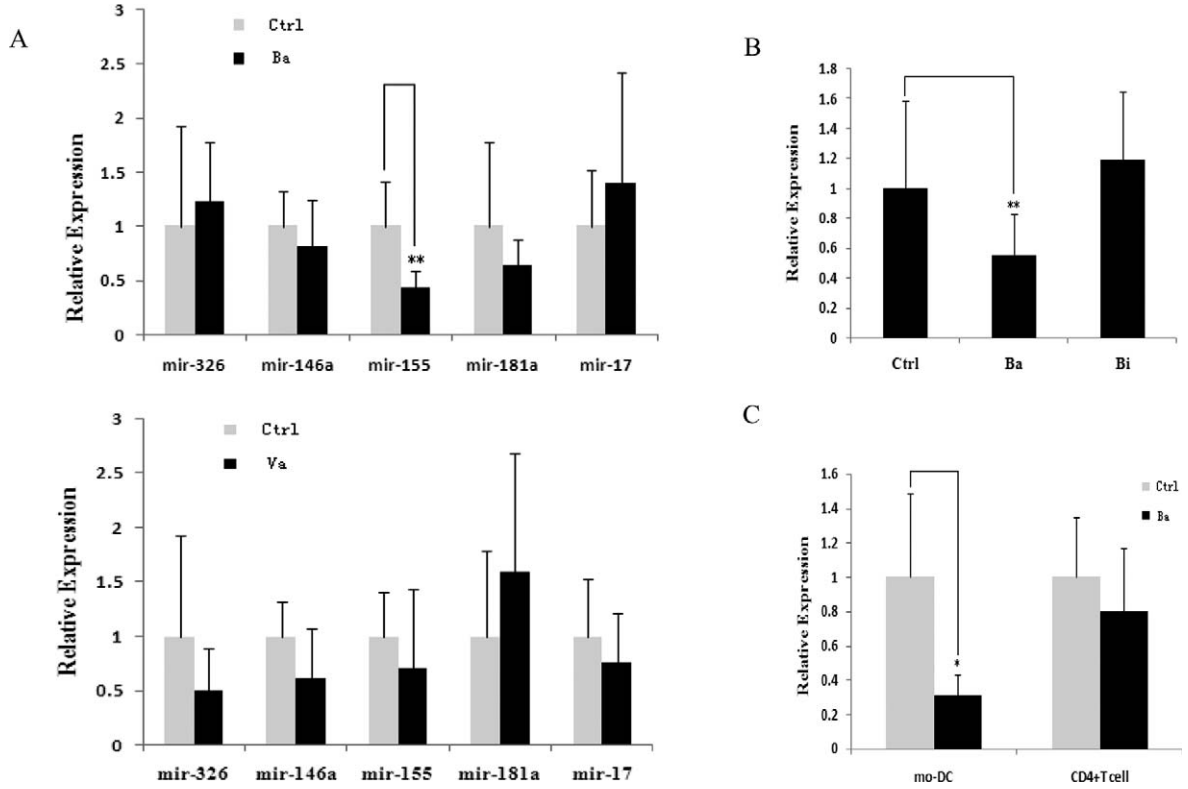

Figure 1. Downregulation of miR-155 in BD patients with active uveitis. (**A**) Quantitative PCR (qPCR) analysis for expression of five miRNAs in PBMCs from healthy controls (Ctrl; $n = 7$), BD patients with active uveitis (Ba; $n = 7$), and VKH patients with active uveitis (Va; $n = 19$–21). (**B**) Expression of miR-155 in PBMCs from healthy controls (Ctrl; $n = 20$), BD patients with active uveitis (Ba; $n = 23$), or patients without active uveitis (Bi; $n = 19$). (**C**) Expression of miR-155 in mo-DCs and $CD4^+$ T cell fraction of PBMCs from BD patients with active uveitis and controls ($n = 7$ per group). The U6 small nuclear RNA was amplified as internal control. Data are the mean ± SD. Ctrl, healthy controls; Ba, BD patients with active uveitis; Va, VKH patients with active uveitis. $^*P < 0.05$, $^{**}P < 0.01$.

Western Blotting for TAB2 Protein

Human mo-DCs (1.5×10^6) were seeded into 24-well plates and transfected with mimics and inhibitors. Forty-eight hours after transfection, cells were lysed with the cell lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, and 100 μM phenylmethanesulfonyl fluoride. Protein concentrations of the extracts were measured with a protein assay (Bio-Rad, Richmond, CA). Equal amounts of the extracts were loaded and subjected to 10% SDS-PAGE, transferred onto polyvinylidene difluoride membrane and incubated before blotting with the indicated antibodies and bands were detected with Luminol/Enhancer Solution (Pierce, Rockford, IL). TAB2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Relative expression levels were quantified using Quantity One software, version 4.52 (Bio-Rad).

3′-UTR Luciferase Reporter Assays

Wild-type and mutated 3′-UTR sequences of TAB2 were cloned into the pMIR-REPORT Luciferase miRNA Expression Reporter Vector (Ambion, Austin, TX) according to the standard protocol. HEK293T cells, obtained from American Type Culture Collection (Manassas, VA), were maintained in Dulbecco's modified Eagle's medium and cultured to 70% confluence in 24-well plates. After 20 hours, the firefly luciferase reporter gene construct (200 ng per well) and pRL-CMV Renilla luciferase construct (4 ng per well) were cotransfected with Lipo2000 (Invitrogen), together with either negative control or the miR-155 mimics. Luciferase activities were measured 24 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Statistical Analysis

Student's *t*-test and one-way ANOVA were applied using SPSS 17.0 software (SPSS Inc., Chicago, IL). Data are shown as mean ± SD. *P* values less than 0.05 were considered significant.

Results

Significantly Decreased Expression of miR-155 Is Observed in BD Patients with Active Uveitis, but Not in VKH Patients with Active Uveitis

Five known immunologically relevant miRNAs (miR-155, miR-146a, miR-326, miR-181a, miR-17) were screened in PBMCs, DCs, and $CD4^+$ T cells from BD patients with active uveitis, VKH patients with active uveitis, and healthy controls (Fig. 1). The results showed that only the miRNA-155 expression was significantly decreased in PBMCs and DCs, but not $CD4^+$ T cells from BD patients with active intraocular inflammation as compared with those without active disease or healthy controls. A further experiment with a larger group of controls and BD patients with active and inactive uveitis confirmed these results. There was no detectable difference in the miRNA expression in the PBMCs between VKH patients with active uveitis and healthy controls. We also investigated whether there was a relationship between the expression of miRNA-155 and serum levels of relevant cytokines in patients. The serum levels of IL-1β and IL-17, however, remained below the detection level in sera from both patients and healthy controls.

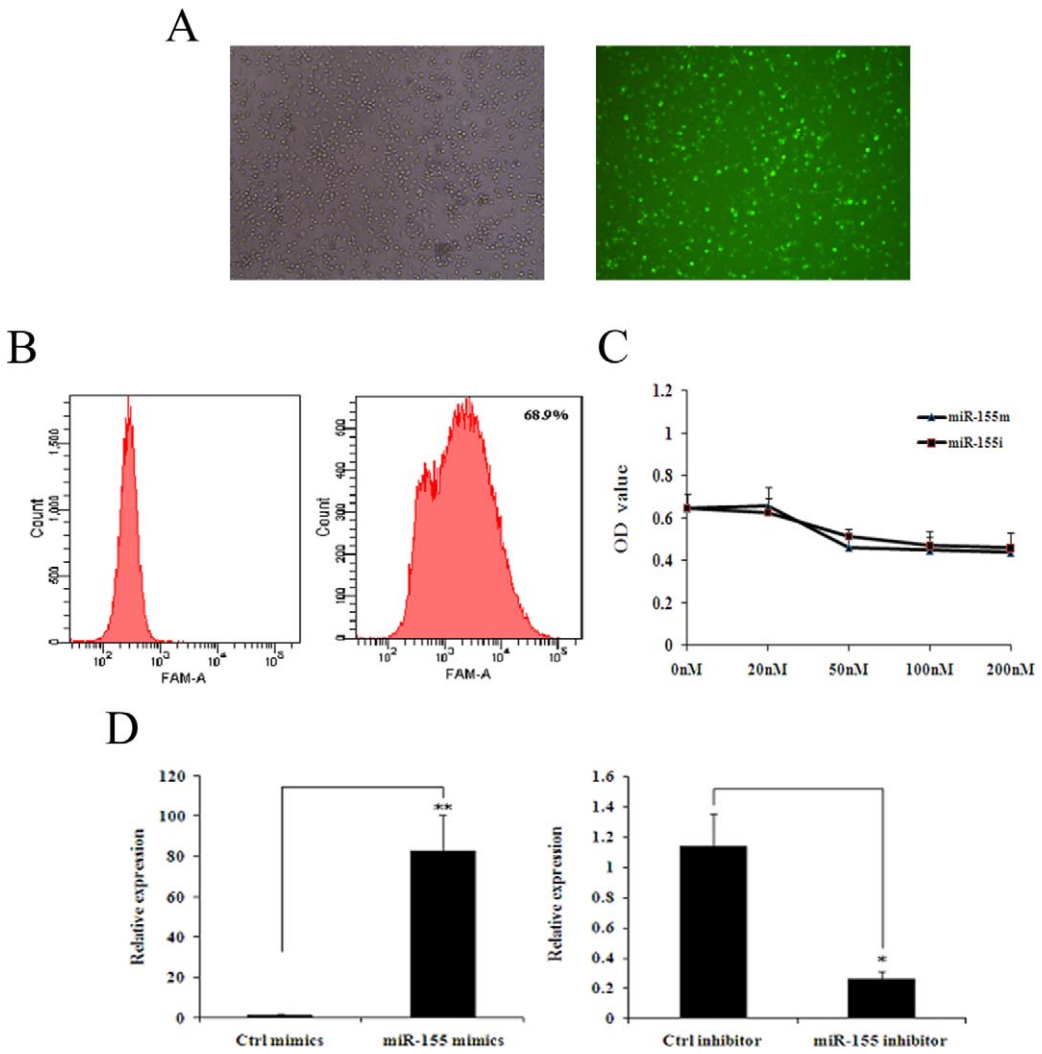

Figure 2. Expression of miRNA-155 in mo-DCs. (**A**) Human mo-DCs transfected with FAM-labeled miRNA mimics control were observed by inverted fluorescent microscopy. (**B**) Human mo-DCs transfected with FAM-labeled miRNA mimics control were detected by flow cytometry. (**C**) Cell viability as measured by CCK8 in DCs cultured in the presence of various concentrations of miR-155 mimics and inhibitor. (**D**) Human mo-DCs were transfected with miR-155 mimics or control mimics (*left*), miR-155 inhibitor or control inhibitor (*right*), as indicated at a final concentration of 100 nM. After 48 hours, miR-155 expression was measured by real-time quantitative PCR and normalized to the expression of U6. Data are the mean ± SD ($n = 5$) of one representative experiment.

miR-155 Inhibits the Production of IL-6 and IL-1β, and Promotes the Production of IL-10 by imDCs, but Has No Effect on DC Maturation

As IL-6, IL-1β, IFN-γ, and IL-10 have been found to play an important role in immune regulation, a further in vitro study was designed to examine whether the miR-155 had an effect on the production of these cytokines. As shown in Figure 2, the different concentrations of mimics or inhibitors did not influence the viability of DCs and in vitro infection of mimics led to stable exogenous gene expression with approximately 70% efficiency in purified DCs, as indicated by the carboxyfluorescein (FAM) reporter fluorescence. The miR-155 mimics and inhibitors were successfully transfected to imDCs, as shown by their overexpression and a decreased expression of miR-155 respectively. DCs overexpressing miR-155 were shown to significantly inhibit the production of IL-6 and IL-1β and promoted the expression of IL-10 as compared with those transfected with an irrelevant sequence control. DCs with a decreased expression of miR-155 were able to produce higher amounts of IL-6 and IL-1β but a lower level of IL-10 as compared with controls (Fig. 3). There was no detectable IFN-γ production either in DCs overexpressing miR-155 or in those with a decreased expression of miR-155. Another experiment was performed to examine whether miR-155 could influence the maturation of DCs. The results showed that neither the transfection of miR-155 mimics nor the transfection of miR-155 inhibitor had an effect on the expression of the tested surface markers including CD80, CD40, CD83, CD86, and HLA-DR (Fig. 4).

miR-155 is Negatively Associated with Intracellular IL-17 Expression of $CD4^+$ T Cells in a T Cell/DC Coculture System

To investigate the intrinsic effect of DCs on the production of IL-17 by $CD4^+$ T cells, an important proinflammatory cytokine, imDCs were transfected with miR-155 mimics or inhibitor and

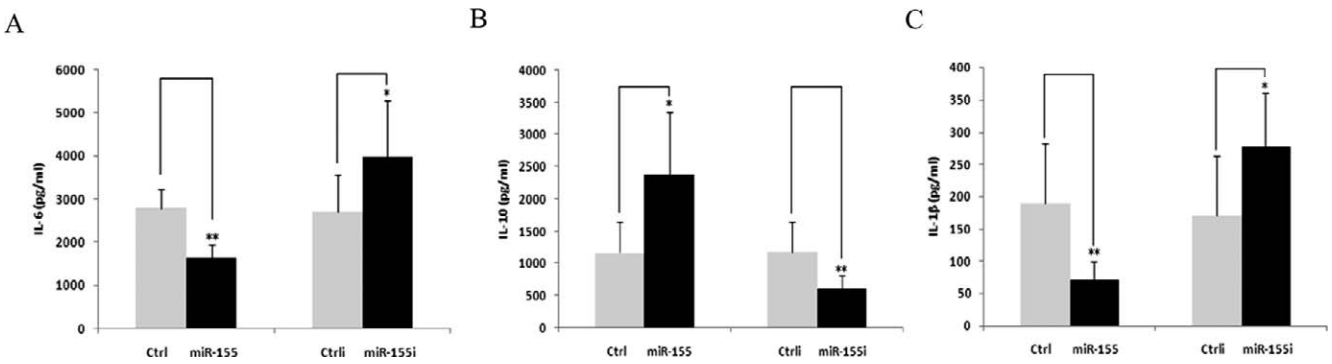

Figure 3. miR-155 negatively regulates LPS-induced cytokine production in mo-DCs. Human imDCs (1.5×10^6) were transfected with control mimics, miR-155 mimics, control inhibitor, and miR-155 inhibitor at a final concentration of 100 nM. After 48 hours, DCs were stimulated with 100 ng/mL LPS for 24 hours. IL-6 (**A**), IL-10 (**B**), and IL-1β (**C**) in supernatants were measured by ELISA ($n = 8$–10 per group). Data are shown as mean ± SD. Ctrl, ctrl mimics; miR-155, miR-155 mimics; Ctrli, ctrl inhibitor; miR-155i, miR-155 inhibitor. $^*P < 0.05$; $^{**}P < 0.01$.

were then cocultured with allogeneic $CD4^+$ T cells. The results showed that imDCs containing miR-155 mimics significantly inhibited intracellular IL-17 expression by $CD4^+$ T cells as compared with those containing an irrelevant sequence control. Coculture of immature DCs transfected with miR-155 inhibitor resulted in a higher expression of IL-17 by $CD4^+$ T cells as compared with cocultures with DCs transfected with an irrelevant sequence control (Fig. 5).

miR-155 Significantly Inhibits the Expression of Functional mRNA Target

To gain insight into the molecular mechanism of miR-155, we used bioinformatics tools to identify its potential target. From TargetScan and miRBase, we discovered miR-155 base pairs with sequences in the 3′-UTR of TAB2 (Fig. 6A). TAB2 plays a

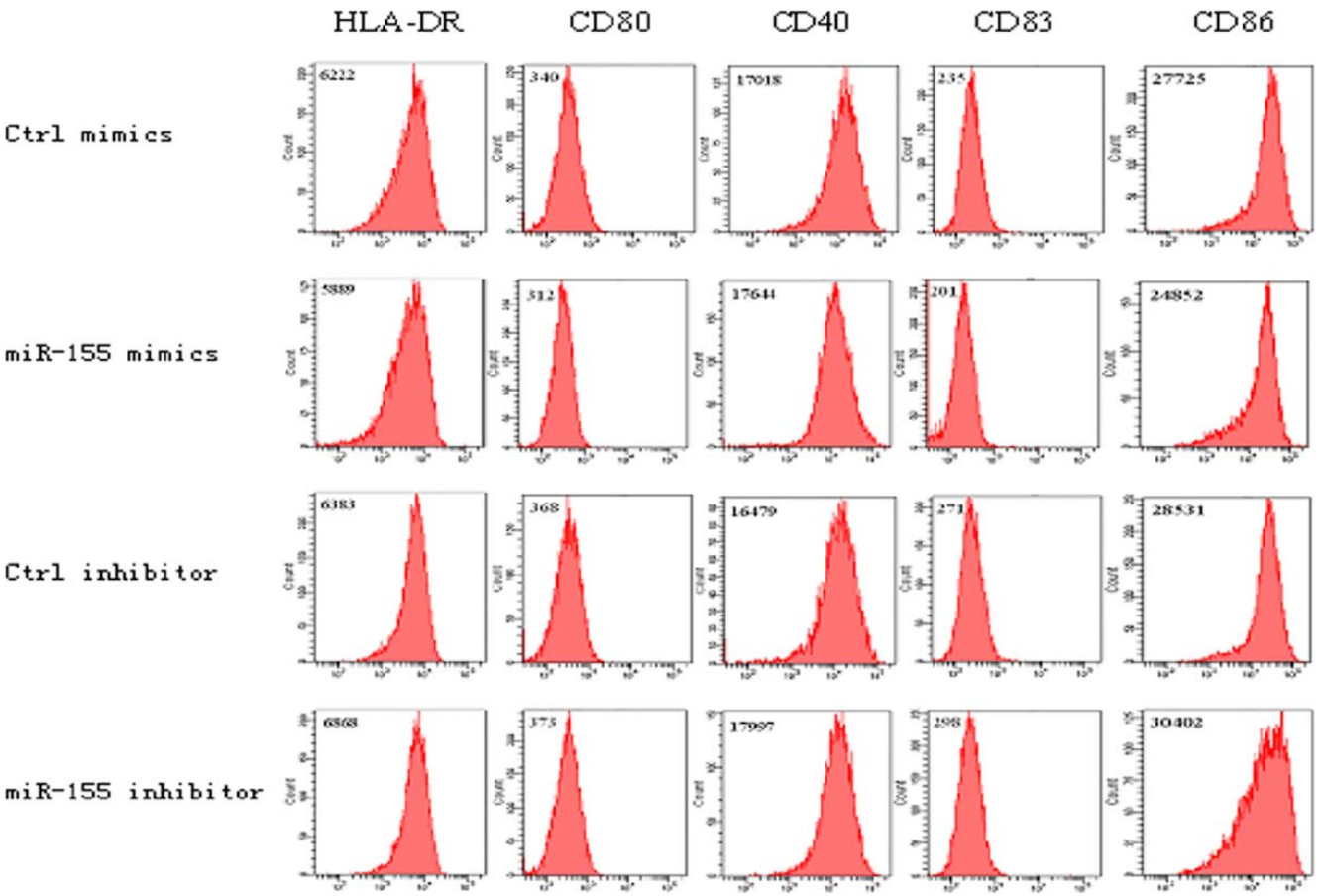

Figure 4. Cell surface marker expression on DCs by miR-155. Human imDCs were transfected with control mimics and miR-155 mimics or control inhibitor and miR-155 inhibitor at a final concentration of 100 nM, respectively. After 48 hours, DCs were stimulated with 100 ng/mL LPS for 24 hours, then stained with specific Ab against CD80, CD40, CD83, CD86, and HLA-DR, and analyzed by flow cytometry. Numbers in histograms indicate the mean fluorescence of DCs in each group. The histograms shown are from a representative experiment. Similar results were obtained in five independent experiments.

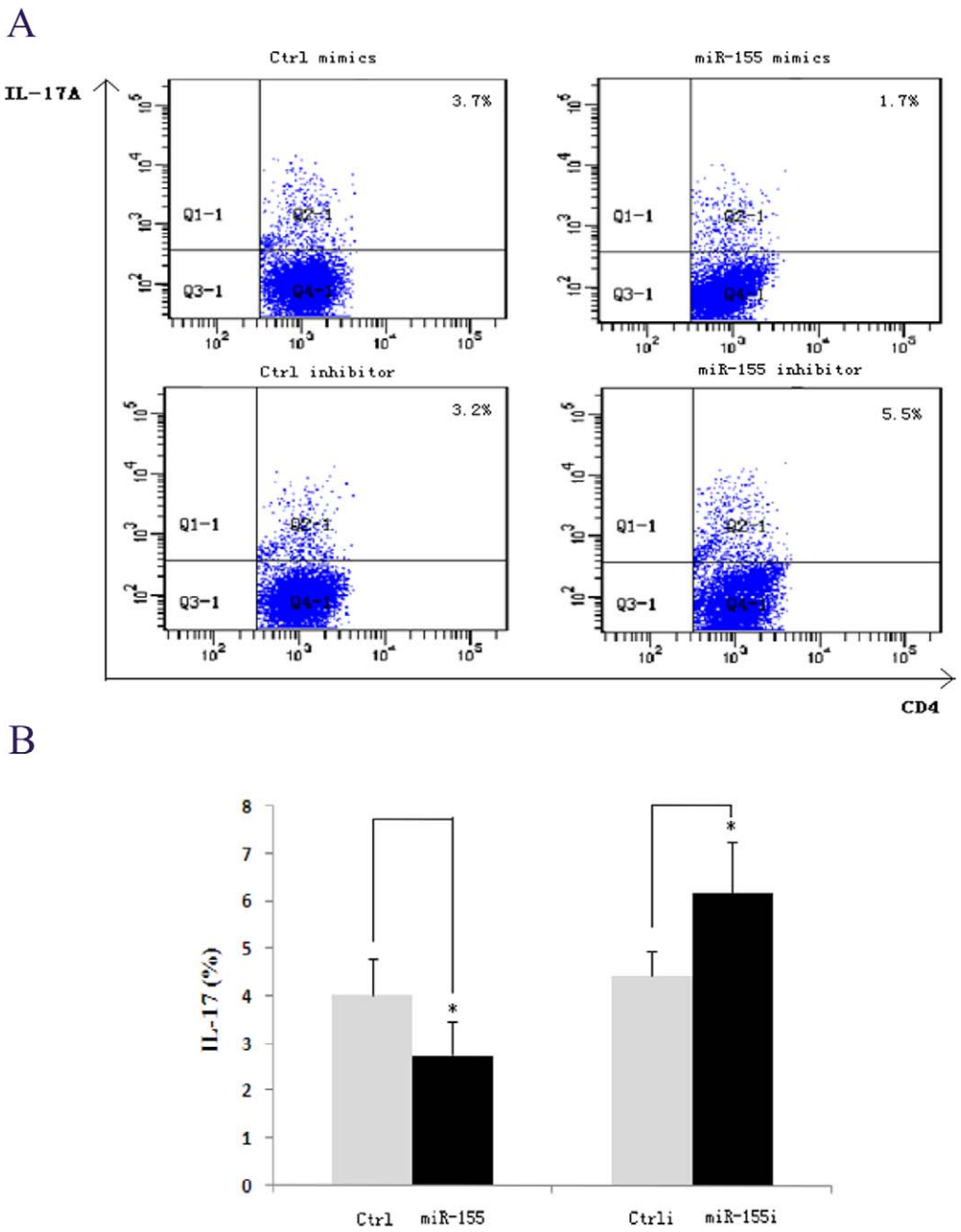

Figure 5. Effect of DC transfection with miR-155 mimics and miR-155 inhibitor on IL-17 expression of $CD4^+$ T cells. Transfected DCs were cocultured with $CD4^+$ T cells, whereafter $CD4^+$ T cells were recovered, stimulated with PMA/ionomycin for 5 hours, and subjected to FACS analysis (1×10^4 cells). (**A**) Representative dot plots from five independent experiments. Numbers indicate percentages of positive cells in that quadrant. (**B**) Quantitative analysis of IL-17–expressing $CD4^+$ T cells. Data are shown as mean ± SD ($n = 5$ per group). Ctrl, ctrl mimics; miR-155, miR-155 mimics; Ctrli, ctrl inhibitor; miR-155i, miR-155 inhibitor. $^*P < 0.05$.

role in the TLR/IL-1 signal transduction cascade and was selected as a candidate in this study.[25] We then tested whether miR-155 could directly repress the identified mRNA targets through 3′-UTR interactions. Our results showed that overexpression of miR-155 significantly inhibited luciferase activity of a reporter containing the wild-type TAB2 3′-UTR but not that of a reporter with a mutated 3′-UTR, which indicated that miR-155 specifically targets via the predicted binding sites (Fig. 6B). A further experiment was also performed to introduce miR-155 mimics and inhibitor into mo-DCs followed by the analysis of TAB2 expression using Western blotting. The results showed that miR-155 suppressed the expression of TAB2 at the protein level (Figs. 6C, 6D). In view of the decreased expression of miR-155 in active BD patients compared with the healthy controls and the direct inhibitory effect of miR-155 on target TAB2, we further investigated the TAB2 protein expression in DCs from BD patients with active uveitis. The result showed that the expression of TAB2 protein was increased in DCs from BD patients with active uveitis compared with healthy controls (Figs. 6E, 6F).

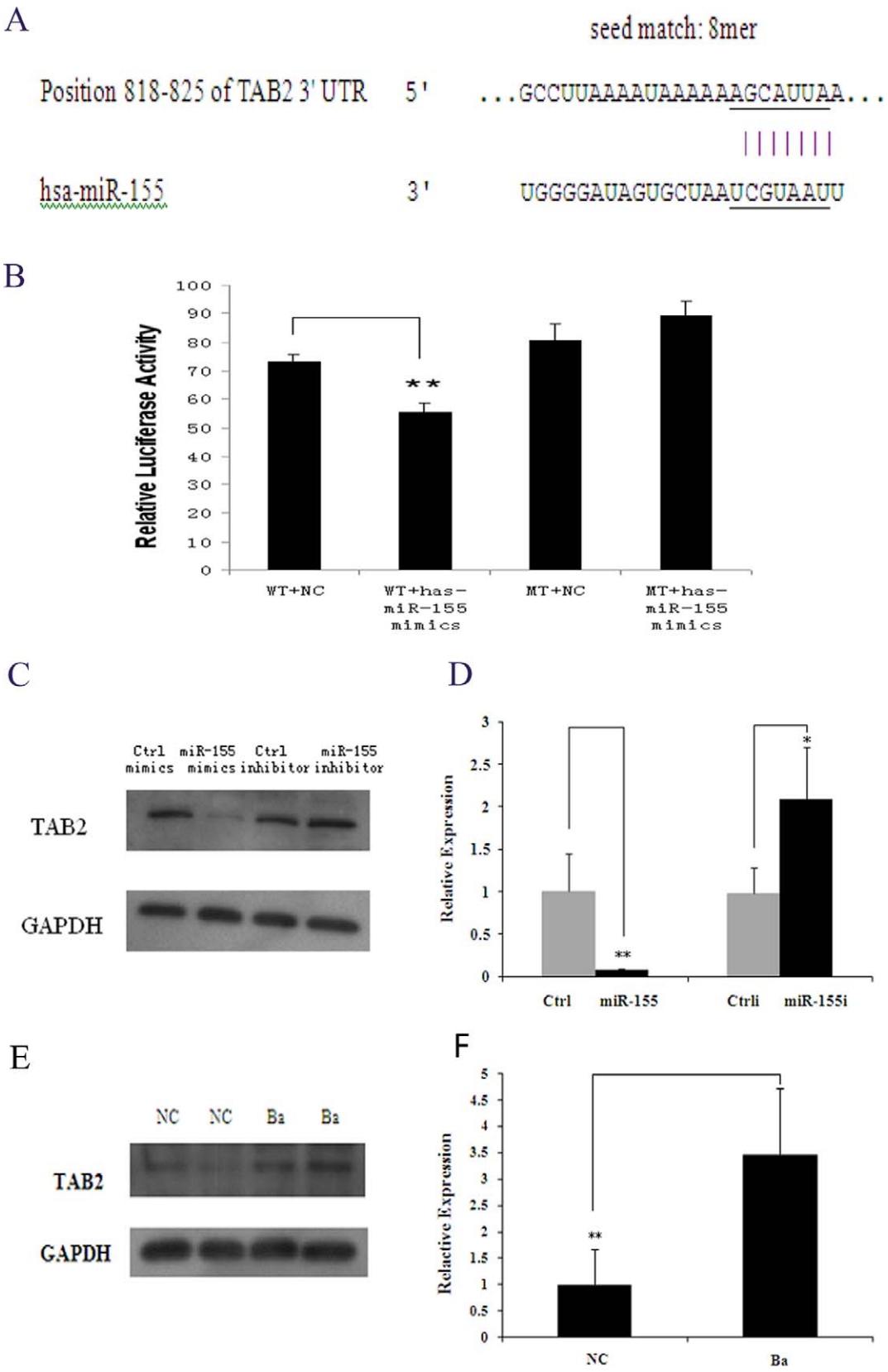

Figure 6. miR-155 targets human TAB2. (**A**) Predicted interaction between the miR-155 seed region and its target sites match on 3′-UTR of human TAB2 mRNA, determined from TargetScan (http://www.targetscan.org). (**B**) HEK293T cells were cotransfected with 200 ng wild-type or mutated TAB2 3′-UTR firefly luciferase reporter plasmids, 4 ng pRL-CMV Renilla luciferase reporter plasmids, together with either negative control or the has-miR-155 mimics. Luciferase activities were measured 24 hours after transfection using the Dual-Luciferase Reporter Assay System. Data are the mean ± SD ($n = 6$) of one representative experiment. Similar results were obtained in at least three independent experiments. (**C**) Human mo-DCs (1.5×10^6) were transfected with control mimics and miR-155 mimics or control inhibitor and miR-155 inhibitor at a final concentration of 100 nM as

indicated. After 48 hours, TAB2 protein was detected by Western blotting. GAPDH served as a loading control. The immunoreactive bands shown are from a representative experiment. Similar results were obtained from four independent experiments. (**D**) The band intensities were quantified and statistical analysis was performed. NC, negative control; WT, wild-type 3′-UTR luciferase reporter; MT, mutated 3′-UTR luciferase reporter. Ctrl, ctrl mimics; miR-155, miR-155 mimics; Ctrli, ctrl inhibitor; miR-155i, miR-155 inhibitor. (**E**) Human mo-DCs (1.5×10^6) were from active BD patients and healthy controls. TAB2 protein was detected by Western blotting. GAPDH served as a loading control. The immunoreactive bands shown are from a representative experiment. Similar results were obtained from five independent experiments. (**F**) The band intensities were quantified and statistical analysis was performed. NC, negative control; Ba, BD patients with active uveitis. $^*P < 0.05$; $^{**}P < 0.01$.

Discussion

Since the discovery of miRNAs, tremendous effort has been devoted to determining their biologic functions and a potential link between miRNAs and various diseases in humans. In the present study, we investigated a possible involvement of miRNAs in the pathogenesis of two uveitis entities, namely BD and VKH. The results showed a significantly decreased expression of miR-155 in PBMCs and mo-DCs of BD patients with active uveitis but not in VKH patients with active uveitis. The miR-155 transfected DCs were shown to inhibit the production of IL-6 and IL-1β in association with an upregulated IL-10 expression. They were also able to significantly inhibit intracellular IL-17 expression of allogeneic $CD4^+$ T cells. Luciferase reporter assays and Western blotting identified TAB2 as the target gene of miR-155. Moreover, we found an increased expression of TAB2 in DCs from BD patients with active uveitis as compared with healthy controls. These results collectively suggest that downregulated miR-155 expression may be involved in the pathogenesis of BD.

The miRNAs are believed to be involved in the physiological or pathological processes in vivo by targeting multiple functionally related proteins or a key protein target.[26,27] A number of miRNAs have been shown to play a critical role in the immune system or inflammatory response by regulating the differentiation of various immune cell subsets.[28–30] In the present study, five known immunologically relevant miRNAs based on miRBase and relevant reports were selected as candidates and their expression was investigated in BD patients, VKH patients, and controls. We found a significantly downregulated expression of miR-155 in the PBMCs and DCs of BD patients with active uveitis. Interestingly, we found that a decreased expression of miR-155 was observed only in active BD patients but not in active VKH patients. This result suggests that downregulated expression of miR-155 may be a unique event in BD patients with active uveitis and that different mechanisms are involved in BD as compared with VKH disease. Our result is generally consistent with earlier reports by Rodriguez et al.[31] They have shown that miR-155 knockout mice suffer from an exaggerated autoimmune response in the lungs with marked leukocyte invasion.[31] However, our result is somewhat different from that reported in the RA and in the experimental autoimmune encephalomyelitis (EAE) model.[18,32] In RA patients, an increased miR-155 expression was observed in synovial fibroblasts compared with those from osteoarthritis patients.[32] In an EAE model, $CD4^+$ T cells were shown to have a higher expression of miR-155 and silencing of miR-155 was to shown to ameliorate disease activity.[18] The reasons as to the controversies are not understood but could be due to the origins of the tested cells or the differences in the disease models employed. Recently, Ishida et al.[33] reported an upregulated expression of miR-142-5p and miR-21 and a downregulated expression of miR-182 in association with an increased IL-17 expression in experimental autoimmune uveoretinitis. It would be interesting to investigate the expression of these miRNAs and their role in BD and VKH disease in future studies.

In view of the decreased expression of miR-155 in active BD patients, we further investigated whether this downregulated miR-155 correlated with serum levels of certain relevant cytokines; however, we did not find any association of miR-155 and the production of relevant cytokines because the serum level of IL-1β and IL-17 remained below the detection limit of the assay we used. Because earlier reports showed that the serum IL-6 level was also undetectable in BD patients and healthy controls,[34] we did not compare the relationship of downregulated miR-155 and the expression of this cytokine. A further study was designed to explore whether miR-155 could influence in vitro cytokine production and the maturation of DCs, a population critical for the induction of immune response and immune tolerance.[35,36] The results showed that overexpression of miR-155 significantly inhibited the LPS-induced production of pro-inflammatory cytokines including IL-6 and IL-1β, but increased the expression of anti-inflammatory cytokines such as IL-10. However, it did not influence the maturation of DCs. These results were, by and large, in agreement with those reported earlier.[31] In the latter study, the expression level of major histocompatibility complex II and costimulatory molecules, important indexes of DC maturation, of bone marrow–derived DCs in miR-155-deficient mice was normal, whereas the stimulatory ability of these DCs for T-cell proliferation was significantly impaired. These results suggest that miR-155 could exert its role in BD pathogenesis possibly through modulating relevant cytokines produced by DCs rather than by regulating their functional status. As DCs exert their function mainly through modulating T cells,[37] we further investigated the effect of DCs with up- or downregulated miR-155 expression on IL-17 production by allogeneic $CD4^+$ T cells. Interestingly, we found that overexpression of miR-155 could significantly inhibit intracellular IL-17 production by $CD4^+$ T cells, whereas downregulated miR-155 expression promoted intracellular IL-17 production by $CD4^+$ T cells. Collectively, these results suggest that a decreased miR-155 expression may lead to the production of certain proinflammatory cytokines and downregulated expression of IL-10, an important anti-inflammatory cytokine, thereby contributing to BD development.

To further explore the mechanisms involved in the cytokine regulation of miR-155, further experiments were performed to identify the target gene for miR-155. Among the potential direct mRNA targets of miR-155, a number of targets have been identified in murine or in human systems, such as TAB2, c-Maf, PU.1, SHIP1, AGTR1, and MMP3.[25,31,32,38–40] In this study, we focused on TAB2 as a target gene of miR-155 in view of its role in the modulation of IL-1β and IL-6, two important cytokines involved in autoimmune and inflammatory disease pathogenesis. As a multifunctional signaling molecule, TAB2 is a part of a molecular complex containing the TNF receptor–associated factor 6 (TRAF6) and has been shown to facilitate IL-1–dependent TRAF6 ubiquitination.[41] It promotes activation of the inflammatory response on TLR4 or IL-1 receptor triggering and activation of the TLR/IL-1 signaling pathway.[25] Our studies used both Luciferase reporter assays and Western blotting experiments to identify TAB2 as a target of miR-155 in BD. These findings are in agreement with an earlier report.[25]

Furthermore, in order to investigate whether the observed decrease in miR-155 expression to active BD patients could directly correlate TAB2 expression, we measured TAB2 protein expression in DCs from active BD patients and found an increased expression of TAB2 in BD patients with active uveitis compared with healthy controls. Based on our results and previous studies, it is likely that a downregulated miR-155 expression leads to a dysregulation in the control of the TLR/IL-1 signaling transduction pathway through at least partially targeting TAB2.

It is worthwhile to point out that there are some limitations in our study. Our result showed a decreased expression of miR-155 in DCs in BD patients with active uveitis as compared with VKH patients with active uveitis. It is not clear whether the decreased miR-155 expression is also present in other uveitis entities, such as idiopathic acute or chronic anterior uveitis, ocular sarcoidosis, and retinal vasculitis. Furthermore, our study focused on only one target gene, TAB2. It is not clear whether the effect of miR-155 observed in this study is mediated solely by TAB2 or by a combination of a number of target genes.

In conclusion, our study revealed that miR-155 expression was downregulated in active BD patients. MiR-155 was able to negatively regulate the production of proinflammatory cytokines by DCs via TLR/IL-1 signaling cascade by targeting TAB2. The downregulated miR-155 expression could be involved in the pathogenesis of BD due to a loss of control concerning the production of proinflammatory cytokines by DCs and $CD4^+$ T cells.

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References

1. Gul A. Behcet's disease as an autoinflammatory disorder. *Curr Drug Targets Inflamm Allergy*. 2005;4:81–83.
2. Direskeneli H. Autoimmunity vs autoinflammation in Behcet's disease: do we oversimplify a complex disorder? *Rheumatology (Oxford)*. 2006;45:1461–1465.
3. Sakane T, Takeno M, Suzuki N, Inaba G. Behcet's disease. *N Engl J Med*. 1999;341:1284–1291.
4. Moorthy RS, Inomata H, Rao NA. Vogt-Koyanagi-Harada syndrome. *Surv Ophthalmol*. 1995;39:265–292.
5. Murakami S, Inaba Y, Mochizuki M, Nakajima A, Urayama A. A nation-wide survey on the occurrence of Vogt-Koyanagi-Harada disease in Japan [in Japanese]. *Nihon Ganka Gakkai Zasshi*. 1994;98:389–392.
6. Rao NA. Mechanisms of inflammatory response in sympathetic ophthalmia and VKH syndrome. *Eye (Lond)*. 1997;11(Pt 2); 213–216.
7. de Smet MD, Dayan M. Prospective determination of T-cell responses to S-antigen in Behcet's disease patients and controls. *Invest Ophthalmol Vis Sci*. 2000;41:3480–3484.
8. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev*. 2006;20:515–524.
9. Liu J. Control of protein synthesis and mRNA degradation by microRNAs. *Curr Opin Cell Biol*. 2008;20:214–221.
10. Bartel DP, Chen CZ. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat Rev Genet*. 2004;5:396–400.
11. Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A*. 2004; 101:2999–3004.
12. Lecellier CH, Dunoyer P, Arar K, et al. A cellular microRNA mediates antiviral defense in human cells. *Science*. 2005;308: 557–560.
13. Lu LF, Liston A. MicroRNA in the immune system, microRNA as an immune system. *Immunology*. 2009;127:291–298.
14. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A*. 2006;103:12481–12486.
15. Haasch D, Chen YW, Reilly RM, et al. T cell activation induces a noncoding RNA transcript sensitive to inhibition by immunosuppressant drugs and encoded by the proto-oncogene, BIC. *Cell Immunol*. 2002;217:78–86.
16. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci U S A*. 2007;104:1604–1609.
17. Thai TH, Calado DP, Casola S, et al. Regulation of the germinal center response by microRNA-155. *Science*. 2007;316:604–608.
18. Murugaiyan G, Beynon V, Mittal A, Joller N, Weiner HL. Silencing microRNA-155 ameliorates experimental autoimmune encephalomyelitis. *J Immunol*. 2011;187:2213–2221.
19. Wang G, Tam LS, Li EK, et al. Serum and urinary cell-free MiR-146a and MiR-155 in patients with systemic lupus erythematosus. *J Rheumatol*. 2010;37:2516–2522.
20. Kurowska-Stolarska M, Alivernini S, Ballantine LE, et al. MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis. *Proc Natl Acad Sci U S A*. 2011;108: 11193–11198.
21. Criteria for diagnosis of Behcet's disease. International Study Group for Behcet's Disease. *Lancet*. 1990;335:1078–1080.
22. Read RW, Holland GN, Rao NA, et al. Revised diagnostic criteria for Vogt-Koyanagi-Harada disease: report of an international committee on nomenclature. *Am J Ophthalmol*. 2001;131:647–652.
23. Hou J, Wang P, Lin L, et al. MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. *J Immunol*. 2009;183: 2150–2158.
24. Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y, Ueno K. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol Pharm Bull*. 1996;19: 1518–1520.
25. Ceppi M, Pereira PM, Dunand-Sauthier I, et al. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proc Natl Acad Sci U S A*. 2009;106:2735–2740.
26. Xiao C, Rajewsky K. MicroRNA control in the immune system: basic principles. *Cell*. 2009;136:26–36.
27. Hoefig KP, Heissmeyer V. MicroRNAs grow up in the immune system. *Curr Opin Immunol*. 2008;20:281–287.
28. Lin YC, Kuo MW, Yu J, et al. c-Myb is an evolutionary conserved miR-150 target and miR-150/c-Myb interaction is important for embryonic development. *Mol Biol Evol*. 2008; 25:2189–2198.
29. Xiao C, Calado DP, Galler G, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell*. 2007;131:146–159.
30. Du C, Liu C, Kang J, et al. MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. *Nat Immunol*. 2009;10:1252–1259.
31. Rodriguez A, Vigorito E, Clare S, et al. Requirement of bic/microRNA-155 for normal immune function. *Science*. 2007; 316:608–611.

32. Stanczyk J, Pedrioli DM, Brentano F, et al. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. *Arthritis Rheum*. 2008;58:1001–1009.
33. Ishida W, Fukuda K, Higuchi T, Kajisako M, Sakamoto S, Fukushima A. Dynamic changes of microRNAs in the eye during the development of experimental autoimmune uveoretinitis. *Invest Ophthalmol Vis Sci*. 2011;52:611–617.
34. Yamakawa Y, Sugita Y, Nagatani T, et al. Interleukin-6 (IL-6) in patients with Behcet's disease. *J Dermatol Sci*. 1996;11:189–195.
35. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*. 2010;11:373–384.
36. Liu YJ, Kanzler H, Soumelis V, Gilliet M. Dendritic cell lineage, plasticity and cross-regulation. *Nat Immunol*. 2001;2:585–589.
37. Liu X, Zhan Z, Xu L, et al. MicroRNA-148/152 impair innate response and antigen presentation of TLR-triggered dendritic cells by targeting CaMKIIalpha. *J Immunol*. 2010;185:7244–7251.
38. O'Connell RM, Rao DS, Chaudhuri AA, et al. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med*. 2008;205:585–594.
39. Costinean S, Sandhu SK, Pedersen IM, et al. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of Emicro-MiR-155 transgenic mice. *Blood*. 2009;114:1374–1382.
40. Sethupathy P, Borel C, Gagnebin M, et al. Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3′ untranslated region: a mechanism for functional single-nucleotide polymorphisms related to phenotypes. *Am J Hum Genet*. 2007;81:405–413.
41. Takaesu G, Kishida S, Hiyama A, et al. TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol Cell*. 2000;5:649–658.