MicroRNA-155 regulates the Th17 immune response by targeting Ets-1 in Behçet's disease

S.Y. Na[1], M.-J. Park[1], S. Park[2], E.-S. Lee[1]

[1]Department of Dermatology and
[2]Department of Microbiology, Ajou University School of Medicine, Suwon, South Korea.

So Young Na, MD, PhD
Mi-Jin Park, MS
Sun Park, MD, PhD
Eun-So Lee, MD, PhD

Please address correspondence to:
Eun-So Lee, MD, PhD,
Department of Dermatology,
Ajou University School of Medicine,
164 World cup-ro,
Yeongtong-gu, Suwon-si,
Gyeonggi-do, 16499 South Korea.
E-mail: esl@ajou.ac.kr

Received on October 28, 2015; accepted in revised form on April 5, 2016.

Clin Exp Rheumatol 2016; 34 (Suppl. 102): S56-S63.

Key words: Behçet's disease, Th17 cells, microRNA-155, Ets-1

Funding: this research was supported by a grant from the Korea Healthcare Technology R&D Project Ministry of Health, Welfare and Family Affairs, Republic of Korea (A101936).

Competing interests: none declared.

ABSTRACT

Objective. *The goal of this study was to investigate whether microRNA-155 might be a potential therapeutic target for Behçet's disease (BD).*

Methods. *Expression levels of miR-155 were quantified using TaqMan microRNA assays in peripheral blood mononuclear cells and in isolated CD4⁺ T cells from BD patients and healthy controls. To identify targets of miR-155, luciferase reporter assays and western blotting were performed. The effect of miR-155 on Th17 polarisation was analysed in patients with active BD by using flow cytometry and enzyme-linked immunosorbent assay.*

Results. *The expression of miR-155 and IL-17 was significantly increased in CD4⁺ T cells of patients with active BD. A luciferase reporter assay and western blot showed that Ets-1 expression was reduced by miR-155 mimics. Furthermore, the expression of Ets-1 was significantly decreased in patients with active BD compared to healthy controls. More importantly, repression of miR-155 in CD4⁺ T cells from active BD patients increased Ets-1 expression and reduced the number of IL-17-expressing T cells and overall IL-17 production.*

Conclusion. *MiR-155 regulates the Th17 immune response by targeting Ets-1. Suppression of miR-155 reduced the amount of pathogenic IL-17-expressing T cells and may be a potential therapeutic strategy for BD.*

Introduction

Behçet's disease (BD) is a chronic inflammatory disease characterised by recurrent oral and genital ulcers, uveitis, and skin lesions (1). Although the pathogenesis and aetiology of BD remain unclear, a critical role for Th17 cells in acute attacks has been reported (2-4). However, the molecular mechanisms underlying Th17 polarisation in BD remain to be elucidated.

MicroRNAs (miRNAs) are small non-coding RNA molecules, which regulate host gene expression by binding to the 3´ untranslated region (UTR) of their target messenger RNA, thus targeting them for degradation or translational repression at the post-transcriptional level (5). Recent studies on miRNAs have demonstrated aberrant expression and/or function of miRNAs in chronic inflammatory and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (MS) (5, 6). To date, miRNA-155 (miR-155) is one of the most common miRNAs implicated in chronic inflammation and autoimmunity (7, 8). Additionally, it has been shown that miR-155 regulates megakaryopoiesis and the expression of inflammatory molecules by targeting Ets-1 in endothelial cells (9, 10). Moisan *et al*. (11) reported that Ets-1 has an inhibitory role on Th17 differentiation in mice. However, it is not known whether Ets-1 is a target of miR-155 in human CD4⁺ T cells or whether it is associated with Th17 differentiation in BD. In this study, the expression and function of miR-155 and its potential as a therapeutic target in BD were investigated in human cells.

Materials and methods

Patients

The study enrolled 46 BD patients, who were treated at the Department of Dermatology of Ajou University Hospital. Eighteen patients diagnosed with recurrent aphthous ulcer (RAU) without any other evident disease were classified as the disease control group, and 23 healthy volunteers were recruited to the healthy control (HC) group. All BD patients met the international criteria for the diagnosis of BD (12), as well as the diagnostic criteria of the BD Research Committee of Japan (13). BD patients were divided into either the group with active disease, when they presented with at least one

BD symptom despite treatment, or the group with inactive disease, when their BD was in a well-controlled state. Enrolled patients did not take high-dose systemic corticosteroids or any other immunosuppressive agent for at least 1 month prior to blood sampling. Demographics and clinical characteristics of patients and controls are summarised in Table I. This study was approved by the Institutional Review Board of Ajou University (AJIRB-GEN-GEN-11-243).

Table I. Demographics and clinical characteristics of patients and controls.

Characteristics	Active BD (n=24)	Inactive BD (n=22)	RAU (n=18)	HC (n=23)
Age, years[1]	44.5 ± 6.3	41.7 ± 8.6	44.4 ± 9.6	39.0 ± 4.7
Sex, no. male/female	6/18	13/9	5/13	6/17
ESR, mm/hr[1]	31.3 ± 23.5	13.7 ± 9.2	21.3 ± 20.5	–
CRP, mg/dL[1]	1.6 ± 4.0	0.3 ± 0.3	0.3 ± 0.6	–
Medications, no. of patients				
Steroids (≤10 mg)[2]	4	5	2	–
Colchicine	10	19	6	–

[1]Mean± standard deviation, [2]Prednisone or equivalent dosage of another steroid. Dosages of other steroids were converted to prednisone equivalents (*e.g.* 40 mg of methylprednisolone was considered equivalent to 50 mg of prednisone).
BD: Behçet's disease; RAU: recurrent aphthous ulcer; HC: healthy control; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

Cell culture and condition of Th17 polarisation

Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Paque PLUS (GE Healthcare, Freiburg, Germany). $CD4^+$ T cells were isolated using the EasySep™ Human CD4 Selection Kit (StemCell Tech., Vancouver, BC, Canada) according to the manufacturer's instructions. PBMCs or $CD4^+$ T cells were resuspended in RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin with fetal bovine serum (GIBCO-BRL) and cultured at 37°C under a 5% CO_2 atmosphere. Cells were activated with anti-CD3 (100 ng/mL, clone HIT3a) and anti-CD28 (200 ng/mL, clone CD28.2), and differentiated into Th17 cells by supplementation with IL-1β (10 ng/mL), IL-6 (20 ng/mL), IL-23 (100 ng/mL), TNF-α (10 ng/mL), and TGF-β (1 ng/mL) (R&D Systems Inc., Minneapolis, MN) for 5 days. During the final 4 h, 2 μM of monensin (eBiosciences, San Diego, CA) was added to the stimulated PBMCs.

Transfection of $CD4^+$ T cells with microRNA

$CD4^+$ T cells were transfected with a miR-155 mimic, miR-155 hairpin inhibitor (Dharmacon, Chicago, IL), or miR-155 locked nucleic acid (LNA) inhibitor (Exiqon, Woburn, MA) using the Neon™ Transfection System (Invitrogen, Carlsbad, CA, USA) or Lipofectamine™ 2000 (Invitrogen). Negative control mimics or inhibitors (Dharmacon) were used as matched controls. MiR-155 LNA inhibitor was used for the long-term inhibition of miR-155 in $CD4^+$ T cells (14).

Flow cytometric analysis

Intracellular cytokine staining was performed using BD Cytofix/Cytoperm (BD Pharmingen, San Jose, CA). Cells were labelled with APC-conjugated anti-CD4 and PE-conjugated anti-IL-17 (BD Biosciences Pharmingen, San Diego, CA) antibodies and then analysed by multi-colour flow cytometry (BD FACS CantoII, San Jose, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

IL-17 concentrations in serum and in culture supernatants were measured using ELISA kits (R&D Systems Inc.) and all samples were processed in duplicate.

RNA preparation and real-time polymerase chain reaction

Total RNA was isolated from fresh and stimulated PBMCs with TRIzol® (Invitrogen). Reverse transcription of RNA was performed using dNTP and oligo (dT) primers (Invitrogen). cDNA production from miRNA was performed using the TaqMan® MicroRNA Reverse Transcriptase Kit (Applied Biosystems, Foster, CA). The obtained cDNA was analysed by real-time PCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the $2^{-\Delta\Delta Ct}$ method. Normalisation was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for mRNA or U6 for miR-155.The calibrator sample (ΔCt calibration) was assigned from the HC group. Relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method. Primers and internal probes for miR-155 (Hs002623), IL-17 (Hs00174383_m1), Ets-1 (Hs00428293_m1), U6 (Hs001973), and GAPDH (Hs99999905_m1) were purchased as assay-on-demand primer-probe sets (Applied Biosystems).

Western blot analysis

Briefly, cells were extracted in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing protease inhibitors (Sigma-Aldrich) and extracts were subjected to western blotting using anti-Ets-1 and anti-IL-17 (Abcam, Cambridge, UK) antibodies and Immobilon Western chemiluminescent HRP Substrate (Merck Millipore, Darmstadt, Germany). We used Image J software for quantification of the results.

Luciferase reporter assay

Jurkat T cells were transfected with either pGL3 Ets-13′-UTR plasmid (kindly provided by Dr G. Marziali from Istituto Superiore di Sanità, Italy (10)) or pGL3-Basic, together with 10, 30, or 60 pmol of either a miR-155 mimic (Dharmacon) or a miRNA mimic negative control (Dharmacon) by using the Neon™ Transfection System (Invitrogen). To normalise for the differences in transfection efficacy, cells were co-transfected with pEGFP-N1 plasmid at a ratio of 1:10. After 48 h, cells were washed and lysed according to the manufacturer's protocol (Promega, Madison, WI). Luciferase activity in the cell lysate was measured using a luminometer (Molecular Devices, Sunnyvale, CA).

Statistical methods

Student's *t*-test was performed and *p*-values lower than 0.05 were considered statistically significant.

Results

Upregulation of IL-17 in patients with BD

To verify Th17-mediated inflammation in BD patients, IL-17 expression was measured using flow cytometry and ELISA. IL-17 levels in sera and in cells were below the detection limit in the resting state in all patients and controls (data not shown). On day 5 after Th17 cell polarisation, intracellular IL-17 expression in PBMCs was increased in all experimental groups. The frequency of IL-17-expressing cells was significantly increased in the active and inactive BD groups, as compared to the RAU and HC groups (Fig. 1A). Secretory IL-17 levels in the supernatant on day 5 after polarisation of Th17 cells were also significantly increased in the active and inactive BD groups, as compared to those in the RAU and HC groups (Fig. 1B). Next, the IL-17 mRNA levels in PBMCs were quantified. In PBMCs obtained on day 0, IL-17 mRNA levels were significantly higher in the group of patients with active BD than in the RAU and HC groups (Fig. 1C). On day 5 after Th17 cell polarisation, IL-17 mRNA levels in PBMCs were also increased in the BD groups relative to the control groups, although a significant difference was observed only between the inactive BD and the HC group. Taken together, these results suggest that inflammation in BD is associated with the upregulation of IL-17.

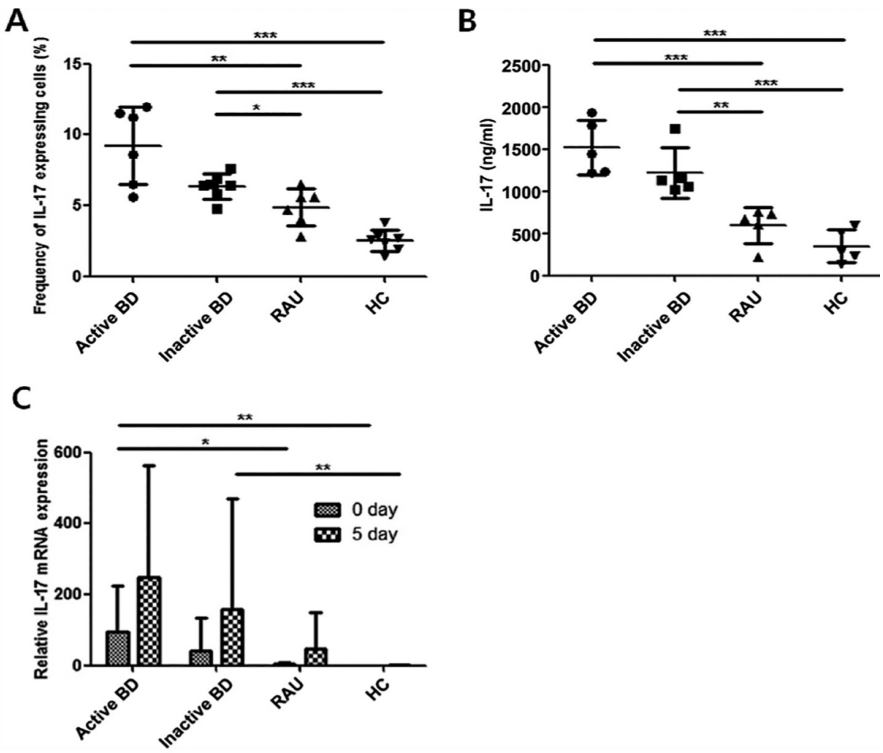

Fig. 1. Upregulation of IL-17 in patients with BD.
PBMCs were cultured in Th17 polarisation conditions for 5 days.
A. The amount of IL-17-expressing cells in PBMCs from active BD (n=6), inactive BD (n=7), RAU (n=6), and HC (n=7) groups after 5 days of cultivation were determined by flow cytometry.
B. IL-17 levels in culture supernatants of PBMCs from active BD (n=5), inactive BD (n=5), RAU (n=5), and HC (n=5) groups after 5 days of cultivation were measured by ELISA.
C. IL-17 mRNA levels in PBMCs from active BD (n=5), inactive BD (n=6), RAU (n=5), and HC (n=7) groups were determined by quantitative real-time PCR. The measured mRNA levels were normalised to those of GAPDH (mean ± SD). *p<0.05, **p<0.01, ***p<0.001.
BD: Behçet's disease; PBMCs: peripheral blood mononuclear cells; RAU: recurrent aphthous ulcer; HC: healthy control; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation.

Suppressed Ets-1 expression in patients with BD

Since Ets-1 was reported as a negative regulator of Th17 differentiation (11), we further investigated Ets-1 expression in PBMCs obtained on day 0 and day 5 from BD patients and control groups. In PBMCs on day 0, Ets-1 mRNA levels were significantly lower in patients with active BD as compared to those in the HC group (Fig. 2A). Although Ets-1 expression was increased after 5 days of culture in the Th17 polarisation condition in all groups, active BD patients showed significantly suppressed Ets-1 mRNA levels compared to those in the HC group. Ets-1 mRNA levels were also significantly decreased in patients with inactive BD compared to those in the RAU and HC groups.

To evaluate Ets-1 protein levels and their relationship with IL-17, western blotting was performed using PBMCs obtained on day 0 and day 5 from patients with active BD and from HCs. As expected, Ets-1 protein levels were significantly decreased in patients with active BD compared to those in the HC group on day 5 (Fig. 2B). In contrast, IL-17 expression was significantly enhanced in patients with active BD compared to that observed in the HC group on day 0 and day 5. Taken together, patients with active BD showed decreased Ets-1 expression and increased IL-17 expression, in contrast to what was observed in HCs.

Ets-1 is a target of miR-155 in T cells

Although Ets-1 was reported as a target of miR-155 in megakaryocytes (10), miRNA targets may differ according to cellular contexts (15). Therefore, we wanted to determine whether Ets-1 mRNA is also a target of miR-155 in T cells. We performed a luciferase reporter assay using the pGL3 Ets-1 3′-UTR plasmid, which contains potential miR-155-binding sites at nucleotides 4570–4592 (Fig. 3A). Jurkat T cells were transfected with different concentrations of miR-155 mimic or miR control, together with pGL3 Ets-1 3′-UTR plasmid. Luciferase activity was significantly repressed by miR-155 co-transfection as compared to miR control co-transfection at a concentration of 60

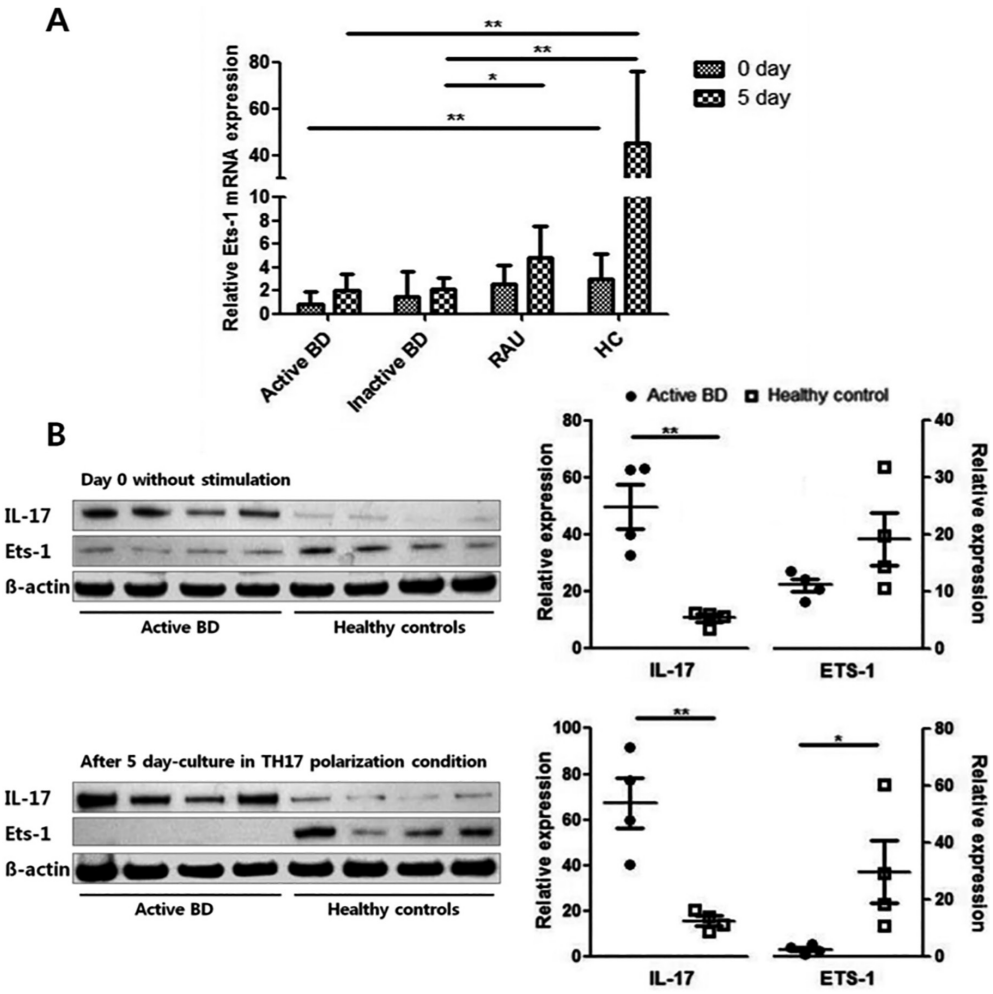

Fig. 2. Suppressed Ets-1 expression in patients with BD.
PBMCs were cultured in Th17 polarisation conditions for 5 days.
A. Expression of Ets-1 in PBMCs from active BD (n=5), inactive BD (n=6), RAU (n=5), and HC (n=7) groups were determined on day 0 and day 5 by quantitative real-time PCR. The measured mRNA levels were normalised to those of GAPDH (mean ± SD).
B. Western blotting and densitometric analysis of Ets-1 and IL-17 were performed for PBMCs from active BD (n=4) and HC (n=4) groups on day 0 and day 5. Protein expression levels were normalised against β-actin levels.
*p<0.05, **p<0.01.
BD: Behçet's disease;
PBMCs: peripheral blood mononuclear cells;
RAU: recurrent aphthous ulcer;
HC: healthy control;
PCR: polymerase chain reaction;
GAPDH: glyceraldehyde-3-phosphate dehydrogenase;
SD: standard deviation.

pmol (p<0.05) (Fig. 3B), indicating that miR-155 targets Ets-1. To further confirm that Ets-1 is a functional target of miR-155, CD4+ T cells from HCs were transfected with a miR-155 mimic or miR-155 hairpin inhibitor. Subsequently, Ets-1 expression was analysed by western blotting. The results indicated that miR-155 significantly suppressed the expression of Ets-1 protein (Fig. 3C), consistent with the results of the luciferase reporter assay. However, Ets-1 expression was not significantly increased in CD4+ T cells transfected with miR-155 hairpin inhibitor compared to matched controls, which may be due to short half-life of the hairpin inhibitor (16). Nevertheless, this result indicates that miR-155 inhibits Ets-1 expression, specifically in CD4+ T cells.

Upregulation of miR-155 in patients with BD

The expression of miR-155 in BD patients was investigated using real-time PCR. MiR-155 levels in PBMCs showed a tendency to increase in patients with active BD as compared to that observed in the control groups, although this observation was not statistically significant in either the resting or the stimulation state (Fig. 4A). In CD4+ T cells, miR-155 expression was significantly increased in patients with active BD compared to that in the RAU and HC groups (Fig. 4B). We also analysed the difference in miR-155 expression with respect to organ involvement. Although there was no statistically significant difference, miR-155 expression in PBMCs showed a tendency to increase in active BD patients with mucocutaneous symptoms than in controls; however, no differences were observed between active BD patients with uveitis and controls. The expression of miR-155 in CD4+ T cells showed no differences with respect to organ involvement (Data are not shown.).

Inhibition of miR-155 in CD4+ T cells impairs IL-17 expression

To evaluate the suppressive effect of miR-155 inhibitor on IL-17 expression, we transfected CD4+ T cells from patients with active BD with miR-155 LNA inhibitor, and the transfected cells were polarised to Th17 cells for 5 days. The frequency of IL-17-expressing CD4+ T cells was significantly decreased in cells transfected with miR-155 LNA inhibitor compared to the cells transfected with miR inhibitor control (Fig. 5A, B). The concentration of IL-17 in the supernatant of the cultures was also significantly decreased in cells transfected with miR-155 LNA inhibitor (Fig. 5C). Overall, a decrease in the number of Th17 cells and in the production of IL-17 was observed upon miR-155 inhibition.

Discussion

In recent years, several studies have demonstrated that Th17 cells play a

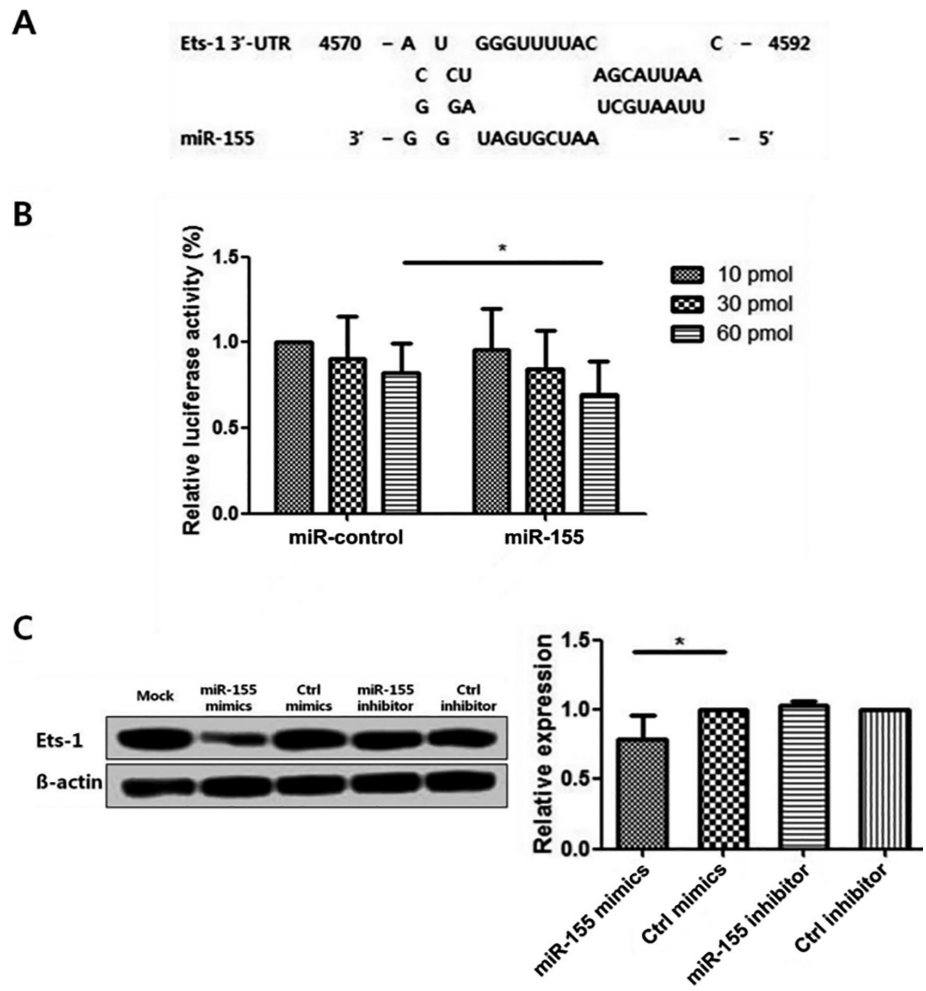

Fig. 3. MiR-155 targets Ets-1 in T cells.
A. Sequence of miR-155 and its potential binding sites in Ets-1 3′-UTR.
B. Relative luciferase activity of a reporter plasmid carrying Ets-1 3′-UTR sites in Jurkat T cells co-transfected with miR-155 mimic or miR control. Data are expressed as mean ± SD of 10 independent experiments.
C. Representative data of western blotting and densitometric analysis of Ets-1 protein levels in $CD4^+$ T cells from the HC group (n=7).
Isolated $CD4^+$ T cells were transfected with 60 pmol of miR-155 mimic, miR-155 hairpin inhibitor, or their matched controls and subjected to western blotting 2 days later. The mock experiment represents the cells that were treated with transfection reagent without oligonucleotides. Ets-1 expression levels were normalised against β-actin levels.
*p<0.05.
MiR-155: microRNA-155;
UTR: untranslated region;
SD: standard deviation;
HC: healthy control.

critical role in the pathology of BD, although the underlying mechanisms of Th17 skewing are not clear. Here, we demonstrate that the expression of Ets-1 is decreased and inversely correlated with IL-17 expression in patients with active BD. In addition, we showed that Ets-1 expression in $CD4^+$ human T cells was inhibited by miR-155, whose levels were increased in BD patients. Moreover, miR-155 suppression led to a significant inhibition of Th17 differentiation of $CD4^+$ T cells in patients with active BD.

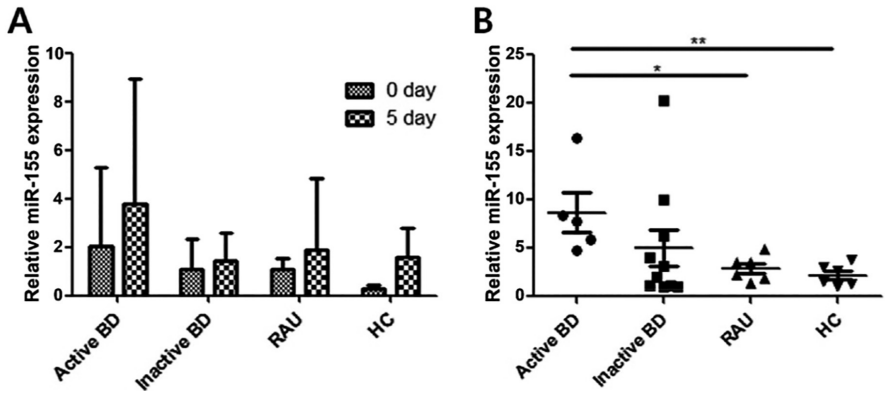

Fig. 4. Upregulation of miR-155 in patients with BD.
A. Expression of miR-155 in PBMCs from active BD (n=5), inactive BD (n=6), RAU (n=5), and HC (n=7) groups before and after 5 day-culture in Th17 polarisation conditions.
B. Expression of miR-155 in $CD4^+$ T cells from active BD (n=5), inactive BD (n=10), RAU (n=6), and HC (n=6) groups. MiR-155 levels were determined by quantitative RT-PCR and normalised to the levels of U6 small nuclear RNA. *p<0.05, **p<0.01.
MiR-155: microRNA-155; BD: Behçet's disease; PBMCs: peripheral blood mononuclear cells; RAU: recurrent aphthous ulcer; HC: healthy control.

Our results suggest that enhanced miR-155 expression might lead to low expression of Ets-1 and an increased frequency of Th17 in BD. The association of miR-155 with Th17 has been previously reported. Yao *et al*. (17) demonstrated that miR-155 is involved in the regulation of Treg/Th17 cell differentiation and IL-17A production. O'Connell *et al*. (18) showed that mice deficient in miR-155 were resistant to experimental autoimmune encephalitis because of the inhibition of Th17 cell development, indicating that miR-155 plays a critical role in inflammatory T cell development. In a mouse model of collagen-induced arthritis, miR-155 knockout mice were protected against arthritis development (19, 20). A study

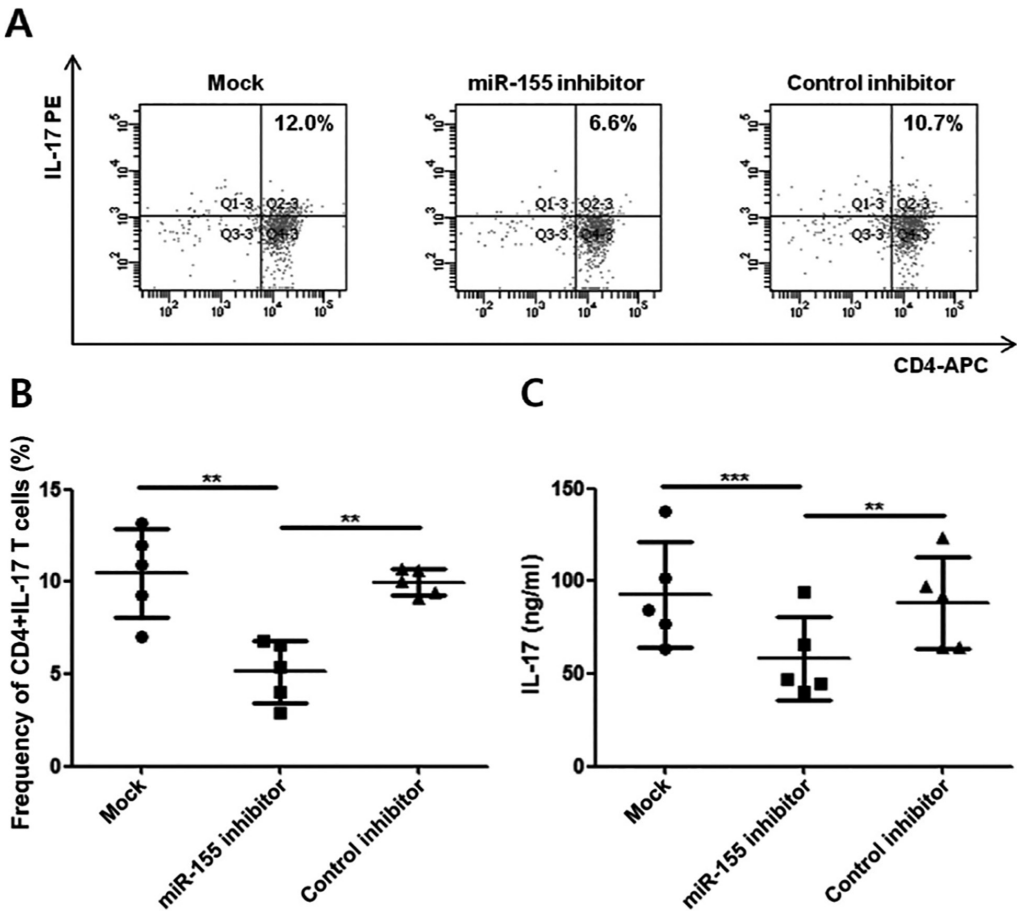

Fig. 5. Repression of miR-155 in CD4+ T cells impairs IL-17 expression.
Isolated CD4+ T cells from active BD (n=5) patients were transfected with miR-155 LNA inhibitor or control inhibitor. Subsequently, transfected cells were cultured in Th17 polarisation conditions for 5 days.
A. Representative FACS plots for a patient with active BD.
B. The proportion of CD4+IL-17-expressing T cells was measured by flow cytometry.
C. IL-17 concentration in culture supernatants of stimulated CD4+ T cells from patients with active BD (n=5) were measured by ELISA. Bars show the relative expression levels (mean ± SD).
**p<0.01, ***p<0.001.
MiR-155: microRNA-155;
BD: Behçet's disease;
LNA: locked nucleic acid;
ELISA: enzyme-linked immunosorbent assay;
SD: standard deviation.

of the underlying mechanisms revealed that the protection resulted from the impaired production of pathogenic autoreactive B and T cells as well as Th17 polarisation. Although Ets-1 was a known target of miR-155 in Th17 cells in mice (21), this observation was not confirmed in human T cells. In this study, luciferase reporter assays and western blotting identified Ets-1 as the target gene of miR-155 in human T cells (Fig. 3). Furthermore, Ets-1 expression had not been analysed in BD patients. Similar to our results, expression of Ets-1 decreased in relapsing MS patients compared to healthy donors (14).

In accordance with the findings of a potential pathologic association between miRNAs and chronic inflammatory and autoimmune diseases, it is reported that specific miRNAs including miR-196a, miR-146, miR-499, and miR-182 play a role in the pathogenesis of BD (22-25). These reports demonstrate that gene polymorphisms of miRNAs may lead to BD development by modulating miRNA expression and by regulating the production of proinflammatory cytokines. It has also been suggested that miRNAs, such as miR-199-3p and miR-720, may act as biomarkers for discriminating between BD patients and HC (26). We detected an upregulation of miR-155 expression in CD4+ T cells of patients with active BD (Fig. 4). A recent report by Zhou *et al*. demonstrated that miR-155 expression is significantly decreased in PBMCs and dendritic cells (DCs) but not in CD4+ T cells from BD patients with active intraocular inflammation, as compared to those without active disease or to HCs (27). They also observed that introduction of miR-155 in DCs inhibited intracellular IL-17 expression in CD4+ T cells. These findings are not in agreement with the findings of our study, where we observed higher expression of miR-155 in BD, promotion of Th17 differentiation, and production of IL-17. Even though the reasons for these discrepancies are not clear, these may be attributed to the method of CD4+ T cell separation and different methods for evaluating the effect of miR-155 on IL-17 expression. In the study by Zhou *et al*., peripheral CD4+ T cells were isolated from PBMCs by negative selection whereas in the current study, they were isolated by positive selection, which may be one of the possible explanations. They also transfected miR-155 in DCs and evaluated the expression of IL-17 in co-cultured CD4+ T cells. In contrast, we directly transfected the miR-155 in CD4+ T cells following their differentiation to Th17 cells and then evaluated IL-17 expression. Additionally, the type of BD manifestation and treatment also could be the reason for the discrepancies. The other study described ocular symptoms alone in BD patients, whereas we enrolled BD patients with oral ulcers (50%) and erythema nodosum-like lesions (41.7%) as the major symptoms. Active uveitis is typically treated with systemic immunosuppressive agents because of its characteristic resistance to treatment and the possibility of blindness. Although Zhou

et al. mentioned that the enrolled BD patients with active ocular inflammation had not received any systemic immunosuppressive agents for at least 1 week before enrolment, the effects of previous treatment could have persisted at the time of blood sampling. In our study, only four patients presented with active uveitis, and they were new patients who had not taken any drugs, including immunosuppressive agents or corticosteroids, prior to enrolment. Although some of the enrolled BD patients used low-dose corticosteroids, its effect was not immunosuppressive, but was anti-inflammatory (28). Additionally low-dose corticosteroids were also used in some of the RAU groups; therefore, we assumed that the effect of the drugs was quite marginal. We also analysed the difference in the expression of miR-155 among active mucocutaneous BD, active ocular BD, RAU, and HC. Although there was no statistically significant difference, the expression of miR-155 in PBMCs showed a tendency to increase in active BD patients with mucocutaneous symptoms than in controls, but no differences were observed between patients with active BD with ocular symptom and the controls. On the contrary, the expression of miR-155 in $CD4^+$ T cells showed no difference with respect to organ involvement. Future studies with large sample sizes are required to clarify this issue.

The main goal of BD treatment is relieving the symptoms and resolving the inflammation to prevent tissue damage and complications. There is some evidence that manipulation of aberrant miRNA levels could inhibit inflammation and disease progression. In mice models of MS, overexpressed miR-326 aggravated the phenotype of experimental autoimmune encephalomyelitis, whereas this aggravation completely disappeared by suppression of miR-326 (14). MiR-155 knockout mice were also protected against arthritis by impaired production of pathogenic B and T cells (19, 20). Glucocorticoids (GCs), as one of the most widely used anti-inflammatory therapy in BD, inhibit the expression of miR-155 in lipopolysaccharide-induced inflammatory responses (29). Additionally, combined therapy with GCs and miR-155 inhibition showed a synergistic effect on the anti-inflammatory effect of GCs. We observed that antagonising miR-155 in $CD4^+$ T cells from patients with active BD resulted in a significant decrease in IL-17-expressing T cells and IL-17 levels (Fig. 5). Blocking miRNA function using compounds such as antimiRs may be an effective therapeutic strategy. Several efforts have been made to deliver antimiRs into cells and tissues in an efficient and stable manner (16). Further animal studies using antimiRs are needed to develop this therapeutic strategy in the context of BD.

In conclusion, we have demonstrated that miR-155 targets Ets-1 in human $CD4^+$ T cells and observed an increased expression of miR-155 in $CD4^+$ T cells from patients with active BD. Inhibition of miR-155 expression in $CD4^+$ T cells from patients with active BD resulted in a decrease in the number of IL-17-producing $CD4^+$ T cells. These results suggest that miR-155 may be a critical factor in the regulation of Th17 differentiation by targeting Ets-1 in human T cells. Therefore, the development of a miR-155 antagonist could be useful as a therapeutic target to regulate the Th17 immune response in BD.

Acknowledgement

We thank G. Marziali (Istituto Superiore di Sanità, Rome, Italy) for providing the reporter plasmid carrying *Ets1* 3′-UTR.

References

1. SAKANE T, TAKENO M, SUZUKI N, INABA G: Behçet's disease. *N Engl J Med* 1999; 341: 1284-91.
2. EKINCI NS, ALPSOY E, KARAKAS AA, YILMAZ SB, YEGIN O: IL-17A has an important role in the acute attacks of Behçet's disease. *J Invest Dermatol* 2010; 130: 2136-8.
3. SUGITA S, KAWAZOE Y, IMAI A, YAMADA Y, HORIE S, MOCHIZUKI M: Inhibition of Th17 differentiation by anti-TNF-alpha therapy in uveitis patients with Behçet's disease. *Arthritis Res Ther* 2012; 14: R99.
4. NA SY, PARK MJ, PARK S, LEE ES: Up-regulation of Th17 and related cytokines in Behçet's disease corresponding to disease activity. *Clin Exp Rheumatol* 2013; 31 (3 Suppl. 77): 32-40.
5. DAI R, AHMED SA: MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. *Transl Res* 2011; 157: 163-79.
6. O'CONNELL RM, RAO DS, BALTIMORE D: microRNA regulation of inflammatory responses. *Annu Rev Immunol* 2012; 30: 295-312.
7. LENG RX, PAN HF, QIN WZ, CHEN GM, YE DQ: Role of microRNA-155 in autoimmunity. *Cytokine Growth Factor Rev* 2011; 22: 141-7.
8. TENG G, PAPAVASILIOU FN: Shhh! Silencing by microRNA-155. *Philos Trans R Soc Lond B Biol Sci* 2009; 364: 631-7.
9. ZHU N, ZHANG D, CHEN S *et al*.: Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. *Atherosclerosis* 2011; 215: 286-93.
10. ROMANIA P, LULLI V, PELOSI E, BIFFONI M, PESCHLE C, MARZIALI G: MicroRNA 155 modulates megakaryopoiesis at progenitor and precursor level by targeting Ets-1 and Meis1 transcription factors. *Br J Haematol* 2008; 143: 570-80.
11. MOISAN J, GRENNINGLOH R, BETTELLI E, OUKKA M, HO IC: Ets-1 is a negative regulator of Th17 differentiation. *J Exp Med* 2007; 204: 2825-35.
12. Criteria for diagnosis of Behçet's disease. International Study Group for Behçet's Disease. *Lancet* 1990; 335: 1078-80.
13. MIZUSHIMA Y: Revised diagnostic criteria for Behçet's disease in 1987. *Ryumachi* 1988; 28: 66-70.
14. 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: 1252-9.
15. THOMSEN S, AZZAM G, KASCHULA R, WILLIAMS LS, ALONSO CR: Developmental RNA processing of 3'UTRs in Hox mRNAs as a context-dependent mechanism modulating visibility to microRNAs. *Development* 2010; 137: 2951-60.
16. THOMAS M, LANGE-GRUNWELLER K, DAYYOUB E *et al*.: PEI-complexed LNA antiseeds as miRNA inhibitors. *RNA Biol* 2012; 9: 1088-98.
17. YAO R, MA YL, LIANG W *et al*.: MicroRNA-155 modulates Treg and Th17 cells differentiation and Th17 cell function by targeting SOCS1. *PLoS One* 2012; 7: e46082.
18. O'CONNELL RM, KAHN D, GIBSON WS *et al*.: MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* 2010; 33: 607-19.
19. BLUML S, BONELLI M, NIEDERREITER B *et al*.: Essential role of microRNA-155 in the pathogenesis of autoimmune arthritis in mice. *Arthritis Rheum* 2011; 63: 1281-8.
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 USA* 2011; 108: 11193-8.
21. HU R, HUFFAKER TB, KAGELE DA *et al*.: MicroRNA-155 confers encephalogenic potential to Th17 cells by promoting effector gene expression. *J Immunol* 2013; 190: 5972-80.
22. ZHOU Q, HOU S, LIANG L *et al*.: MicroRNA-146a and Ets-1 gene polymorphisms in ocular Behçet's disease and Vogt-Koyanagi-Harada syndrome. *Ann Rheum Dis* 2014; 73: 170-6.
23. QI J, HOU S, ZHANG Q *et al*.: A functional variant of pre-miRNA-196a2 confers risk for Behçet's disease but not for Vogt-Koyanagi-Harada syndrome or AAU in ankylosing

spondylitis. *Hum Genet* 2013; 132: 1395-404.
24. YU H, LIU Y, BAI L, KIJLSTRA A, YANG P: Predisposition to Behçet's disease and VKH syndrome by genetic variants of miR-182. *J Mol Med* (Berl) 2014; 92: 961-7.
25. HATEMI G, SEYAHI E, FRESKO I, TALARICO R, HAMURYUDAN V: Behçet's syndrome: a critical digest of the 2014-2015 literature. *Clin Exp Rheumatol* 2015; 33 (Suppl. 94): S3-14.
26. ERRE GL, PIGA M, CARRU C *et al*.: Global microRNA profiling of peripheral blood mononuclear cells in patients with Behçet's disease. *Clin Exp Rheumatol* 2015; 33 (Suppl. 94): S72-9.
27. ZHOU Q, XIAO X, WANG C *et al*.: Decreased microRNA-155 expression in ocular Behçet's disease but not in Vogt Koyanagi Harada syndrome. *Invest Ophthalmol Vis Sci* 2012; 53: 5665-74.
28. CONN DL: Resolved: Low-dose prednisone is indicated as a standard treatment in patients with rheumatoid arthritis. *Arthritis Rheum* 2001; 45: 462-7.
29. ZHENG Y, XIONG S, JIANG P *et al*.: Glucocorticoids inhibit lipopolysaccharide-mediated inflammatory response by downregulating microRNA-155: a novel anti-inflammation mechanism. *Free Radic Biol Med* 2012; 52: 1307-17.