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Aberrant expression of microRNAs in T cells from patients with ankylosing spondylitis contributes to the immunopathogenesis

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Summary

Ankylosing spondylitis (AS) is a chronic inflammatory disorder characterized by dysregulated T cells. We hypothesized that the aberrant expression of microRNAs (miRNAs) in AS T cells involved in the pathogenesis of AS. The expression profile of 270 miRNAs in T cells from five AS patients and five healthy controls were analysed by real-time polymerase chain reaction (PCR). Thirteen miRNAs were found potentially differential expression. After validation, we confirmed that miR-16, miR-221 and let-7i were over-expressed in AS T cells and the expression of miR-221 and let-7i were correlated positively with the Bath Ankylosing Spondylitis Radiology Index (BASRI) of lumbar spine in AS patients. The protein molecules regulated by miR-16, miR-221 and let-7i were measured by Western blotting. We found that the protein levels of Toll-like receptor-4 (TLR-4), a target of let-7i, in T cells from AS patients were decreased. In addition, the mRNA expression of interferon (IFN)-γ was elevated in AS T cells. Lipopolysaccharide (LPS), a TLR-4 agonist, inhibited IFN-γ secretion by anti-CD3$^+$anti-CD28 antibodies-stimulated normal T cells but not AS T cells. In the transfection studies, we found the increased expression of let-7i enhanced IFN-γ production by anti-CD3$^+$anti-CD28$^+$ lipopolysaccharide (LPS)-stimulated normal T cells. In contrast, the decreased expression of let-7i suppressed IFN-γ production by anti-CD3$^+$anti-CD28$^+$ LPS-stimulated AS T cells. In conclusion, we found that miR-16, miR-221 and let-7i were over-expressed in AS T cells, but only miR-221 and let-7i were associated with BASRI of lumbar spine. In the functional studies, the increased let-7i expression facilitated the T helper type 1 (IFN-γ) immune response in T cells.

Keywords: ankylosing spondylitis, interferon gamma, let-7i, microRNA, T cells, Toll-like receptor-4

Introduction

Ankylosing spondylitis (AS) is a chronic inflammation arthritis that affects both axial and peripheral skeletons and soft tissues. It is conceivable that human leucocyte antigen (HLA)-B27 is the most important risk factor for AS [1], whereas misregulation of T cells could contribute to the inflammatory responses in AS patients [2]. The misfolded HLA-B27 heavy chain homodimer in an animal model has supported the importance of HLA-B27 in the pathogenesis of AS [3]. Subsequent studies have revealed that the activation of Th17 cells is also critical for sustaining the inflammatory responses in AS patients clinically [4–6]. Recently, genetic studies of AS patients have also suggested that T cells might play an essential role in the immunopathogenesis of AS [7]. Dysregulated CD4$^+$ and CD8$^+$ T cells were found in peripheral blood [8,9] and inflammatory joints [10,11] of the AS patients. Moreover, increased intracellular nitric oxide (NO) production and delayed calcium responses were observed in T cells from peripheral blood of AS patients [12].

MicroRNAs (miRNAs) are small, non-coding RNA molecules that regulate the expression of multiple target genes at the post-transcriptional level and hence play critical roles in modulating innate and adaptive immune responses. Altered miRNA expression has been implicated in the

pathogenesis of different forms of arthritis, including rheumatoid arthritis (RA) and osteoarthritis (OA). Many studies have demonstrated that altered expression of miRNAs in synovia, peripheral blood mononuclear cells (PBMCs) or T cells from patients with RA or OA is associated with innate immunity, inflammation, osteoclastogenesis and cartilage synthesis [13–20]. However, the roles of aberrant expressed miRNAs in the pathogenesis of AS remain unclear. We hypothesized that aberrant expression of miRNAs in the T cells of AS patients may alter expression of the downstream target molecules that may contribute to the pathogenesis of AS.

Indeed, our study demonstrated that miR-16, miR-221 and let-7i were over-expressed in AS T cells, and the latter two were associated with radiographic change. Transfection studies suggest that increased expression of let-7i enhanced interferon (IFN)-γ production but suppressed Toll-like receptor-4 (TLR-4) expression in AS T cells.

Material and methods

Patients and controls

Twenty-seven HLA-B27-positive patients fulfilling the 1984 modified New York criteria for the classification of ankylosing spondylitis [21] were recruited for this study. Twenty-three age- and sex-matched healthy volunteers served as a control group. Each participant signed informed consent forms approved by the local institutional review board and ethics committee of Buddhist Dalin Tzu Chi General Hospital, Chia-Yi, Taiwan (no. 09801019). Blood samples were collected at least 12 h after the last dosage of immunosuppressants to minimize the drug effects. The grade of sacroiliitis was identified according to the New York criteria [22] and the lumbar spine involvement was graded by the Bath Ankylosing Spondylitis Radiology Index (BASRI) [23] in AS patients.

Isolation of RNA from T cells

Heparinized venous blood obtained from AS patients and healthy volunteers was mixed with one-fourth volume of 2% dextran solution (MW 464 000 daltons; Sigma-Aldrich, St Louis, MO, USA) and incubated at room temperature for 30 min. Leucocyte-enriched supernatant was collected and layered over a Ficoll-Hypaque density gradient solution (specific gravity 1·077; Pharmacia Biotech, Uppsala, Sweden). After centrifugation at 250 ***g*** for 25 min, mononuclear cells were aspirated from the interface. Then, T cells were purified further by anti-human CD3 magnetic beads using IMag Cell Separation System (BD Bioscience, Franklin Lakes, NJ, USA). The T cell concentration was adjusted to 1×10^6/ml in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/l L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml) (10% FBS-RPMI) for further analysis. Total RNA including miRNA from the T cells was extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA), according to the manufacturer's protocol. The RNA concentration was quantified using a NanoDrop Spectrophotometer.

Reverse transcription (RT) of miRNAs

We converted all miRNAs into corresponding cDNAs in a one-step RT reaction by the method developed by Chen *et al.* [24]. Briefly, 10 μl reaction mixture containing miRNA-specific stem-loop RT primers (final 2 nM each), 500 μM deoxyribonucleotide (dNTP), 0·5 μl Superscript III (Invitrogen, Carlsbad, CA, USA), and 1 μg total RNA were used for the RT reaction. The pulsed RT reaction was performed in the following conditions: 16°C for 30 min, followed by 50 cycles at 20°C for 30 s, 42°C for 30 s and 50°C for 1 s. After RT the products were diluted 20-fold before further analysis.

Measurement of microRNA (miRNA) expression by real-time PCR

A real-time PCR-based method was used to quantify the expression levels of miRNA in this study using the protocol described previously [25]. One microlitre of prepared RT product was used as template for PCR. Then 1 × SYBR Master Mix (Applied Biosystems, Foster City, CA, USA), 200 nM miRNA-specific forward primer and 200 nM universal reverse primer was added for each PCR reaction. All reactions were performed in duplicate on an ABI Prism 7500 Fast real-time PCR system (Applied Biosystems). The condition for quantitative PCR is 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 63°C for 32 s. The expression of the U6 small nuclear RNA was used as endogenous control for data normalization. The threshold cycle (Ct) is defined as the cycle number at which the change of fluorescence intensity crosses the average background level of the fluorescence signal.

First, T cells purified from five AS patients and five healthy controls were analysed for the expression profile of 270 human miRNAs by real-time PCR. We then validated the expression levels of those potentially aberrant expressed miRNAs in T cells from in another 22 AS patients and 18 healthy controls.

Western blotting of cell lysates

T cells were lysed with 1% NP-40 (Sigma-Aldrich) in the presence of a proteinase inhibitor cocktail (Sigma-Aldrich). Seventy micrograms of the cell lysates were electrophoresed and transferred to a polyvinylidene difluoride (PVDF) sheet (Sigma-Aldrich). After blocking, the membranes were incubated with the primary antibodies followed by horseradish

peroxidase (HRP)-conjugated secondary antibodies. Mouse monoclonal anti-c-kit, anti-Bcl-2 and anti-TLR-4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-β-actin was purchased from Sigma-Aldrich as an internal control. Goat anti-rabbit and goat anti-mouse immunoglobulin (Ig)G antibodies as secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The complexes formed were visualized after a chemiluminescence reaction (ECL; GE Healthcare, Little Chalfont, UK). The intensity of the respective band was semi-quantified by Image J (version 1·42; http://rsb.info.nih.gov/ij).

Expression levels of let-7i in T cells from patients with SLE or RA

Eight patients fulfilling the 1982 American College of Rheumatology (ACR) revised criteria for the classification of SLE [26], nine patients fulfilling the 1987 ACR revised classification criteria for RA [27] and 14 healthy volunteers were recruited. The expression level of let-7i in T cells from these patients was measured by the methods described above.

Transfection of miRNA mimics or inhibitors into Jurkat cells

Fresh isolated human T cells or Jurkat cells (1×10^6/ml) purchased from the American Type Culture Collection (Manassas, VA, USA) were electroporated with 1 μg of scrambled oligonucleotides, miRNA mimics (Ambion) or miRNA inhibitors (Ambion) using the Gene Pulser MXcell electroporation system (Bio-Rad Laboratories, Hercules, CA, USA), with the conditions developed by Jordan *et al.* [28]. The expression of miRNA in miRNA-mimic or miRNA inhibitor transfected Jurkat cells was analysed after culturing for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. Because the endogenous TLR-4 protein expression in Jurkat cells is minimal, ionomycin (250 ng/ml; Sigma-Aldrich) and 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) were added to activated Jurkat cells for another 24 h. These cells were then lysed by Western blotting for analysing the expression of TLR-4.

Measurement of mRNA expression

The expression levels of TLR-4 and IFN-γ mRNA were quantified by real-time PCR using a one-step RT–PCR kit (TaKaRa, Shiga, Japan) on an ABI Prism 7500 Fast real-time PCR system (Applied Biosystems). The primers used for TLR-4 were 5′-CGAGGCTTTTCTGAGTCGTC-3′ (forward) and 5′-TGAGCAGTCGTGCTGGT- ATC-3′ (reverse). The primers used for IFN-γ were forward 5′-CTTTAAAGATGACCA- GACCATCCA-3′ and reverse 5′-ATCTCGTTTCTTTTTGTTGCTATTGA-3′. Conditions for the quantitative PCR were 42°C for 5 min and 95°C for 10 s for RT, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Expression of 18S ribosomal RNA was used as endogenous control for data normalization. The normalized mRNA level was defined by the equation: 39 – Ct after normalization by the expression of 18S ribosomal RNA.

Functional analysis of let-7i in human T cells by transfection with let-7i mimic or inhibitor?

T cells isolated from healthy volunteer or AS patients (1×10^6/well) were cultured in the following three conditions: (i) in culture medium only, (ii) in an anti-human CD3 antibody (1 μg; BioLegend, San Diego, CA, USA) precoated plate + 1 μg anti-human CD28 antibody (BioLegend) and (iii) in an anti-human CD3 antibody precoated plate + 1 μg anti-human CD28 antibody + 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich) for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. Furthermore, let-7i mimic, let-7i inhibitor or scrambled oligonucleotide-transfected T cells (3×10^6/well) were also cultured in an anti-human CD3 antibody precoated plate + 1 μg anti-human CD28 antibody + 100 ng/ml LPS for 24 h at 37°C. Then, the cells were pelleted down by centrifugation at 300 ***g***. The supernatants were collected and stored at −80°C for the measurement of IFN-γ by enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences), according to the manufacturer's protocol.

Statistical analysis

All data are represented as the mean ± standard deviation (s.d.). Univariate and multivariate linear regression was applied to calculate the correlation coefficient and significance among different parameters using STATA software (StataCorp, College Station, TX, USA). Statistical significance was assessed by Mann–Whitney *U*-test and a *P*-value less than 0·05 was considered statistically significant.

Results

Patients and controls

The demographic and clinical data of the AS patients were recorded and are summarized in Table 1.

Identification and verification of differential expression of miRNAs in T cells from AS patients and healthy controls

The expression profile of 270 miRNAs in T cells from five AS patients and five healthy controls is shown in Fig. 1a. Each scatter-spot represents the average of normalized miRNA levels of T cells from five AS patients and normal

Table 1. Demographics and clinical data of the ankylosing spondylitis (AS) patients and healthy volunteers.

	AS patients ($n = 22$)	Healthy volunteers ($n = 18$)	*P*-value
Age (years, mean ± s.d.)	42·6 ± 10·2	40·1 ± 9·3	0·33
Sex (F : M)	8:14	5:13	0·74
HLA-B27 (+)	100% (22/22)	–	
C-reactive protein (mg/dl)	0·50 ± 0·68	–	
BASRI-lumbar spine	1·9 ± 1·4	–	
Sacroiliitis (grading)	2·6 ± 1·1	–	
Medication			
NSAID	91% (20/22)	–	
Salazopyrine	91% (20/22)	–	
Anti-TNF therapy	5% (1/22)	–	

BASRI: Bath Ankylosing Spondylitis Radiology Index; NSAID: non-steroidal anti-inflammatory drugs; TNF: tumour necrosis factor; s.d.: standard deviation; –: not determined.

controls. We noted that the expression of eight microRNAs, including miR-150, miR-16, miR-342-5p, miR-221, let-7i, miR-99b, let-7b and miR-513-5p, were significantly higher and five microRNAs including miR-218, miR-409-3p, miR-30e, miR-199a-5p and miR-215 were significantly lower in AS T cells than in normal T cells (fold change >4·5 and $P < 0·05$; Fig. 1b). Then, we chose only the five most differentially expressed miRNAs (defined as fold change >6 and $P < 0·05$), including miR-150, miR-16, miR-342-5p, miR-221 and let-7i for further validation. In the second step, T cells from another 22 AS patients and 18 healthy controls were compared. We confirmed that the expression levels of miR-16, miR-221 and let-7i (fold change: 2·34, 2·38 and 3·17, respectively; all the *P* values < 0·05) were significantly higher in AS T cells than in normal T cells (Fig. 1c).

Correlations of miR-16, miR-221 and let-7i expression with the clinical parameters of AS patients

We then intended to correlate different clinical parameters with the expression levels of miR-16, miR-221 and let-7i in AS T cells by univariate and multivariate linear regression analysis. We found that the expression of miR-221 ($P = 0·022$) and let-7i ($P = 0·031$) were associated positively with BASRI of lumber spine. The expression of miR-16 ($P = 0·086$) was associated positively with BASRI of lumbar spine (Fig. 2). After adjusting for age and gender, the expression of miR-221 (fold change = 1·58, $P = 0·033$) and let-7i (fold change = 1·75, $P = 0·029$), but not miR-16 (fold change = 1·67, $P = 0·059$), were still correlated positively with BASRI of lumbar spine, which reflects inflammatory activity in the lumbar spine (Table 2). However, expression of miR-16, miR-221 and let-7i did not correlate with serum C-reactive protein levels or sacroiliitis by radiography in AS patients (Table 2).

Expression of proteins regulated by miR-16, miR-221 and let-7i in T cells from AS patients and healthy controls

Several studies have demonstrated that miR-16, miR-221 and let-7i regulate the protein expression of Bcl-2, c-kit and

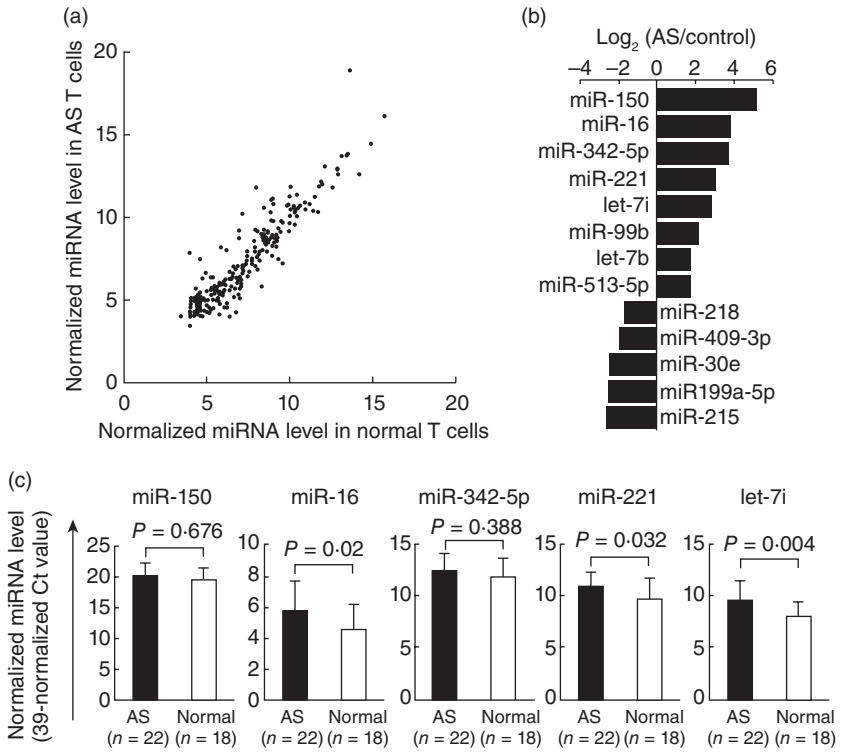

Fig. 1. Comparison of microRNAs (miRNAs) expression in T cells from patients with ankylosing spondylitis (AS) and healthy controls. (a) The expression profile of 270 miRNAs measured by real-time polymerase chain reaction (PCR). Each scatter-spot represents the average of normalized miRNA level in T cells from five AS patients and five healthy controls for each miRNA. The threshold cycle (Ct) is defined as the cycle number at which the change of fluorescence intensity crosses the average background level of the fluorescence signal. The normalized miRNA level was defined as [39 – Ct after normalization with the internal control (U6 small nuclear RNA)]. (b) Eight miRNAs were found potentially over-expressed and five mRNAs were under-expressed in AS T cells (fold change >4·5; *P*-value < 0·05). (c) The five most potentially differentially expressed miRNAs (defined as fold change >6) were validated further by real-time PCR in T cells from another 22 AS patients and 18 compatible healthy controls. Increased expression of miR-16, miR-221 and let-7i in T cells from AS patients was confirmed after validation.

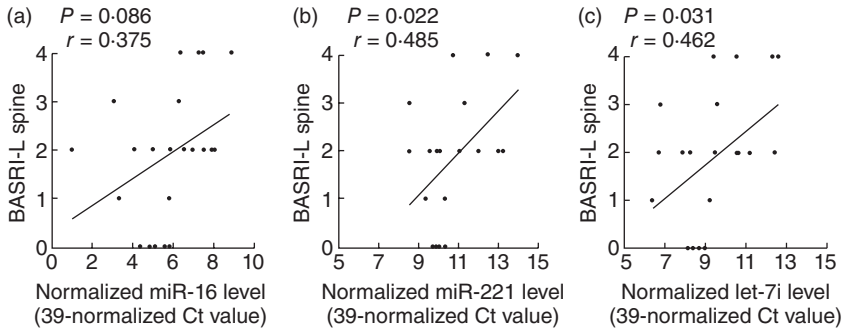

Fig. 2. The correlation of the three over-expressed miRNAs in ankylosing spondylitis (AS) T cells with Bath Ankylosing Spondylitis Radiology Index (BASRI) of lumbar spine. (a) miR-16, (b) miR-221 and (c) let-7i.

TLR-4, respectively [29–31]. To identify the potential biological/pathological relationships between the increased expression of these miRNAs and their target proteins in AS T cells, we compared the protein expression of Bcl-2, c-kit and TLR-4 in AS patients and healthy controls by Western blotting (Fig. 3). The expression level of TLR-4 (Fig. 3c), but not Bcl-2 (Fig. 3b), was significantly lower in AS T cells than in normal T cells. Although miR-221 was over-expressed in AS T cells and its expression level was correlated significantly with BASRI of lumbar spine in AS patients, the expression of c-kit was undetectable by Western blotting in both normal and AS T cells (data not shown). We speculated that miR-221 may play a physiological role in suppressing the protein expression of c-kit in T cells. Thus, we transfected miR-221 inhibitor or scrambled oligonucleotide into AS T cells. The expression level of miR-221 decreased dramatically (fold change: 0·035, $P < 0·05$) after miR-221 inhibitor transfection (Fig. 4a). However, the expression of c-kit remained undetectable by Western blotting (Fig. 4b).

Expression level of let-7i in T cells from healthy controls, patients with SLE or RA and activated Jurkat cells

The above results suggest that increased expression of let-7i in AS T cells. We then analysed the expression of let-7i in other systemic autoimmune diseases, including patients with SLE and RA, for identifying the specificity in AS T cells. We found that the expression of let-7i was not changed in T cells from these patients compared with controls (Fig. 5a). Another interesting finding is that let-7i expression in Jurkat cells was decreased after activation by ionomycin + PMA (Fig. 5b). This may indicate that activated T cells enhance TLR-4 expression. However, further investigation is required to confirm it.

Let-7i mimic transfection suppressed TLR-4 protein expression

For confirming further the roles of let-7i on TLR-4 protein expression, we transfected let-7i mimic or scrambled oligonucleotides into Jurkat cells by eletroporation to detect the effects on TLR-4 mRNA and protein expression. The expression levels of let-7i increased dramatically (fold change: 395·78, $P < 0·05$) after let-7i mimic transfection (Fig. 6a). Increased let-7i expression did not suppress the mRNA expression of TLR-4 in Jurkat cells (Fig. 6b), whereas the protein expression of TLR-4 in both Jurkat and normal T cells was suppressed significantly by Western blotting, as shown in Fig. 6c,d.

Let-7i inhibitor transfection enhanced TLR-4 expression

Conversely, we transfected let-7i inhibitor or scrambled oligonucleotides into Jurkat cells. As expected, the expression

Table 2. Univariate and multivariate liner regression models for assessing the correlations among different clinical parameters and miR-16, miR-221 and let-7i expression levels in T cells from 22 patients with ankylosing spondylitis (AS).

	Univariate analysis Fold change (95% confidence interval)			Multivariate analysis† Fold change (95% confidence interval)		
	miR-16	miR-221	let-7i	miR-16	miR-221	let-7i
Age (per 10 years)	1·09 (0·64–1·85)	1·18 (0·77–1·80)	1·13 (0·68–1·89)	0·75 (0·40–1·42)	0·85 (0·52–1·40)	0·77 (0·43–1·38)
Sex (male/female)	0·98 (0·29–3·33)	1·17 (0·43–3·16)	1·20 (0·37–3·88)	0·69 (0·20–2·37)	0·86 (0·33–2·25)	0·83 (0·27–2·56)
BASRI-L spine (per 1 score)	1·42* (0·95–2·13)	1·45** (1·06–1·98)	1·52** (1·04–2·21)	1·67 (0·98–2·86)	1·58 (1·04–2·40)	1·75 (1·07–2·87)
Sacroiliitis (per 1 grade)	1·07 (0·61–1·88)	1·03 (0·65–1·62)	1·08 (0·63–1·85)	–	–	–
CRP (per 1 mg/dl)	1·69 (0·73–3·90)	1·38 (0·69–2·77)	1·79 (0·81–3·96)	–	–	–

*$P = 0·086$; **$P < 0·05$; †After analysis with multivariate linear regression model adjusted for age and gender, only BASRI-L spine was correlated significantly with increased expression of miR-221 and let-7i. The expression of miR-16 was found ($P = 0·059$) to correlate with BASRI-L spine. BASRI-L spine: Bath Ankylosing Spondylitis Radiology Index of lumbar spine; CRP: C-reactive protein.

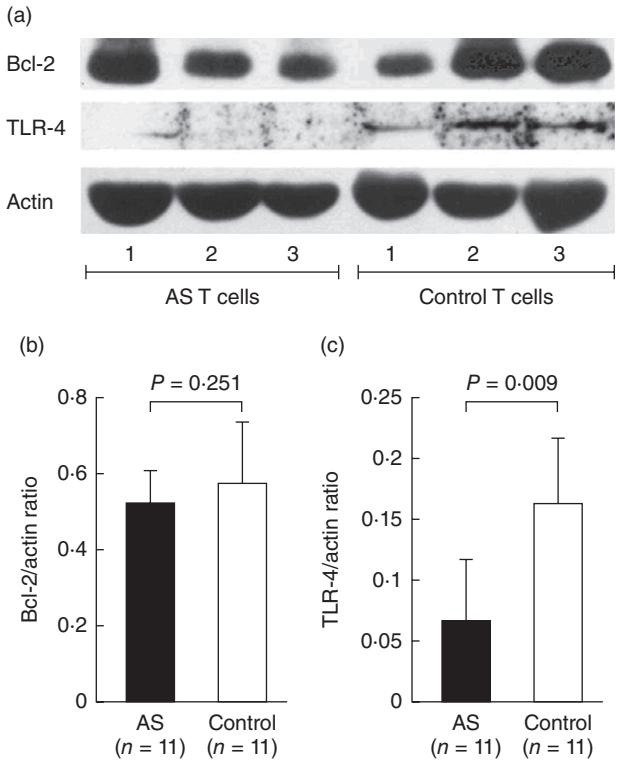

Fig. 3. The protein targeted by miR-16 (Bcl-2) and let-7i [Toll-like receptor (TLR)-4] in T cell lysates were detected by Western blotting. (a) Protein expression of Bcl-2, TLR-4 molecules in T cell lysates from three ankylosing spondylitis (AS) patients and three healthy controls were shown as representative. The expression levels of (b) Bcl-2 and (c) TLR-4 in T cell lysates from 11 AS patients and 11 healthy controls normalized to the actin expression are shown.

level of let-7i was decreased dramatically (fold change: 0·006, $P < 0·05$) after let-7i inhibitor transfection (Fig. 7a). The decreased let-7i expression did not increase TLR-4 mRNA expression significantly in Jurkat cells (Fig. 7b), but enhanced significantly the protein expression of TLR-4 in Jurkat and AS T cells, as shown in Fig. 7c,d by Western blotting. These results confirmed that let-7i inhibited protein translation rather than mRNA degradation of TLR-4 in Jurkat cells.

Functional studies of let-7i on IFN-γ secretion in anti-CD3$^+$anti-CD28-activated T cells co-culture with LPS

Bacterial LPS, a TLR-4 agonist, has been proved to play a crucial role in AS pathogenesis [32], and José *et al.* [33] have reported that LPS could inhibit the T cell response through TLR-4 in mice. We deduced that LPS might exert an inhibitory role on the T cell response in humans, which is involved in the immunopathogenesis of AS. In this study, we demonstrated that there was no difference between the IFN-γ secretion in anti-CD3$^+$anti-CD28-activated T cells from healthy controls and AS patients (46·9 ± 12·0 pg/ml *versus* 58·0 ± 46·0 pg/ml, $P = 0·88$). The addition of 100 ng/ml LPS could suppress IFN-γ secretion effectively in anti-CD3$^+$anti-CD28- activated normal T cells but not AS T cells (6·5 ± 8·2 pg/ml *versus* 73·6 ± 38·8 pg/ml, $P < 0·05$; Fig. 8a). We proposed that the increased expression of let-7i may contribute to the increased production of IFN-γ in AS T cells. Therefore, we transfected let-7i mimic, let-7i inhibi-

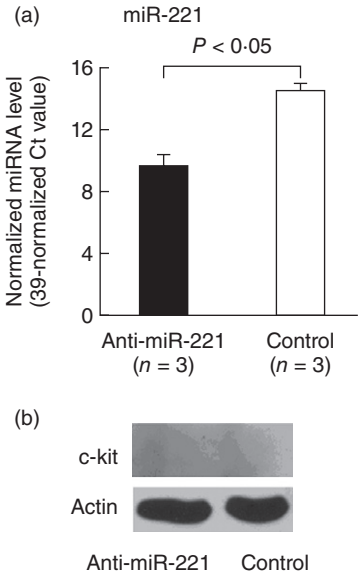

Fig. 4. Effect of miR-221 inhibitor transfection on c-kit protein expression in ankylosing spondylitis (AS) T cells. We transfected miR-221 inhibitor into AS T cells via electroporation. (a) The expression level of miR-221 was decreased dramatically after miR-221 inhibitor transfection for 24 h. (b) The protein expression levels of c-kit were undetectable in AS T cells transfected with miR-221 inhibitor or scrambled oligonucleotides.

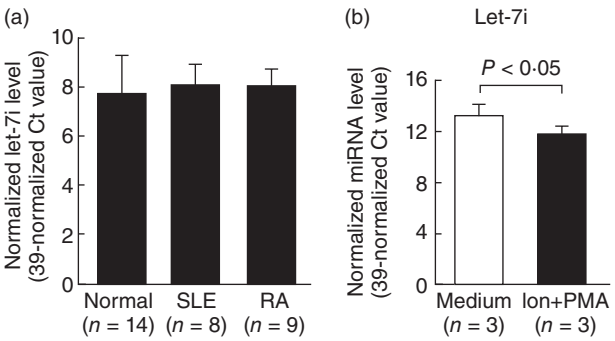

Fig. 5. The expression level of let-7i in T cells from healthy controls, patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) and activated Jurkat cells were compared. (a) Comparison of expression levels of let-7i in T cells from 14 controls, eight SLE and nine RA patients. (b) The expression levels of let-7i were decreased significantly in Jurkat cells after stimulation with 250 ng/ml ionomycin and 10 ng/ml phorbol myristate acetate (PMA) for 24 h.

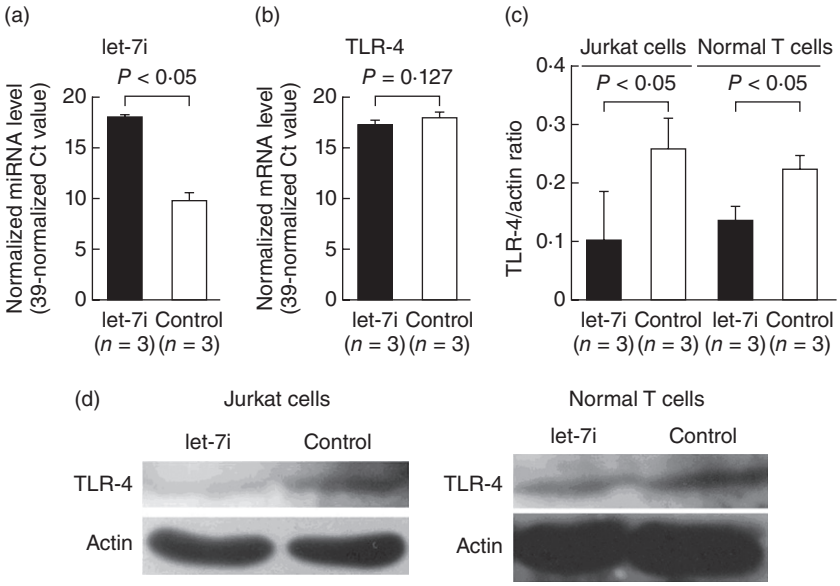

Fig. 6. Effect of let-7i mimic transfection on Toll-like receptor (TLR)-4 mRNA and protein expression. Let-7i mimic was transfected into Jurkat cells via electroporation. (a) After let-7i mimic transfection for 24 h, the expression level of let-7i was increased dramatically in the Jurkat cells compared with the scrambled oligonucleotides transfection group. (b) The TLR-4 mRNA expression levels did not change after let-7i transfection. (c) Jurkat cells were cultured with 250 ng/ml ionomycin and 10 ng/ml phorbol myristate acetate (PMA) and normal T cells were cultured with anti-CD3$^+$anti-CD28 antibodies for 24 h after let-7i mimic transfection. The protein expression of TLR-4 was inhibited significantly in Jurkat and normal T cells after let-7i mimic transfection. (d) A representative case showing increased TLR-4 protein expression after let-7i mimic transfection into Jurkat (left) and normal T cells (right).

tor or scrambled oligonucleotides into normal and AS T cells. In the scrambled oligonucleotide-transfected control groups, we found that IFN-γ production was increased in anti-CD3$^+$anti-CD28$^+$ LPS-stimulated AS T cells compared with normal T cells (87·8 ± 73·1 pg/ml *versus* 27·9 ± 18·4 pg/ml, $P = 0{\cdot}0283$; Fig. 8b). The transfection of let-7i mimic promoted IFN-γ production in anti-CD3$^+$ anti-CD28$^+$ LPS-stimulated normal T cells compared with those transfected with scrambled oligonucleotides (74·9 ± 18·9 pg/ml *versus* 27·9 ± 18·4 pg/ml, $P = 0{\cdot}009$). In contrast, transfection of let-7i inhibitor suppressed IFN-γ production by anti-CD3$^+$anti-CD28$^+$ LPS-stimulated AS T cells compared with those transfected with scrambled oligonucleotides (14·5 ± 26·7 pg/ml *versus* 87·8 ± 73·1 pg/ml, $P = 0{\cdot}047$).

Expression level of IFN-γ mRNA in T cells from AS patients and its correlation with BASRI score of lumbar spine

Because the increased expression of let-7i in anti-CD3$^+$ anti-CD28$^+$ LPS-stimulated T cells could enhance IFN-γ production *in vitro* (Fig. 8b), we compared the mRNA expression of IFN-γ in non-stimulated T cells from AS patients and controls. Indeed, mRNA expression of IFN-γ is increased significantly in resting T cells from AS patients (Fig. 9a). However, we noted no significant correlation between the expression levels of let-7i or BASRI of lumbar spine with the mRNA expression levels of IFN-γ in AS T cells (Fig. 9b,c). It is possible that the IFN-γ expression can be affected by viral or intracellular pathogen infection other

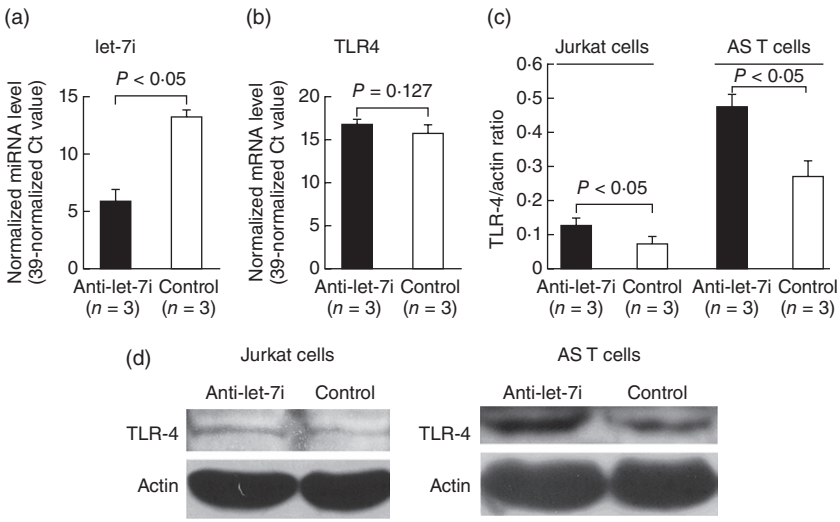

Fig. 7. Effect of let-7i inhibitor transfection on Toll-like receptor (TLR)-4 mRNA and protein expression. We transfected let-7i inhibitor into Jurkat cells via electroporation. (a) After let-7i inhibitor transfection for 24 h, the expression level of let-7i was decreased dramatically in the Jurkat cells compared with the scrambled oligonucleotide transfection groups. (b) The TLR-4 mRNA expression levels did not change after let-7i transfection. (c) The protein expression of TLR-4 was increased significantly in Jurkat and ankylosing spondylitis (AS) T cells after let-7i inhibitor transfection. (d) Representative case showing increased TLR-4 protein expression after let-7i inhibitor transfection into Jurkat (left) and AS T cells (right).

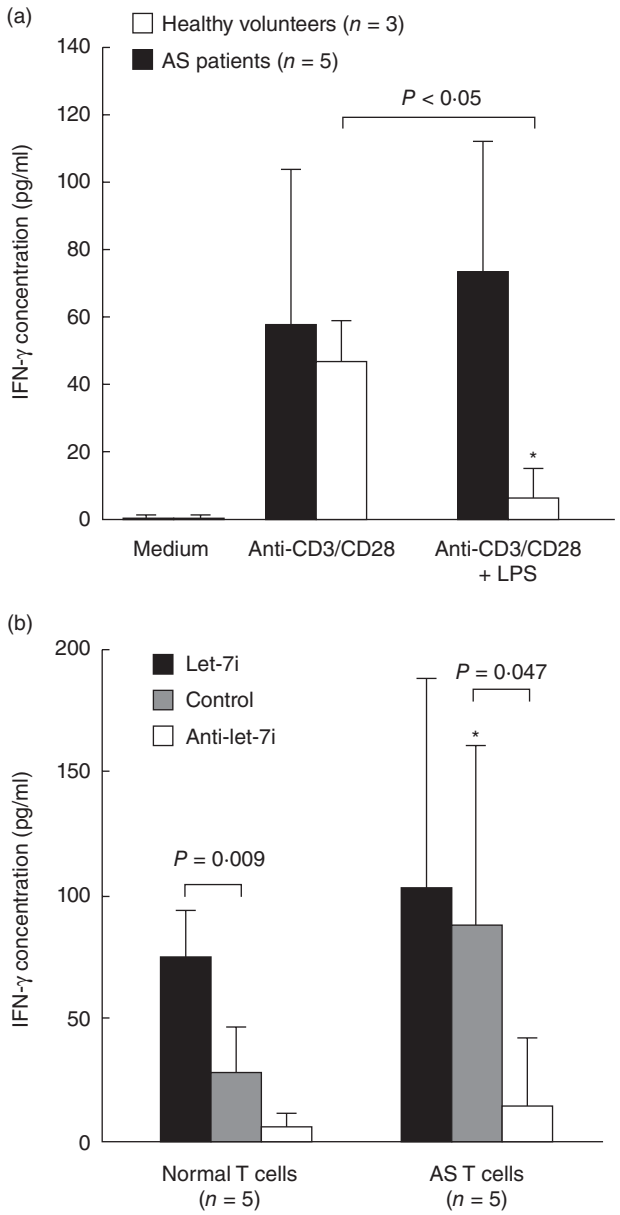

Fig. 8. Functional analysis of let-7i in anti-CD3$^+$anti-CD28-activated normal and ankylosing spondylitis (AS) T cells via transfection with let-7i mimic and inhibitor. (a) Lipopolysaccharide (LPS) (100 ng/ml) potently suppress interferon (IFN)-γ production in anti-CD3$^+$ anti-CD28 activated T cells from healthy volunteers but not AS patients. *The IFN-γ concentration was lower in anti-CD3$^+$anti-CD28$^+$ LPS-stimulated normal T cells compared with AS T cells. ($P < 0{\cdot}05$) (b) The transfection of let-7i mimic increased IFN-γ production in anti-CD3$^+$anti-CD28$^+$ LPS-activated T cells from healthy volunteers, but not AS patients. The transfection of let-7i inhibitor suppressed IFN-γ production by anti-CD3$^+$anti-CD28$^+$ LPS-stimulated AS T cells, but not normal T cells. *In the scrambled oligonucleotides transfected control groups, the IFN-γ concentration was higher in anti-CD3$^+$anti-CD28$^+$ LPS-stimulated AS T cells compared with normal T cells ($P < 0{\cdot}05$).

than disease activity *per se*, and other bone destructive/formation factors such as MMP1 and BMPs, etc. may probably play a role in the syndesmophyte formation in AS spine [34]. We conclude that the let-7i expression level did not affect the IFN-γ mRNA expression directly and was not relevant to the BASRI of lumbar spine in AS patients.

Discussion

Our study demonstrated that the expression of three miRNAs (miR-16, miR-221 and let-7i) was increased in T cells from AS patients compared to those from healthy controls. Clinically, the increased expression of the two miRNAs (miR-221 and let-7i) showed an association with BASRI lumbar spine in AS patients. These results provided an alternative view: that misregulated T cells contribute to the pathological changes in patients with AS via aberrant expression of certain miRNAs.

Previous studies have detected increased TLR-4 protein expression in peripheral blood mononuclear cells, especially monocytes from AS patients by flow cytometry and real-time PCR [35–37]. In contrast, using Western blotting in this study we found that TLR-4 expression specifically in AS T cells was suppressed by let-7i. As TLR-4 is expressed abundantly on monocytes, we proposed that the decreased expression of TLR-4 in AS T cells could be masked easily by the abundant amount of TLR-4 on monocyte or other cell types from AS patients. Moreover, in the cell transfection studies, we found that there were discrepancies between mRNA and protein expressions of TLR-4 due to the effect of let-7i (Figs 6 and 7). As TLR-4 is the prime cellular pattern recognition sensor for microbial pathogens, TLR-4 activation via LPS leads to production of proinflammatory cytokines in innate immune systems [38]. Interestingly, TLR-4 is also expressed on T cells [39], which might have a different immunoregulatory function in the adaptive immune system, as shown in our study. José *et al*. [33] have reported that LPS signalling through TLR-4 could suppress T cell receptor-dependent extracellular signal-regulated kinase 1/2 (ERK1/2) activation in CD4$^+$ T cells in the murine model. Similar to their findings, in this study we demonstrated that LPS could exert an inhibitory signal on the T cell response in humans. Clinical observations revealed that there was a link between AS development with chronic prostatitis in men or pelvic inflammatory disease in women. It is purposed that the microbe infection is from a source of damage-associated molecular pattern molecules (DAMPs) involved in AS pathogenesis. These DAMPs could activate TLRs to elicit the inflammatory reaction and ectopic enchondral bone formation in AS spine [32]. Although bacterial infection such as *Chlamydia* could cause chronic arthritis [40], it is still premature to conclude that bacterial infection can cause AS [41]. Conversely, evidence suggests that AS disease activity became worse, following the different bacterial infections such as *Salmonella*,

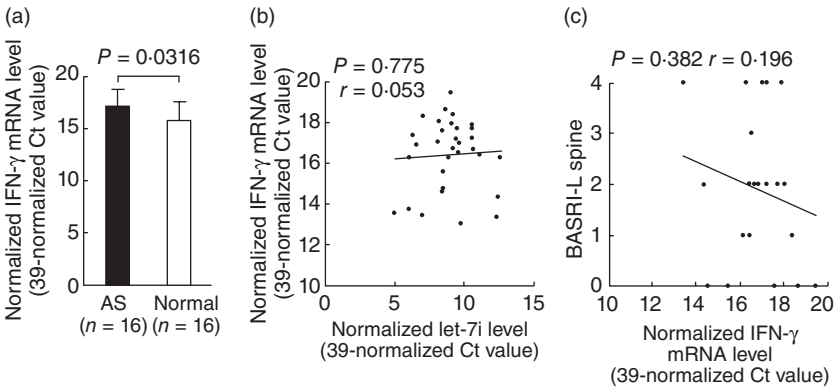

Fig. 9. Comparison of interferon (IFN)-γ mRNA expression between ankylosing spondylitis (AS) and normal T cells and correlations among IFN-γ mRNA with let-7i mRNA and BASRI of lumbar spine in AS patients. (a) The mRNA expression levels of IFN-γ in AS T cells were significantly higher than normal T cells. (b)Correlation between expression levels of IFN-γ mRNA and let-7i. (c) Correlation between expression levels of IFN-γ mRNA and BASRI of lumbar spine in AS patients.

Yersinia, *Campylobacter* and *Chlamydia* [42–46]. Although molecular mimicry between the bacterial components and self-peptides was considered to play a role [47], our results may provide an alternative explanation, that the bacterial LPS could suppress IFN-γ production in activated normal T cells. However, this regulatory mechanism was abrogated by the over-expressed let-7i in AS T cells (Fig. 8a).

IFN-γ is a key proinflammatory cytokine which has been shown to be elevated in serum from AS patients [48]. Although we found no correlation between let-7i and the mRNA expression of IFN-γ in AS patients (Fig. 9b), contradictory to the finding that let-7i may regulate IFN-γ production (Fig. 8b) it is possible that various factors, such as viral or bacterial infection, trigger IFN-γ gene expression to confound our results. In addition, we demonstrated that the increased expression of let-7i enhanced IFN-γ production in anti-CD3$^+$anti-CD28$^+$ LPS-stimulated T cells, but the mRNA expression of IFN-γ was compared in non-stimulated T cells from AS patients and controls. However, we still demonstrated that the IFN-γ mRNA expression levels were increased in AS T cells. Therefore, we propose that the increased let-7i expression in AS T cells activate the Th1 immune responses upon LPS stimulation. Although we showed that increased let-7i expression in T cells could suppress TLR-4 expression, it is premature to conclude that decreased TLR-4 expression on AS T cells contributed directly to this phenomenon. Instead, other molecule(s) involving the T cell signalling pathway targeted by let-7i might play an essential role. Selbach *et al.* demonstrated [49] that one miRNA can translationally repress hundreds of target genes. Nevertheless, the downstream molecular mechanism of increased let-7i expression stimulating a T helper type 1 (Th1) (IFN-γ) immune response requires more detailed studies.

O'Hara *et al.* [50] demonstrated that let-7i expression was suppressed by nuclear factor-kappa B (NF-κB), and many medications used for AS treatment have the potential to suppress NF-κB activity [51]. However, AS is a chronic inflammatory disease; the elevation of NF-κB DNA binding activity in lymphocytes could persist even after several months of adequate therapy [52]. In addition, we observed two newly diagnosed AS patients in this study who had not yet been treated with immunosuppressant. Their T cell let-7i expression levels appeared to be no different from those of the treated AS patients. Therefore, we consider that the increased expression of let-7i was irrelevant to treatment with immunosuppressive drugs. Therefore, the increased let-7i expression is a direct effect from AS disease *per se* and is involved in AS pathogenesis.

In contrast, the expression of Bcl-2 targeted by miR-16 remained unchanged in AS T cells compared with normal T cells (Fig. 3b). This is because other molecules and signalling pathways may compensate Bcl-2 expression that was suppressed by miR-16. In T cell lineage, the expression of c-kit target by miR-221 is limited to the progenitor T cells, and lost gradually upon differentiation [53]. Thus the expression of c-kit could not be detected in T cells from peripheral blood in our study (Fig. 4b). In addition to AS T cells, over-expression of miR-16 was also found in peripheral mononuclear cells from RA patients [16] and activated normal T cells [54]. It is possible that the increased expression of miR-16 and miR-221 in AS patients may trigger inflammatory reactions. The inter-relationships among these three miRNAs and their respective target molecules require further investigation. Recently, the expression of miRNAs was under the control of epigenetic mechanisms such as DNA methylation. The disruption of this network could cause pathological conditions such as malignant changes in gastrointestinal, urological and haematological systems [55–57]. Undoubtedly, investigation of the methylation status of the promoter region in miR-16, miR-221 and let-7i genes is important in elucidating the immunopathogenesis of AS. Conversely, the pathological roles of other altered expressed miRNAs, including miR-99b, let-7b, miR-513-5p, miR-218, miR-409-3p, miR-30e, miR-199a-5p and miR-215 in AS T cells (Fig. 1b), are now under investigation.

In conclusion, we found three highly expressed miRNAs: miR-16, miR-221 and let-7i in T cells from AS patients, among which let-7i and miR-221 were found to be correlated positively with BASRI for lumbar spine. The increased expression of let-7i in AS T cells contributes to the

immunopathogenesis of AS via enhancing the Th1 (IFN-γ) inflammatory response.

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Disclosure

None.

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