

# In Vivo Peripheral Blood Proinflammatory T Cells in Patients with Ankylosing Spondylitis

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**ABSTRACT. Objective.** Previous reports have shown an increase in peripheral blood mononuclear cells' (PBMC) Th17 cell subpopulation and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) secretion after *in vitro* stimulation with anti-CD3/CD28 or phorbol myristate acetate/ionomycin in ankylosing spondylitis (AS). The aim of our study was to determine whether there is a Th17 polarization not subjected to *in vitro* stimulation in patients with AS.

**Methods.** Nonstimulated PBMC were analyzed from 46 patients with AS, including 7 (15.2%) receiving tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) inhibitors, 20 patients with rheumatoid arthritis, and 25 healthy controls. The surface phenotype of freshly isolated PBMC was determined by flow cytometry. Th1, Th2, Th17, and Treg subsets were defined as CD3+CD4+IFN- $\gamma$ +, CD3+CD4+IL-4+, CD3+CD4+IL-17A+, and CD3+CD4+FoxP3+, respectively. Serum cytokines and interleukin 8 (IL-8) levels were quantified by Luminex technology.

**Results.** The percentages of Th17 and Th1 cells in AS were higher than in healthy controls ( $7.4\% \pm 1.8\%$  vs  $0.7\% \pm 0.2\%$  and  $4.0\% \pm 1.3\%$  vs  $1.1\% \pm 0.3\%$ , respectively;  $p < 0.0001$ ). Th17 and Th1 cell subsets in patients taking TNF- $\alpha$  inhibitors were lower than in those naive to such therapeutics and similar to healthy controls. Serum levels of IL-6, IL-17A, TNF- $\alpha$ , and IL-8 were significantly higher in patients with AS compared to controls.

**Conclusion.** The percentages of Th17 and Th1 cells in PBMC without *in vitro* stimulation, as well as cytokine and IL-8 levels, were significantly increased in patients with AS compared with healthy controls. These T cell subsets and cytokine profiles of patients with AS taking TNF- $\alpha$  inhibitors were similar to those of healthy controls. (J Rheumatol First Release Feb 15 2012; doi:10.3899/jrheum.110862)

## Key Indexing Terms:

TH17

ANKYLOSING SPONDYLITIS

TUMOR NECROSIS FACTOR- $\alpha$

One of the most important changes in immunology concepts in recent years is the emergence of Th17 cells as a new subpopulation of CD4+ T cells characterized by the production of proinflammatory cytokines, particularly interleukin 17 (IL-17A)<sup>1,2</sup>, but also IL-6, IL-17F, IL-22, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and granulocyte-macrophage colony-stimulating factor. Moreover, Th17 cells have been related to autoimmune diseases<sup>3</sup>. They also stimulate the formation of osteoclasts and

consequently bone resorption, and they recruit neutrophils and monocytes<sup>4,5,6</sup>. Although the cause of ankylosing spondylitis (AS) is still unknown, a number of studies have clearly demonstrated a significant role of TNF- $\alpha$  in the pathogenesis of the disease<sup>7</sup>. The participation of TNF- $\alpha$  in the pathogenesis of AS has been demonstrated by the clinical improvement observed with the use of TNF- $\alpha$  blockers in these patients<sup>8,9,10,11</sup>. However, the effect of this treatment on the effector T cell subpopulations in circulating blood is still unknown.

Reports have dealt with the possible role of Th17 cells in AS and related spondyloarthritis (SpA). Indeed, sera of patients with AS have increased levels of IL-17 and TNF- $\alpha$ <sup>12,13</sup> as well as Th17 cells after *in vitro* stimulation<sup>14,15</sup>. It is important, however, to determine whether there is a polarization to a Th17 response not subjected to *in vitro* stimulation, by either anti-CD3/CD28 antibodies or phorbol myristate acetate/ionomycin.

The aim of our study was to evaluate the phenotype of freshly isolated circulating CD4+ T cells from patients with AS, including juvenile and adult-onset forms, as well as those naive to TNF- $\alpha$  blockers and those receiving such biologic agents, and compare them with healthy controls and patients with rheumatoid arthritis (RA).

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## MATERIALS AND METHODS

We studied peripheral blood samples of 46 patients with AS diagnosed according to the modified New York criteria<sup>16</sup>, including 28 with juvenile-onset (60.9%; 25 males) and 18 with adult-onset (39.1%; 13 males) disease; 7 (15.2%) received TNF- $\alpha$  inhibitors (6 of them infliximab and 1 etanercept). Twenty-six patients (54.2%), including 1 receiving a TNF- $\alpha$  blocker, scored  $\geq 4$  on the BASDAI (Bath Ankylosing Spondylitis Disease Activity Index)<sup>17,18</sup>. We included as controls 20 patients with RA<sup>19</sup> as a joint autoimmune disease as well as 25 healthy controls. Demographic and clinical characteristics of the study population are shown in Table 1.

The study was approved by the Committees of Medical Ethics of our institutions. Only participants who gave informed consent to participate were recruited.

**Analysis of the CD4+ T cell subpopulations in mononuclear cells by flow cytometry.** Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). In order to analyze the distribution of Th1, Th2, Th17, and Treg subpopulations, cells were first labeled with both antigen-presenting cell-anti-human CD3 and PE-Cy5-anti-human CD4 monoclonal antibodies (BD PharMingen, San Diego, CA, USA) in 4 separated tubes at room temperature in the dark for 20 min. Cells were washed and permeabilized with cytofix/cytoperm solution (BD Biosciences, San Jose, CA, USA) at 4°C for 20 min. Intracellular staining was performed with a PE-Cy7-anti-human interferon- $\gamma$  (IFN- $\gamma$ ), PE-anti-human IL-4, PE-anti-human IL-17A, and FITC-anti-human FoxP3 monoclonal antibodies (eBioscience, San Diego, CA, USA). T cell subsets were analyzed with a flow cytometry FACSARIA system (BD Biosciences). Isotype controls were used to set the threshold and gates in the cytometer. An electronic gate was made for CD3+CD4+ cells and a total of 50,000 events were recorded for each sample and analyzed with the FACSDiva software (BD Biosciences). Results are expressed as the relative percentage of IFN- $\gamma$ , IL-4, IL-17A, or FoxP3-expressing cells present in each gate.

**Cytokine and IL-8 quantitation by Luminex in serum.** Serum concentrations of IL-6, IL-12, IL-17A, IFN- $\gamma$ , TNF- $\alpha$ , and IL-8 were measured by Luminex bead-based technology according to the manufacturer's recommendation (Bio-Rad Laboratories Inc., Hercules, CA, USA). The detection limit of the experimental system was 2.6, 3.5, 3.3, 6.4, 6.0, and 1.0 pg/ml, respectively. Results were analyzed using Bio-Plex v 4.1 software.

**Statistical analysis.** Continuous variables were expressed as mean  $\pm$  SD. Binomial variables were expressed as frequency and percentages. Chi-square, ANOVA, and t tests were used for comparisons between groups. The p values  $< 0.05$  were considered statistically significant. SPSS version 16 software was used.

## RESULTS

**Increased percentage of Th1, Th17, and Treg cells in peripheral blood from patients with AS.** When comparing the per-

centages of cell populations between juvenile and adult-onset patients with AS, no differences were found (data not shown) and thus both groups were joined for the remaining analyses, here denoted the patients with AS. The percentage of Th17 cells was significantly higher in patients with AS compared with healthy controls [ $7.4\% \pm 1.8\%$  vs  $0.7\% \pm 0.2\%$  ( $p < 0.0001$ ); Figure 1].

A certain trend of higher numbers of Th17 cells was observed in patients with RA compared with healthy controls ( $p = 0.06$ ; Figure 2). A clear difference was found, however, in the Th1 cell subpopulation, with an increased percentage in both patients with AS ( $4.0\% \pm 1.3\%$ ) and those with RA ( $4.2\% \pm 1.5\%$ ) compared with healthy controls [ $1.1\% \pm 0.3\%$  ( $p < 0.0001$ ); Table 2]. These results showed a quantitative increase in the Th17 cell subpopulation of peripheral blood from patients with AS, while greater percentages of the Th1 cell subpopulation were similar in both inflammatory diseases. There were no statistically significant differences in the Th2 cell population percentage in the 3 groups under study [AS  $1.3\% \pm 0.4\%$ , RA  $1.1\% \pm 0.3\%$  ( $p = 0.49$ ), and healthy controls  $1.0\% \pm 0.4\%$  ( $p = 0.10$ )]. The percentage of Treg cells was also investigated according to FoxP3 expression. These cells were increased in patients with AS compared with healthy controls [ $7.3\% \pm 1.3\%$  vs  $5.3\% \pm 1.7\%$  ( $p = 0.01$ ); Table 2 and Figure 2].

**Cell populations in patients treated with TNF- $\alpha$  inhibitors.** Seven patients with AS were receiving treatment with biological agents: 6 with infliximab and 1 with etanercept. When the analysis was made on the percentages of CD3+CD4+ subpopulations, we found lower percentages of Th17 cells in these patients compared with AS patients who received other treatments [ $0.8\% \pm 0.2\%$  vs  $7.4\% \pm 1.8\%$  ( $p < 0.0001$ )], and percentages similar to those found in controls [ $0.7\% \pm 0.2\%$  ( $p = 0.85$ ); Figure 3]. Further, it was observed that patients treated with TNF- $\alpha$  blockers had lower percentages of Th1 cells than healthy controls [ $0.7\% \pm 0.2\%$  vs  $1.1\% \pm 0.3$  ( $p = 0.02$ )]. No significant difference was found in Treg cells [AS patients with biological treatment  $5.7\% \pm 1.8\%$ ; controls  $5.3\% \pm 1.7\%$  ( $p = 0.74$ )].

Serum cytokines and IL-8 levels were quantified in 11

Table 1. Demographic and clinical characteristics of the study population. Values are expressed as mean  $\pm$  SD.

Characteristic	AS, n = 39	AS with Anti-TNF- $\alpha$ , n = 7	RA, n = 20	Healthy Controls, n = 25	p*
Age, yrs	32 $\pm$ 13	28 $\pm$ 11	47 $\pm$ 14	32 $\pm$ 8	$< 0.0001$
Sex, M/F	32/7	6/1	5/15	18/7	NS
Age at disease onset, yrs	17 $\pm$ 2	16 $\pm$ 1	43 $\pm$ 3	—	$< 0.0001$
CRP	14 $\pm$ 3.2	10.4 $\pm$ 6.4	11.7 $\pm$ 3	—	0.8
BASDAI	4.4 $\pm$ 2.4	2.5 $\pm$ 3.4	—	—	$< 0.0001$
DAS28	—	—	3.8 $\pm$ 1.3	—	ND

\* ANOVA. AS: ankylosing spondylitis; RA: rheumatoid arthritis; NS: no significant difference; ND: not determined; CRP: C-reactive protein; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; DAS28: 28-joint Disease Activity Score; TNF: tumor necrosis factor.

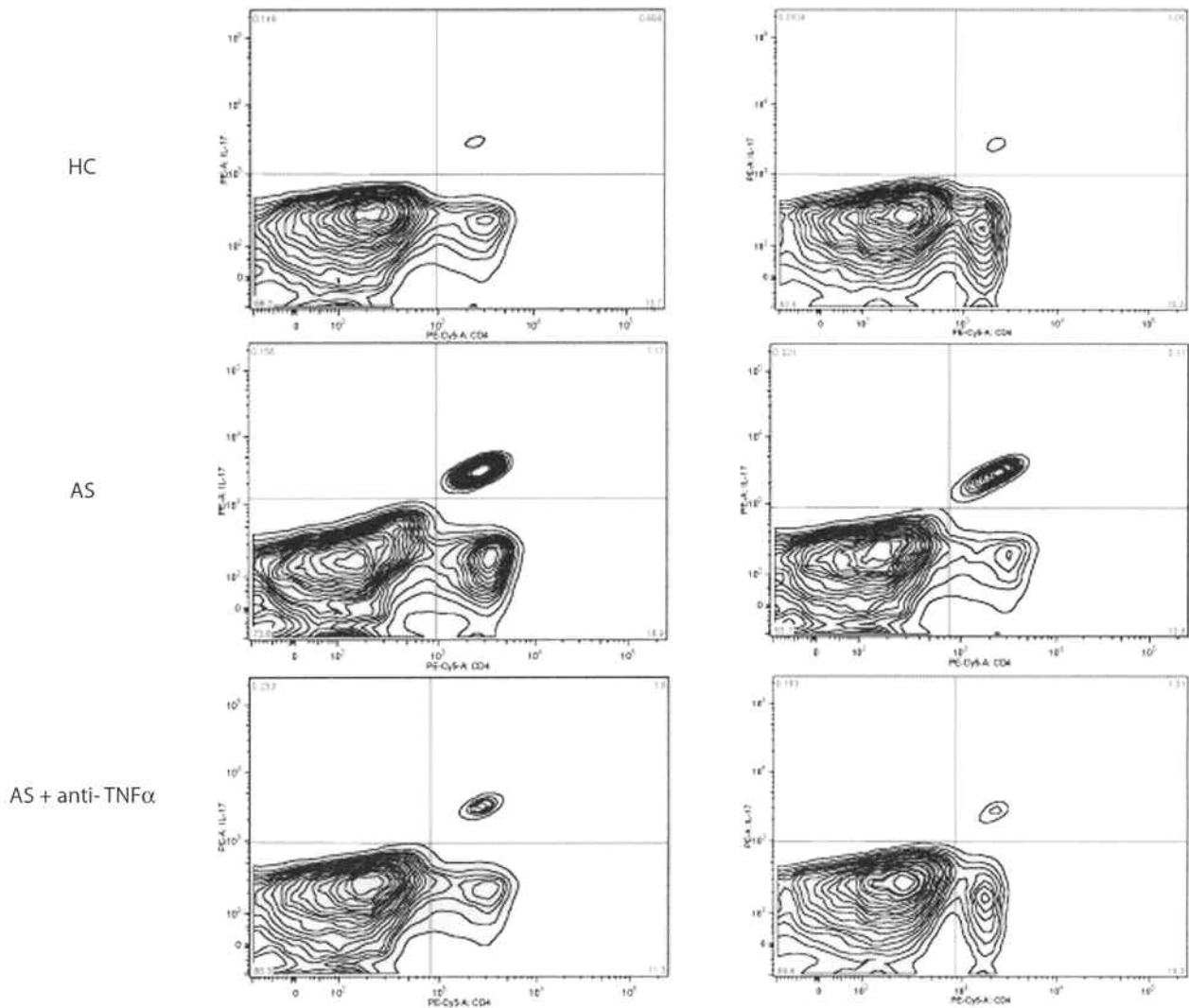


Figure 1. Flow cytometry analysis of the Th17 subpopulations. Representative contour showing *ex vivo*-detected CD4+ T cells producing IL-17A in human peripheral blood lymphocytes from healthy controls (HC), patients with ankylosing spondylitis (AS), and patients with AS treated with TNF- $\alpha$  inhibitors.

selected patients with AS who had BASDAI scores  $\geq 6$  and Th17 levels  $\geq 8\%$ ; in 5 patients with AS under treatment with TNF- $\alpha$  inhibitors (1 patient with active disease and a missing serum measurement were excluded); and in 8 healthy controls. Elevated serum levels of IL-6, IL-12, IL-17A, IFN- $\gamma$ , TNF- $\alpha$ , and IL-8 were found in patients with AS. In all instances, cytokines and IL-8 were significantly higher compared with patients receiving TNF- $\alpha$  blockers and healthy controls (Table 3).

## DISCUSSION

We identified an *in vivo* increase of IL-17-producing CD4+ lymphocytes in peripheral blood from patients with AS. This spontaneous production of cytokines has been reported in bacterial infections<sup>20</sup>. The persistence of this stimulus could promote the differentiation of T cell subpopulations associated with autoimmune inflammatory disease, such as the Th17 cells.

This group of patients also showed an increase in Th1 and Treg cell subpopulations. Reports have shown an increase in the percentage of Th17 cells in mononuclear cells in patients with AS and those with RA<sup>15</sup>, while another study shows an increase solely in SpA, very much like our own results<sup>14</sup>. These apparent contradictions could be due to the fact that Th17 cells in these studies were quantified after *in vitro* stimuli.

Even though the use of anti-CD3/CD28<sup>21</sup> antibodies provides a more physiological stimulus for T cells than phorbol myristate acetate/ionomycin<sup>22</sup>, both send intracellular signals that trigger T lymphocytes. This activation, however, does not reflect the state of T cell subpopulations in basal conditions.

On the other hand, patients treated with TNF- $\alpha$  blockers showed a decrease not only in IFN- $\gamma$ -producing and IL-17A-producing CD4+ T cells, but also in cytokine levels in serum. It is known that patients with AS have increased levels of TNF- $\alpha$  in serum<sup>13</sup>. This cytokine is a powerful proinflammatory mediator, with the ability to produce inflammation

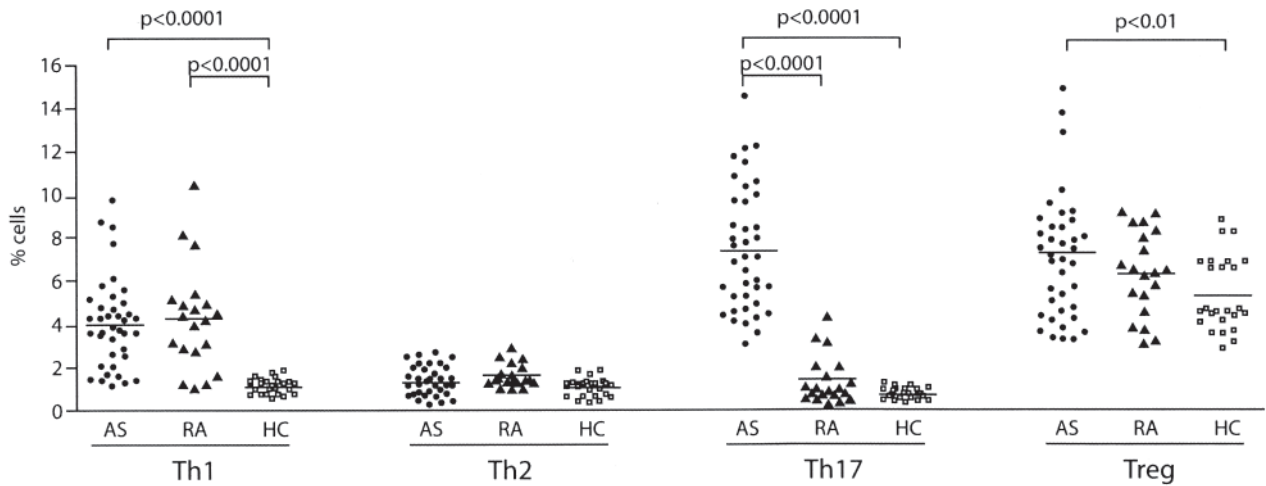


Figure 2. Comparison of the T cell subpopulations between patients with ankylosing spondylitis (AS), patients with rheumatoid arthritis (RA), and healthy controls (HC). Percentages of T cell subpopulations (Th1, Th2, Th17, and Treg) in peripheral blood of HC, patients with AS, and patients with RA.

Table 2. Comparison between groups on T cell subsets in peripheral blood. Values are expressed as mean  $\pm$  SD percentage.

Subpopulation	AS, n = 39	AS with Anti-TNF- $\alpha$ , n = 7	RA, n = 20	Healthy Controls, n = 25	p*
Th1	4.0 $\pm$ 1.3	0.7 $\pm$ 0.2	4.2 $\pm$ 1.5	1.1 $\pm$ 0.3	< 0.0001
Th2	1.3 $\pm$ 0.4	1.3 $\pm$ 0.6	1.1 $\pm$ 0.3	1 $\pm$ 0.4	0.2
Th17	7.4 $\pm$ 1.8	0.8 $\pm$ 0.2	1.4 $\pm$ 1.1	0.7 $\pm$ 0.2	< 0.0001
Treg	7.3 $\pm$ 1.3	5.7 $\pm$ 1.8	6.3 $\pm$ 1.9	5.3 $\pm$ 1.7	0.03

\* One-way ANOVA. AS: ankylosing spondylitis; TNF: tumor necrosis factor; RA: rheumatoid arthritis.

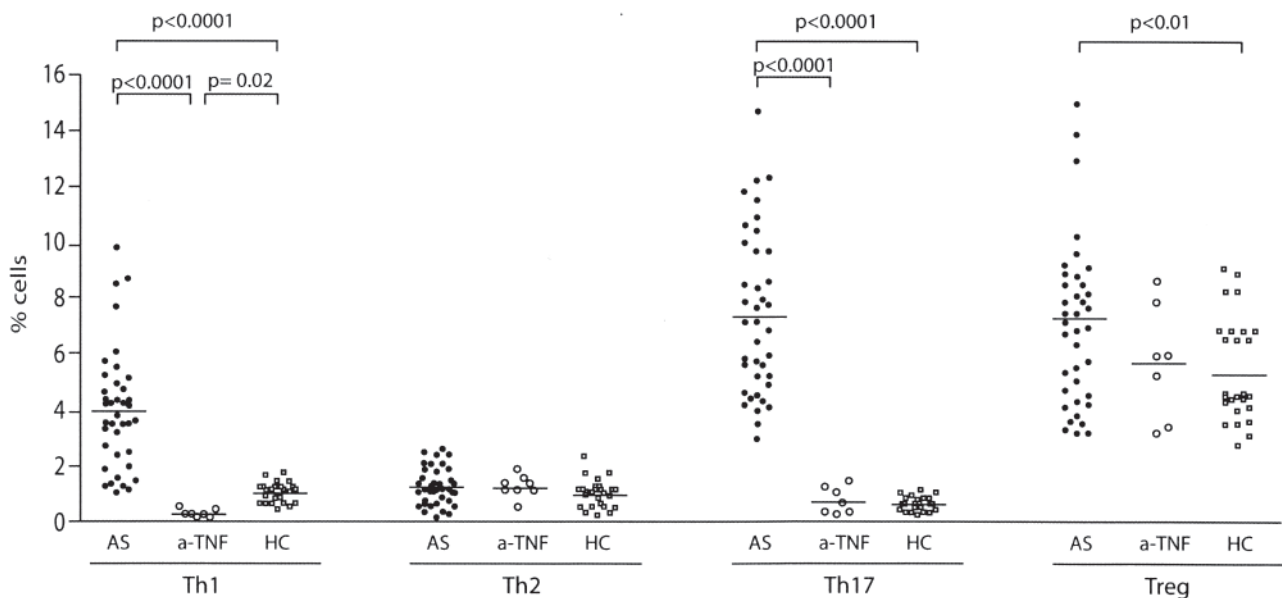


Figure 3. Comparison of the T cell subpopulations between patients with ankylosing spondylitis (AS) and healthy controls (HC). Percentages of T cell subpopulations (Th1, Th2, Th17, and Treg) in peripheral blood of HC, patients with AS, and patients with AS treated with TNF- $\alpha$  inhibitors.

Table 3. Comparison of serum cytokines and IL-8 levels among groups. Values are expressed as mean  $\pm$  SD.

Cytokine	AS, n = 11	AS with Anti-TNF- $\alpha$ , n = 5	Controls, n = 8	p*
IL-6, pg/ml	535.2 $\pm$ 84.2	4.4 $\pm$ 0.6	2.5 $\pm$ 0.2	< 0.0001
IL-8, pg/ml	1114.1 $\pm$ 425.9	6.1 $\pm$ 1.2	2.3 $\pm$ 0.2	0.03
IL-12, pg/ml	10.3 $\pm$ 1.8	3 $\pm$ 0.2	3.2 $\pm$ 0.1	0.001
IL-17A, pg/ml	25 $\pm$ 3.2	2.1 $\pm$ 0.5	3.3 $\pm$ 0.2	< 0.0001
IFN- $\gamma$ , pg/ml	96.1 $\pm$ 16	20.2 $\pm$ 3.1	42.1 $\pm$ 2.3	0.001
TNF- $\alpha$ , pg/ml	126.3 $\pm$ 7.6	4.7 $\pm$ 0.8	6 $\pm$ 0.4	< 0.0001

\* One-way ANOVA. AS: ankylosing spondylitis; TNF: tumor necrosis factor; IFN: interferon.

through direct and indirect mechanisms. The clinical improvement of patients with AS who use TNF- $\alpha$  inhibitors indicates the participation of TNF- $\alpha$  in the pathogenesis of AS<sup>9,10,11</sup>. In animal models of arthritis, it has been shown that the use of TNF- $\alpha$  inhibitors, with the soluble fraction receptor or with the monoclonal antibody, results in a decrease in Th1 and Th17 cells in joints, suggesting common pathways for these cell populations<sup>23</sup>. Further, these studies suggest that an explanation for this decrease in T cell subpopulations is that the use of these biological agents prevents cellular migration from the lymphatic nodes to the periphery. However, it has been reported that TNF- $\alpha$  takes part in the differentiation of dendritic cells, which in turn are able to favor the differentiation of Th1 and Th17 effector T cell subpopulations characterized by the production of inflammatory cytokines<sup>24</sup>, and that the use of TNF- $\alpha$  inhibitors in patients with AS prevents the maturation of MHC-II myeloid dendritic cells and decreases the number of T cells producing TNF- $\alpha$ , IFN- $\gamma$ , and IL-2<sup>25,26</sup>.

The balance between effectors and Treg cells in the periphery depends on many variables, such as the concentration and kind of antigen, the target organ, and the cytokines produced by the same T cells and by the antigen-presenting cells, which in turn stimulate the secretion of cytokines by other cell populations, creating a microenvironment that modulates cell differentiation and defines the kind of immune response. In the cell plasticity model proposed by Lohr, *et al*<sup>27</sup>, a kinetic model of the polarization of immune cell response is shown. In this model, the inflammatory response initiated by the production of IL-6 triggers the differentiation of Th17 cells which, in turn, gives rise to the development of Th1 cells, suggesting the participation of both Th cells in an acute stage of the disease. This phase is followed by the inhibition of Th1 cells and the predominance of Th17 cells, accompanied by the increase of clinical manifestations, which in turn triggers yet again the increase of Th1 cells, a change that together with the increase in Treg cells decreases the number of Th17 cells. It is in this third and last phase that the predominance of the Treg cell population leads to recovery. Within the framework of AS, which is a chronic inflammatory disease with a persistent proinflammatory environment, the conditions for the differentiation of Th1 and Th17 cells are always favorable, and thus a feedback mechanism is created that leads to the perpetuation

of the inflammatory process. It is possible that the increase of Treg cells is caused by an attempt by the peripheral tolerance mechanisms to control the immune response and that the suppressing capacity of Treg cells is surpassed; or it might be that the effector cells are not susceptible to regulation, as seen in other diseases<sup>28,29</sup>. Another possibility is that the increase of these cells is related to the common differentiation pathways of Treg and Th17 cells, suggesting that cells identified as CD4+FoxP3+ could become IL-17-producing cells<sup>30</sup>.

Our results suggest a role for Th17 cells in the pathogenesis of AS, and that treatment with TNF- $\alpha$  inhibitors decreases peripheral cell populations characterized by the production of proinflammatory cytokines, such as Th1 and Th17 cell subpopulations.

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