Intracytoplasmic Cytokine Expression and T Cell Subset Distribution in the Peripheral Blood of Patients with Ankylosing Spondylitis

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ABSTRACT. ***Objective.*** To determine the role of inflammatory mediators in the pathogenesis of ankylosing spondylitis (AS), we investigated peripheral blood lymphocyte subsets and their intracellular cytokine production.

Methods. The percentages of T and B lymphocytes, natural killer (NK) cells, activated T lymphocytes, CD4+ T helper (Th), and CD8+ T cytotoxic (Tc) cells were determined by flow cytometry in 42 patients with AS compared to 52 healthy controls. In order to assess circulating Th1/Th2 and Tc1/Tc2 subsets, we used a whole-blood cytometric assay based on the intracellular interferon-γ, interleukin 4 (IL-4), and IL-10 expression of the cells.

Results. In the peripheral blood, the frequencies of CD4+ T helper and CD56+ NK cells were higher in AS (54.8% and 16.2%, respectively) compared to controls (45.3% and 10.8%) ($p < 0.05$). The frequencies of Th0 (1.9% vs 0.8%) and Tc0 (2.1% vs 0.8%) cells were higher, while that of Tc1 cells was lower (26.6% vs 40.1%) in patients with AS versus controls ($p < 0.05$). The percentage of IL-10-producing Tc cells was significantly higher in AS (18.4%) versus controls (8.5%) ($p < 0.05$). Finally, the active phase of AS was associated with significantly lower percentage of IL-10-producing Tc cells in the peripheral blood (6.6%) compared to patients with inactive AS (23.1%).

Conclusion. Our results provide further evidence for an altered T cell subset distribution and intracytoplasmic cytokine balance in AS. (First Release Nov 1 2008; J Rheumatol 2008;35:2372–5; doi:10.3899/ jrheum.070839)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS
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CD8+ CELLS

Ankylosing spondylitis (AS) is a progressive rheumatic disease associated with chronic inflammation of the sacroiliac joints, axial skeleton, large proximal joints, and entheses[1]. Inflammatory sites in AS contain granulation tissue infiltrated by leukocytes, eventually leading to cartilage erosion and ossification of the involved joints[2].

Cytokines are involved in most of the described inflammatory and destructive processes. Macrophage-derived proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), mediate inflammation and joint destruction, as shown by the efficacy of TNF-α inhibitors in the treatment of spondyloarthropathies[3]. However, many fewer data are available on the role of T cell-derived cytokines in AS.

CD4+ and CD8+ T cells can be divided into subsets based on their intracellular cytokine production patterns[4,5]. Type 1 helper (Th1) and cytotoxic (Tc1) T cells predominantly produce interferon-γ (IFN-γ) and interleukin 2 (IL-2). These cell subsets are primarily involved in cell-mediated immune responses. Type 2 helper (Th2) and cytotoxic (Tc2) T cells readily express IL-4, IL-5, and IL-10, and they play a pivotal role in humoral and atopic responses[6,7]. Th0 and Tc0 cells producing both type 1 and type 2 cytokines may be precursors of differentiated helper, cytotoxic, and memory T cells[8]. Over the last decade it became obvious that the functional polarization of T lymphocyte subsets is implicated in the pathogenesis of various types of arthritis.

Regarding assessments of circulating cytokines, IFN-γ levels were not significantly higher in sera of patients with AS than in those of subjects with noninflammatory low back pain[9]. Moreover, IL-4 could not be detected in the sera of patients with AS[10]. Using the polymerase chain reaction method, a Th2-biased cytokine pattern was found in reactive

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arthritic compared to rheumatoid arthritic synovial tissues[11]. Within peripheral blood lymphocytes (PBL) isolated from patients with AS, the percentage of IFN-γ-producing T cells was lower in the patients than in HLA-B27-negative healthy controls[12]. Nevertheless, these results may have limitations, as serum concentrations of cytokines may only indirectly reflect the inflammation process within the sacroiliac joints or the entheses[13].

To further analyze the role of these T cell subsets in the pathogenesis of AS, we analyzed T cells *ex vivo* by a rapid-flow cytometric assay in whole blood. This method provides an environment close to physiological, thus preventing artificial effects. Our goal was to assess PBL subsets including the percentage of T, B, and NK cells and the ratio of activated T cells as determined by CD69/CD3 and HLA-DR/CD3 expression. CD69 is an early and HLA-DR a late activation marker of T cells. We also determined the Th0-Th1-Th2 and Tc0-Tc1-Tc2 profiles of PBL in patients with AS in comparison to healthy individuals.

MATERIALS AND METHODS

Patients and controls. Forty-two patients with AS (29 men and 13 women, mean age 44.2 yrs, range 21–63 yrs) undergoing regular followup at our institution were included. The diagnosis of AS was based on the modified New York criteria[14,15] and all the patients were HLA-B27-positive. Twenty-two of the 42 patients with AS were in active state of disease with elevated C-reactive protein (CRP) > 4.6 mg/l and Bath AS Disease Activity Index (BASDAI) > 4[14]. No patient had CRP > 10 mg/l. Most patients (37/42, 88%) received nonsteroidal antiiflammatory drugs (NSAID). Among the 22 patients with active disease (BASDAI > 4), 8 had peripheral and 14 only axial involvement, while all the 20 inactive patients had only axial involvement. Thus, 7 patients were treated with 15–17.5 mg per week methotrexate and one patient with 2000 mg per day sulfasalazine. No patient received biologicals at the time of or 6 months prior to the study. We recruited 52 healthy controls from the clinical staff (32 men, 20 women, mean age 31.5 yrs, range 20–56 yrs). Informed consent was obtained from each patient and control.

Measurement of intracellular cytokine expression of Th and Tc lymphocytes in whole blood. Evaluation of intracellular cytokines was performed as described[16]. Briefly, as resting lymphocytes do not contain substantial amount of cytokines[17], we stimulated T cells in whole blood by phorbol-myristate-13-acetate (PMA, 25 ng/ml; Sigma) and ionomycin (1 μg/ml; Sigma) for 4 h at 37°C. In order to block the excretion of newly synthetized cytokines, brefeldin-A (10 μg/ml; Sigma) was also added to the cells. After stimulation, the whole-blood samples were incubated separately with quantum red-labelled anti-CD3, anti-CD4, or anti-CD8 monoclonal antibodies in the dark for 30 min at room temperature. Erythrocytes were then lysed for 10 min (FACS Lysing Solution; Becton Dickinson) followed by a washing step and the permeabilization of plasma membrane of leukocytes for another 10 min (FACS Permeabilizing Solutions; Becton Dickinson). Finally, cytoplasmic IFN-γ and IL-4 were detected by FITC-labeled anti-IFN-γ and phycoerythrin-labeled IL-4 (Becton Dickinson) and the samples were fixed with paraformaldehyde and analyzed using a Coulter Epics XL flow cytometer. The proportion of IFN-γ+ and IL-4+ cells was determined by assessing 5000 CD4+ and CD8+ T cells. FITC-labeled anti-IL-10 (Becton Dickinson) was used to assess IL-10 content of the cells.

Statistical analysis. Data are presented as mean value ± standard deviation (SD). The differences between healthy controls and patients with AS were calculated by Student's independent t- and Mann-Whitney tests. p values ≤ 0.05 were considered statistically significant.

RESULTS

Lymphocyte subpopulations in peripheral blood. The percentages of CD4+ (Th) (54.8% ± 9.9% vs 45.3% ± 7.7%; p < 0.05) and CD56+ (NK) cells (16.2% ± 4.9% vs 10.8% ± 5.7%; p < 0.05) were significantly higher within the PBL population of patients with AS in comparison to controls (Table 1). There was no difference in other T cell subsets including CD3+ (total T), CD8+ (Tc), and CD19+ (B) cells between patients and controls (Table 1). The frequency of CD69+/CD3+ and HLA-DR+/CD3+ activated peripheral blood T cells was also similar in patients with AS and controls (data not shown).

Intracellular cytokine expression in peripheral lymphocytes of patients with AS. We defined the Th0, Th1, Th2, Tc0, Tc1, and Tc2 subsets with respect to their CD4 or CD8 expression and their intracellular IFN-γ and IL-4 expression (Table 2). Among Th cells, AS was associated with a Th0 response, as the frequency of these undifferentiated cells was significantly higher in patients with AS in comparison to controls (1.88% ± 0.97% vs 0.82% ± 0.71%; p < 0.05; Table 2). There were no differences in the ratio of Th1 (25.5% ± 8.2% vs 24.9% ± 7.9%) and Th2 cells (0.48% ± 0.55% vs 0.45% ± 0.39%) between patients with AS and controls (p = non-significant, NS; Table 2).

Regarding Tc cells, AS reflected a Tc0 response, as the frequency of Tc0 (2.11% ± 1.02% vs 0.76% ± 0.55%) cells was significantly higher, while that of Tc1 cells (26.6% ± 11.8% vs 40.1% ± 15.4%) was significantly lower in AS in comparison to controls (p < 0.05; Table 2). The frequency of Tc2 cells did not differ in AS and controls (0.36% ± 0.22% vs 0.44% ± 0.35%; p = NS; Table 2).

The percentage of IL-10-producing regulatory T cells was also assessed. While the frequency of IL-10-producing Tc cells was increased in patients with AS compared to controls (18.4% ± 9.2% vs 8.5% ± 6.1%; p < 0.05), the percentage of IL-10-producing Th cells did not differ in patients and controls (14.2% ± 6.1% vs 12.8% ± 10.1%; p = NS; Table 3).

Finally, we compared patients with active and inactive AS with regard to these T cell subsets. The percentages of Th0, Th1, Th2, Tc0, Tc1, and Tc2 cells were similar in patients with active (n = 22) versus inactive disease (n = 20) (data not shown). However, the percentage of IL-10-producting CD8+ Tc cells was lower in active (6.6% ± 0.8%) in comparison to inactive patients (23.1% ± 6.8%; p < 0.05).

DISCUSSION

In comparison to rheumatoid arthritis, very little information is available on the role of specific T cell subsets and the production of intracellular cytokines by T cells in AS. Therefore, we aimed to determine the role of distinct T cell subsets and cytokines produced by these cells in AS.

Our data suggest that the frequency of CD4+ helper and CD56+ NK cells in the peripheral blood of patients with AS

Table 1. Lymphocyte subpopulations in the peripheral blood of patients with AS and healthy controls. Percentages of lymphocyte subpopulations were determined by flow cytometry in 42 patients with ankylosing spondylitis (AS) and 52 healthy controls.

	CD3+ (%)	CD4+ (%)	CD8+ (%)	CD56+ (%)	CD19+ (%)
AS patients (n = 42)	74.7 ± 8.3	54.8 ± 9.9	20.4 ± 4.5	16.2 ± 4.9	10.2 ± 4.2
Controls (n = 52)	70.5 ± 7.3	45.3 ± 7.7	19.6 ± 5.9	10.8 ± 5.7	11.7 ± 4.1
p	NS	< 0.05	NS	< 0.05	NS

Values are mean ± SD. NS: nonsignificant.

Table 2. Th0, Th1, Th2, Tc0, Tc1, and Tc2 subsets based on intracellular cytokine expression in the peripheral blood of patients with AS and healthy controls. Percentages of lymphocyte subpopulations were determined by flow cytometry in 42 patients with ankylosing spondylitis (AS) and 52 healthy controls.

	Th0 (%) (CD4+/IFNγ+/IL-4+)	Th1 (%) (CD4+/IFNγ+)	Th2 (%) (CD4+/IL-4+)	Tc0 (%) (CD8+/IFNγ+/IL-4+)	Tc1 (%) (CD8+/IFNγ+)	Tc2 (%) (CD8+/IL-4+)
AS patients (n = 42)	1.88 ± 0.97	25.5 ± 8.2	0.48 ± 0.55	2.11 ± 1.02	26.6 ± 11.8	0.36 ± 0.22
Controls (n = 52)	0.82 ± 0.71	24.9 ± 7.9	0.45 ± 0.39	0.76 ± 0.55	40.1 ± 15.4	0.44 ± 0.35
p	< 0.05	NS	NS	< 0.05	< 0.05	NS

Values are mean ± SD. NS: nonsignificant.

Table 3. Percentage of IL-10-producing Th and Tc cells in the peripheral blood of patients with AS and healthy controls. Percentages of lymphocyte subpopulations were determined by flow cytometry in 42 patients with ankylosing spondylitis (AS) and 52 healthy controls.

	Th-IL-10 (%) (CD4+/IL-10+)	Tc-IL-10 (%) (CD8+/IL-10+)
AS patients (n = 42)	14.2 ± 6.1	18.4 ± 9.2
Controls (n = 52)	12.8 ± 10.1	8.5 ± 6.1
p	NS	< 0.05

Values are mean ± SD. NS: nonsignificant.

is significantly higher than in that of healthy controls. We have not found any publications considering this issue. There is evidence that the recruitment of CD8+ T cells into the site of inflammation may play an important role in joint damage associated with AS[18]. We observed the accumulation of CD4+ Th cells in peripheral blood, which may relate to the concomitant migration of CD8+ lymphocytes into the affected joints.

We also assessed Th and Tc lymphocyte subsets by determining their intracytoplasmic cytokine production[17,19,20]. The intracellular cytokine content of peripheral blood CD4+ Th cells indicated an accumulation of Th0 cells in the peripheral blood of patients with AS, while the percentage of Th1 and Th2 cells, as well as the Th1/Th2 ratio, was similar in patients and controls. Regarding CD8+ Tc cells, we observed a decrease in Tc1/Tc2 ratio in patients with AS compared to controls. However, this change was not due to the preponderance of type 2 cytokines, but rather to the lower frequency of IFN-γ-producing Tc cells. These results are partly in accord with the report of Rudwaleit, *et al*, who found decreased production of IFN-γ by both CD4+ and CD8+ T cells in AS[12]. The reason for the partial discrepancy between their results and ours may be the different environment of stimulated cells, as they separated the peripheral mononuclear cells and froze them until use, while we used the fresh whole-blood method for detection of cytokine production after *in vitro* stimulation.

We found significantly increased percentages of Th0 and Tc0 cells in patients with AS compared to controls. These pluripotent precursor cells have the potential to differentiate into either IFN-γ- or IL-4-producing Th or Tc cells, which is determined predominantly by the local cytokine environment. Thus, the increased frequency of Th0 and Tc0 cells in AS could reflect the perpetuated proliferation of uncommitted cells, but the role of these cell populations in AS needs to be further elucidated.

We also assessed the ratio of IL-10-producing Th and Tc cells in the peripheral blood. In patients with AS, we observed an increased percentage of IL-10-producing Tc cells compared to controls, while the frequencies of IL-10-producing Th cells were similar in patients and controls. IL-10 is not a purely type 2 cytokine; moreover, IL-4 and IL-10 may have disparate effects in arthritis. For example, in rheumatoid arthritis, a disease characterized by a Th1 dominance, both IL-10 and IL-4 may exert chondroprotective properties, while IL-10, but not IL-4, inhibits mononuclear cell traffic into the synovial tissue[21]. It has been suggested that T cell-derived IL-10 may be involved in the immunoregulatory functions of T cells. Chou, *et al* detected higher IL-10 concentration in the supernatants of activated peripheral blood mononuclear cells derived from patients with active compared to inactive AS[22]. Zou, *et al*[23] analyzed the effect of anti-TNF-α therapy on T cell subpopulations.

Infliximab treatment induced a significant decrease in the number of CD4+ and CD8+ T cells expressing IFN-γ and TNF-α upon stimulation; however, there was no change in the number of IL-10- or IL-4-producing T cells during treatment[23]. These results suggest that increased IL-10 production by Tc cells may be one of the antiinflammatory feedback mechanisms in AS. In order to test this hypothesis, we also studied the percentage of IL-10-producing cells in patients with active and inactive disease. Significantly lower percentages of IL-10-producing Tc cells were detected in patients with active compared to inactive AS, indicating that active AS may be associated with a defective antiinflammatory response driven by IL-10-producing Tc cells.

Very little information has been published regarding the distribution and cytokine production of T cell subsets in AS. Although we tested a relatively small number of patients, our results may provide additional data to understand the role of Th and Tc cells as well as T cell-derived cytokines in the perpetuation and regulation of inflammation associated with AS.

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