Effect of All-transretinoic Acid on Th17 and T Regulatory Cell Subsets in Patients with Ankylosing Spondylitis

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ABSTRACT. ***Objective.*** We compared Th17 and T regulatory cells in patients with ankylosing spondylitis (AS) and in healthy controls. The effect of all-transretinoic acid (ATRA) was studied on cultured CD4+ T cells of patients with AS compared to controls.

Methods. Eighteen patients with AS and 18 age- and sex-matched healthy controls were included. CD4+ T cells were separated and cultured in conditions of anti-CD3 and anti-CD28 stimulation with and without ATRA. Intracellular and secreted cytokines, transcription factors, and gene expression were evaluated after 72 h.

Results. The frequency of CD4+IL-17+ T cells was significantly higher in patients with AS compared to controls, and ATRA could significantly decrease it. The frequency of forkhead box protein 3 (FOXP3)+ retinoic acid-related orphan receptor γt (RORγt) negative T-bet negative CD4+ cells was significantly lower in cases compared to controls. Intracellular and secreted interferon-γ (IFN-γ) was not significantly different between cases and controls. ATRA significantly increased intracellular IFN-γ in cases but not in controls. Tumor necrosis factor-α (TNF-α) secretion was significantly higher and interleukin 10 secretion was significantly lower in culture supernatant of cases compared to controls. ATRA could significantly decrease TNF-α secretion in cases.

Conclusion. Our findings favor a pathogenic role for Th17 cells in AS. Th1 cells did not seem to contribute in the pathogenesis of this disease. The effect of ATRA as an immunomodulator on deviated immune cells was associated with decreased inflammatory markers. This association could be a reason for a clinical trial of ATRA in patients with AS. (J Rheumatol First Release March 1 2013; doi:10.3899/jrheum.121100)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS ALL-TRANSRETINOIC ACID
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Ankylosing spondylitis (AS) is a chronic progressive inflammatory disease with worldwide distribution. It mainly affects the axial skeleton and the prevalence ranges between 0.1% and 1.4%. Men are more affected than women and the disease usually occurs before the third decade of life[1]. The association of the disease with the HLA-B27 gene has been clearly shown; however, the exact pathogenic mechanism in AS is not known. The involvement of genes including the interleukin 23 (*IL23R*) gene implies that other mechanisms might be responsible in this disease. The *IL23R* gene encodes a cytokine receptor important in the Th17 cell subset of T cells[2,3] and the polymorphisms in this receptor are revealed to be associated with AS[4].

Since the Th17 cell subset was identified, numerous studies have focused on the involvement of this distinct Th subset in autoimmune diseases. The Th17 cell subset is the main source of IL-17 secretion and its key transcription factor could be retinoic acid-related orphan receptor γt (RORγt). Elevated serum levels of IL-17[5,6] and increased frequency of IL-17-positive CD4+ T cells[7] have been reported in patients with AS. Studies have discovered the

plasticity of Th17 cells and their potential to change to other subsets of Th cells[8,9], including forkhead box protein 3 (FoxP3)+ regulatory T cells.

CD4+ T regulatory cells (Treg), which express the transcription factor FoxP3, are a distinct Th subset able to suppress autoimmune processes[10]. These Treg cells can be induced in the periphery and they have been shown to have antiinflammatory effects.

Further, there is evidence that the active metabolite of vitamin A, all-transretinoic acid (ATRA), can divert the balance of Th17 cells into Treg cells by expression of FoxP3 and suppression of IL-17 in CD4+ T cells[11]. ATRA has been shown to have important regulatory roles in cellular proliferation and differentiation and to ameliorate various autoimmune models and diseases such as inflammatory arthritis[12], type 1 diabetes[13], experimental autoimmune uveoretinitis[14], and experimental autoimmune encephalomyelitis[15].

We aimed to compare CD4+ T cell cytokine secretion and transcription factors in a homogenous group of AS patients with those in healthy controls, and to investigate the effect of ATRA on Th17 and Treg cell differentiation of CD4+ T cells of our study groups. For proper comparison of the 2 groups, vitamin A dietary intake and blood levels were measured.

MATERIALS AND METHODS

Patients and controls. Eighteen patients with AS, diagnosed based on modified New York criteria[16], and 18 healthy age-matched and sex-matched controls were included. Patients with AS were recruited from the Iranian Ankylosing Spondylitis Association and healthy controls were employees of Tehran University of Medical Sciences. A questionnaire on demographic data, disease information, and medications was completed and the presence of any acute disease was ruled out by physical examination and history. These data were recorded for all patients: number of entheses, number of swollen joints, the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score[17,18], the Bath Ankylosing Spondylitis Functional Index (BASFI)[18,19], total back pain scores, the Bath Ankylosing Spondylitis Global Score (BAS-G)[20], and the Bath Ankylosing Spondylitis Metrology Index (BASMI)[21]. BASDAI and BASFI are recommended by the international Ankylosing Spondylitis Assessment working group consensus[22] in clinical evaluation of patients with AS and have been shown to be specific to disease and sensitive to change[23]. C-reactive protein (CRP) levels and HLA-B27 positivity were determined for patients. HLA-B27 subtypes were defined by sequence-specific primers using commercial kits (Olerup SSP AB).

Patients who had at least 2 of the following criteria were considered to have active AS: BASDAI score ≥ 4, total back pain score ≥ 4 measured by the numerical rating score, or morning stiffness lasting ≥ 1 h[24].

The study protocol was approved by the ethics board of Tehran University of Medical Sciences and written informed consent was obtained from all participants.

Cells and culture. Peripheral blood mononuclear cells were separated from whole blood by Ficoll-Hypaque gradient centrifugation (Lymphoflot, Biotest). CD4+ T cells were isolated by Dynabeads Untouched Human CD4 T Cell kit (Invitrogen), with a purity > 95% and viability > 98%. Cells were then cultured for 72 h in 24-well and 96-well plates coated with 2 μg/ml anti-CD3 (OKT3) supplemented with 1 μg/ml anti-CD28 (CD28.2).

ATRA. ATRA (Sigma-Aldrich), dissolved in dimethyl sulfoxide under argon gas as stock, was prepared for culture with RPMI culture medium supplemented with 10% fetal bovine serum and 1% L-glutamine 1 μM. The concentration was based on preliminary experiments, and CD4+ T cells were cultured in conditions with or without ATRA[25].

Antibodies. All monoclonal antibodies and their isotype controls (eBioscience) were used according to the manufacturer's recommendations. The antibodies used were as follows: anti-human CD3 (clone OKT3), anti-human CD28 (clone CD28.2), FITC anti-human CD69, FITC anti-human FoxP3, phycoerythrin (PE) anti-mouse/human RORγt, PerCP-Cy5.5 anti-mouse/human T-box21 (T-bet), FITC anti-human IL-17, PE anti-human interferon-γ (IFN-γ), PE-Cy5 anti-human CD45RA, and FITC anti-human CD4.

Intracellular cytokines and transcription factors. After 72 h culture, cells were stimulated for 4 h with 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich). Brefeldin A (3 μg/ml) was added at the final 3 h of stimulation. PMA-stimulated and ionomycin-stimulated cells for measurement of intracellular cytokines and other cells for measurement of intracellular transcription factors were then fixed and permeabilized by eBioscience fixation and permeabilization buffers. Cells were stained by appropriate monoclonal antibodies and were analyzed using BD flow cytometer and Flowjo software. In preliminary studies, intracellular staining was performed on *ex-vivo* unstimulated cells and the results were too low to be analyzed.

Secreted cytokines. Secreted cytokines from stimulated CD4+ T cells after 72 h of culture were measured in cell-free supernatants using eBioscience ELISA kits according to the manufacturer's instructions. The detection limit for IL-17 was 2 pg/ml and for IL-10, tumor necrosis factor-α (TNF-α), and IFN-γ was 4 pg/ml.

Gene expression. Gene expression was investigated in anti-CD3 and anti-CD28 stimulated CD4+ T cells cultured for 72 h in 24-well plates, with or without ATRA. Messenger RNA was isolated using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was transcribed into complementary DNA (cDNA) using the RevertAid First Strand cDNA Synthesis kit (Fermentase). Real-time PCR was performed using SYBR Green with the cycling program of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 60°C. Results were normalized to β-actin levels according to the ΔΔCt method, and relative gene expression was reported.

Vitamin A status. Dietary vitamin A intake was assessed by a validated food frequency questionnaire[26] and 24-h recall forms for 3 consecutive days. The data were analyzed by FPII software (ESHA Research). For measurement of vitamin A in the blood, serum retinol binding protein (RBP) level and transthyretin levels were assessed by ELISA[27].

Statistical analysis. All analyses were performed by SPSS 17.0 software. All variables were examined by 1-sample Kolmogorov-Smirnov test to test their normal distribution. Parametric variables were described by mean ± SEM and nonparametric variables by median. Independent-sample T test and paired-sample T test were used to compare means between parametric variables. Mann-Whitney U test was used for statistical comparisons of nonparametric data (data from real-time PCR). Correlations were determined by Pearson correlation. P values < 0.05 were considered significant.

RESULTS

Patients and controls. Eighteen patients with AS (13 men, 5 women) with a mean age of 34 ± 2 years and 18 age-matched and sex-matched healthy controls with a mean age of 33 ± 1 years were included in the study. Body mass index was not significantly different between cases and controls. Mean disease duration was 10 ± 1 years and age at disease onset was 23 ± 1 years. Of the patients studied, 12 (66.7%) were using nonsteroidal antiinflammatory drugs (NSAID), 7 (38.9%) were using disease-modifying

medication (sulfasalazine), and none were using corticosteroids or anti-TNF drugs.

HLA-B27 was positive in 12 patients (66.7%) and 2 controls (11.1%). The characteristics of patients and subtypes of HLA-B27 in HLA-B27-positive patients are shown in Table 1. HLA-B27*02 was the most frequent subtype in this population, followed by HLA-B27*05 and HLA-B27*04.

Dietary macronutrient intake and dietary vitamin A intake were not significantly different between patients and controls. RBP levels were significantly lower (p = 0.038) in patients (41.8 ± 8.2 μg/ml) compared to controls (69.8 ± 9.9 μg/ml), but there were no significant differences in transthyretin levels between cases and controls.

IL-17 and RORγt transcription factor in patients and controls. White blood cell counts, lymphocyte counts, and CD4+ T cell number and percentages were not statistically different between cases and controls.

The frequency of IL-17+ IFN-γ–negative cells was significantly higher in cases compared to controls and most IL-17+ cells in patients with AS and healthy controls were memory cells and CD45RA-negative (Figure 1A). Frequency of IL-17/IFN-γ double-positive cells was not significantly different between cases and controls (Table 2). Of IL-17+CD4+ T cells, 26.3 ± 2.5% in patients and 32.3 ± 2.6% in controls coexpressed IFN-γ. The difference was not statistically significant between the 2 groups.

Secreted levels of IL-17 in culture supernatants of CD4+ T cells were not significantly different between cases and controls (734 ± 143 pg/ml in cases vs 902 ± 138 pg/ml in controls; p = 0.407).

In patients with AS who were using NSAID, frequency of IL-17+ IFN-γ–negative cells was significantly lower (p = 0.003) with a mean of 0.7% ± 0.03% compared to patients who were not using NSAID, with a mean of 1.2% ± 0.1%.

For patients with AS, no correlation was found between percentage of total IL-17+CD4+ T cells or IL-17+ IFN-γ-negative CD4+ T cells and CRP, BAS-G, BASDAI, BASFI, and BASMI and no relation with activity of the disease.

Frequency of CD4+ T cells expressing RORγt transcription factor was not statistically different between cases and controls (19.3% ± 2.6% in cases, 20.8% ± 2.3% in controls; p = 0.679; Figure 1B). Percentage of cells expressing RORγt did not correlate with disease indices.

IFN-γ and T-bet transcription factor in patients and controls. Frequency of IFN-γ+ IL-17–negative cells in CD4+ T cell population was not statistically different between cases and controls (Table 2, Figure 1C). In patients with AS, there were no significant correlations between the percentage of IFN-γ+ cells and disease indices (e.g., BASMI, BASDAI, BASFI, and CRP).

Secreted levels of IFN-γ in culture supernatants of CD4+ T cells were not significantly different between cases and controls (50.3 ± 15.8 ng/ml in cases vs 58.1 ± 15.3 ng/ml in controls; p = 0.727; Figure 1D).

Mean percentages of CD4+ T cells expressing T-bet transcription factor were 4.6% ± 0.8% in cases and 3.6% ± 0.5% in controls (Figure 1E). Percentages of T-bet expressing CD4+ T cells were not significantly different between cases and controls.

IL-10 and FoxP3 transcription factor in patients and

Table 1. Demographic data and characteristics of the patients with ankylosing spondylitis.

Patient/ sex/age	Disease Duration, yrs	BASDAI Score	BASFI Score	BASMI Score	Disease Activity	Treatment	CRP	HLA-B27 Subtypes
1/M/38	8	6.83	4.50	4.80	Active	NSAID + DMARD	+	HLA-B*27:02
2/M/53	20	5.50	2.10	3.00	Active	—	–	Negative
3/M/27	3	0.50	0.60	1.00	Not active	NSAID	–	HLA-B*27:02
4/F/44	7	6.83	6.50	4.20	Active	—	–	Negative
5/M/28	9	1.00	0.20	2.40	Not active	NSAID + DMARD	–	HLA-B*27:04
6/F/26	5	1.67	4.80	2.60	Not active	NSAID + DMARD	–	HLA-B*27:02
7/M/31	11	4.67	6.50	6.40	Active	NSAID	–	HLA-B*27:05
8/F/40	16	2.83	0.70	1.80	Not active	—	–	Negative
9/F/21	7	2.50	0.80	2.00	Not active	DMARD	–	HLA-B*27:02
10/M/27	13	4.83	0.50	1.40	Active	NSAID + DMARD	–	HLA-B*27:02
11/M/36	9	3.00	7.60	5.20	Not active	NSAID	+	HLA-B*27:04
12/M/49	16	3.00	3.10	1.60	Not active	NSAID	–	HLA-B*27:04
13/M/42	27	5.17	5.90	6.20	Active	NSAID + DMARD	–	HLA-B*27:04
14/M/25	3	2.17	0.40	2.60	Not active	NSAID + DMARD	–	HLA-B*27:04
15/M/26	10	2.00	0.30	0.60	Not active	—	–	Negative
16/M/29	7	8.33	7.80	4.60	Active	NSAID	+	Negative
17/F/36	10	4.83	1.30	1.00	Active	—	–	Negative
18/M/35	7	1.50	1.40	1.20	Not active	NSAID	–	HLA-B*27:02

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; BASMI: Bath Ankylosing Spondylitis Metrology Index; CRP: C-reactive protein; NSAID: nonsteroidal antiinflammatory drugs; DMARD: disease-modifying antirheumatic drug.

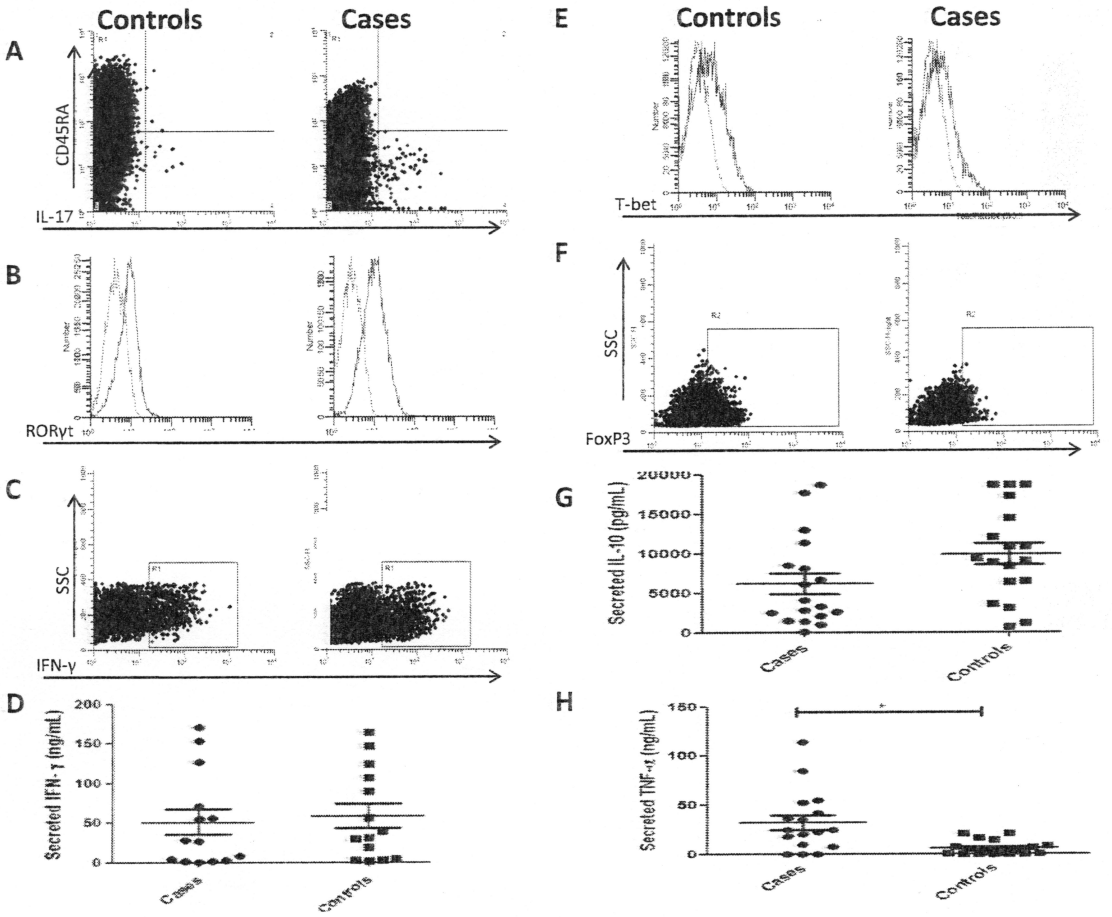

Figure 1. Comparison between cases and controls of cytokines and transcription factors in CD4+ T cell cultures after 72 h. A. Representative dot plots showing higher IL-17+ CD45RA-negative CD4+ T cells in cases compared to controls. B. Representative histograms showing similar retinoic acid-related orphan receptor-γ t (RORγt) expression in cases and controls. C and D. Similar intracellular IFN-γ expression and cytokine secretion in CD4+ T cells in cases and controls. E. Representative histograms showing similar T-bet expression in cases and controls. F. Representative dot plots showing lower FoxP3+CD4+ T cells in RORγt-negative T-bet-negative cell population in cases compared to controls. G. Secreted IL-10 was shown to be significantly lower in cases compared to controls. H. Secreted TNF-α was significantly higher in cases compared to controls. Typical examples of intracellular staining are shown and cells are gated first according to their forward scatter and side scatter. Bars show the mean values and SEM.

controls. The percentages of FoxP3+ RORγt-negative Tbet-negative cells were significantly lower in cases (9.7% ± 1.2%) compared to controls (16.1% ± 3.0%; p = 0.048; Figure 1F), but they had no significant correlation with disease indices.

Secreted IL-10 in culture supernatant of CD4+ T cells was lower in cases (6172 ± 1339 pg/ml) compared to controls (9911 ± 1387 pg/ml), but the differences were not statistically significant (p = 0.061; Figure 1G).

TNF-α in patients and controls. TNF-α secretion in culture supernatant of CD4+ T cells was significantly higher in cases (64.2 ± 23.8 ng/ml) compared to controls (5.6 ± 1.7 ng/ml; p = 0.026; Figure 1H).

Disease activity. Eight patients were discovered to have active disease. Total entheses count, number of joints with arthritis, CRP, BAS-G, and BASDAI were significantly higher in patients with active disease compared to patients with inactive disease.

Percentages of IL-17+, IFN-γ+, FoxP3+, RORγt+, and T-bet+ CD4+T cells were not significantly different between patients with active or inactive disease.

Effect of ATRA on CD4+ T cells. Mean percentage of IL-17+ IFN-γ-negative CD4+ T cells decreased significantly after 72 h of ATRA treatment in culture in patients (0.19% ± 0.08% decrease in cases; p = 0.042) but not in controls (Figure 2A). Secreted levels of IL-17 also decreased significantly in cases after addition of ATRA (243 ± 104 pg/ml decrease; p = 0.034), but not in controls. In patients who were not using NSAID, ATRA significantly reduced the frequency of IL-17+ IFN-γ-negative CD4+ T cells (0.48% ±

Table 2. Intracellular cytokine staining results in cases and controls.

	Mean ± SEM	Mean Difference*	95% CI of the Difference	p
IL-17+ IFN-γ – CD4+ T cells (%)				
Cases	0.9 ± 0.06	0.36	0.20 to 0.53	< 0.0001
Controls	0.5 ± 0.04			
IL-17+ IFN-γ+ CD4+ T cells (%)				
Cases	0.3 ± 0.04	0.05	–0.06 to 0.17	0.336
Controls	0.2 ± 0.03			
IL-17– IFN-γ+ CD4+ T cells (%)				
Cases	9.0 ± 0.8	1.11	–1.4 to 3.7	0.389
Controls	7.9 ± 0.9			

* Between case and control groups. IFN: interferon.

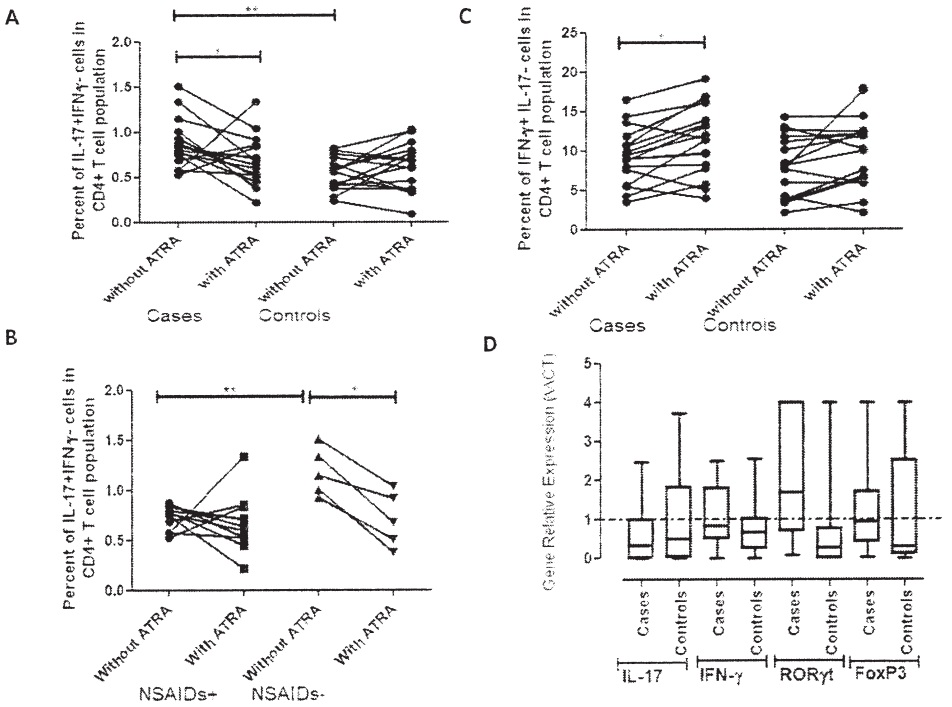

Figure 2. Effect of all-transretinoic acid (ATRA) on cytokines and transcription factors in CD4+ T cells of patients with AS and healthy controls. A. Cases had significantly higher percentage of IL-17+ IFN-γ-negative CD4+ T cells compared to controls and ATRA significantly reduced the frequency of IL-17+ IFN-γ-negative CD4+ T cells in cases. B. The frequency of IL-17+ IFN-γ-negative CD4+ T cells was significantly higher in patients who were not using NSAID, and ATRA significantly reduced the frequency of IL-17+ IFN-γ-negative CD4+ T cells in these patients. C. The frequency of IFN-γ+ IL-17-negative CD4+ T cells was not significantly different between cases and controls, and ATRA could increase this percentage only in patients and not in controls. D. CD4+ T cells were cultured for 3 days in the presence of anti-CD3 and anti-CD28 and gene expression level was determined by real-time PCR. The results were normalized to β-actin. Boxes and lines show median and 5%-95% percentiles. *p < 0.05. **p < 0.005. RORγt: retinoic acid-related orphan receptor-γt; FoxP3+: forkhead box protein 3+.

0.07% decrease; p = 0.003; Figure 2B) and secreted IL-17 in culture supernatant of CD4+ T cells (213 ± 48 pg/ml decrease; p = 0.012).

The percentage of IL-17+IFN-γ+CD4+ T cells did not change significantly after the addition of ATRA to the cultures. The percentage of IFN-γ+CD4+ T cells rose significantly after ATRA was added to the cultures of cases (2.1% ± 0.6% increase; p = 0.004), but not in controls (Figure 2C).

The amount of secreted IFN-γ and IL-10 did not change significantly after addition of ATRA to the cultures, in cases

or controls. Secreted TNF-α decreased significantly in culture supernatants of cases (56.7 ± 21.1 ng/ml decrease; p = 0.016), but not in controls.

ATRA significantly increased the percentage of FoxP3+ RORγt-negative T-bet-negative cells in controls (p = 0.042) but not in cases. RORγt expression in stimulated CD4+ T cells increased after 72 h of culture in patients but not in controls. The increase in RORγt expression was in FoxP3+RORγt+CD4+ T cells. T-bet expression did not change significantly after ATRA treatment in cases or controls.

The effect of ATRA on CD4+ T cell gene expression was analyzed by real-time PCR. There were no significant differences between cases and controls regarding effects of ATRA on change of expression of IL-17 and IFN-γ. IL-17 gene expression was reduced to half after ATRA treatment in culture (median value 0.5). However, IFN-γ expression after addition of ATRA was reduced to 0.8 and 0.6 times the value before ATRA treatment in cases and controls, respectively. ATRA could significantly increase RORγt expression in cases (1.7 times; p = 0.001) compared to controls (0.7 times). Change in FoxP3 expression was not significantly different between the 2 groups (Figure 2D).

DISCUSSION

A number of studies have focused on the role of Th17 cells in autoimmune diseases and AS. Increased frequency of Th17 cells in patients with AS has been reported[7], but concurrent evaluation of cytokines and their transcription factors and the effect of ATRA as an immune modulator have not been evaluated. Vitamin A dietary intakes and transthyretin levels of our cases were not significantly different from the controls, while RBP levels were significantly lower in cases compared to controls. These results indicated vitamin A deficiency in patients in comparison to the controls. Of the patients, only 66.7% were positive for HLA-B27, and this was compatible with previous studies performed in the Iranian population[28,29]. There are now more than 40 variants of HLA-B27 reported in the literature. The subtypes identified in the patients were among those reported in all populations including Iranian patients with AS[1,28,30].

Overall, the frequencies of IL-17+ cells in the CD4+ T cell population were comparable to those found in the study by Jandus, *et al*[31] and Shen, *et al*[7]. In accord with these studies[7,31], we also observed that the frequency of IL-17+CD4+ T cells was higher in patients with AS compared to controls. We could further show that this increased frequency was in the IL-17+IFN-γ-negative CD4+ T cell population.

Regarding IL-17/IFN-γ double-producing T cells, there are conflicting results in literature, with some studies reporting more and some less pathogenicity of these cells *in vivo*[32]. However, in our study, these cells were not significantly different between cases and controls and their frequency (around 20%) was comparable to data reported by Shen, *et al*[7].

In our study, frequency of RORγt-expressing CD4+ T cells was not significantly different between cases and controls. This could imply that other transcription factors such as STAT3 can contribute to the increased frequency of IL-17–producing cells in patients with AS[33].

The finding of lower levels of IL-17+ IFN-γ-negative CD4+ T cells in patients who were using NSAID might imply that NSAID can be effective in reducing IL-17+ T cells in AS. However, no effect of NSAID was observed on other cytokines and transcription factors, and to confirm these findings, longitudinal data on patients with AS before and after the institution of NSAID should be obtained.

In our study, as in the study by Shen, *et al*[7], there were no significant differences between IFN-γ+CD4+ T cells in cases and controls. This was confirmed by the similar expression of T-bet in cases and controls and the results of ELISA. It can be concluded that Th1 cells might not be involved too much in the pathogenesis of AS in patients with uncomplicated AS.

FoxP3+ Treg cells have been reported to have normal numbers in patients with AS[34]. We observed that FoxP3+ RORγt-negative T-bet-negative CD4+ T cells were significantly lower in cases compared to controls. Decreased FoxP3+CD4+ T cells along with decreased IL-10 secretion in culture supernatants can be indicative of lower Treg cells in patients with AS compared to healthy controls.

Disease activity was defined based on clinical and patient-rated scores. In our study, similar to the study by Taylan, *et al*[6], there was no significant difference between cytokine levels in patients with active versus inactive AS. Even CRP levels were not significantly different between these 2 groups of patients. This could imply that disease activity based on these scores might not reflect the real inflammatory status of the patients.

The control of autoimmunity by retinoids and derivatives of vitamin A has been previously reported[11]. ATRA was shown to induce the expression of FoxP3 and suppress the expression of IL-17 in CD4+ T cells in mice[11]. It has been proposed in a study on mice that there is a self-correcting mechanism of ATRA for transforming growth factor-β (TGF-β), to regulate both proinflammatory and antiinflammatory immunity. ATRA is capable of inhibiting the IL-6-driven induction of proinflammatory Th17 cells and promoting antiinflammatory Treg cell differentiation[35]. ATRA, in the presence of TGF-β, was also reported to induce the generation of CD4+CD25+FoxP3+ Treg cells with stable suppressive activity from human naive T cells[36].

We found that ATRA could suppress IL-17+ IFN-γ-negative CD4+ T cells *in vitro* in patients with AS and not in healthy controls. It was also shown that ATRA could decrease IL-17 in patients who were not using NSAID and

had increased levels of IL-17. IL-17+IFN-γ+CD4+ T cells that were not significantly different between cases and controls did not change significantly after addition of ATRA. This could be explained by the effect of ATRA on deviated immune response and its effect on balancing the immune system. Xiao, *et al* also reported that ATRA had no effect on FoxP3 and IL-17 expression in mouse cells in neutral conditions[15]. Another study also revealed that retinoid signaling is limited to the sites of inflammation[37].

The increase in RORγt expression after the addition of ATRA was in RORγt+FoxP3+CD4+ T cells, and this group can be an intermediate one that eventually changes to FoxP3+ T cells. It has been suggested that the cells coexpressing both these transcription factors cannot efficiently change into Th17 cells and direct and indirect interaction of FoxP3 and RORγt attenuate the function of RORγt[38]. A study on mice showed that ATRA not only enhances *de novo* generation of Treg cells but also suppresses *de novo* differentiation of Treg cells into Th17 cells by downregulating IL-6 receptors[39]. Future studies investigating this double-positive population of cells would be useful.

Nozaki, *et al* found that in patients with rheumatoid arthritis[40], ATRA decreased TNF-α and IFN-γ secretion. Similarly, we found decreased levels of TNF-α after ATRA treatment in culture, but we observed increased levels of IFN-γ-producing cells in patients with AS after ATRA treatment. The reason might be that different diseases were studied.

In support of our findings, an orally administered synthetic retinoid called Am80 was studied in experimental autoimmune myositis, and was found to increase IFN-γ and IL-10 production while decreasing TNF-α[41]. The increase in IFN-γ+ cells after addition of ATRA to the cultures in our study might also be due to the existence of transitional Th subsets capable of producing IFN-γ and antiinflammatory cytokines. In a study on mice, IFN-γ+FoxP3+ cells were shown[38]. Further, there are some studies that have shown a protective role of IFN-γ against autoimmune diseases[42,43,44,45].

Our findings favor a pathogenic role for IL-17–secreting CD4+ T cells in AS. Increased IL-17+CD4+ T cells and decreased Treg cells might be responsible for the pathogenesis of AS. Lower RBP levels in patients with AS need to be further studied by assessing vitamin A levels in these patients. ATRA could be a subject for a clinical trial in patients with AS; it may be able to restore the normal function of the immune system.

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