PREFERENTIAL TYPE 1 CHEMOKINE RECEPTORS AND CYTOKINE PRODUCTION OF CD28\(^{+}\) T-CELLS IN ANKYLOSING SPONDYLITIS

- Extended Report -

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**Short title:** Chemokine receptors and cytokines in ankylosing spondylitis

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**KEY WORDS**
Ankylosing spondylitis, chemokines, chemokine receptors, cytokines, Tumor necrosis factor-alpha
ABSTRACT

Objective: To examine serum levels of type 1 and type 2 chemokines and lymphocytic expression of chemokine receptors, and to compare the results with lymphocytic cytokine production in AS patients.

Methods: Twelve AS patients (44.9±14.7 years) and 27 healthy controls (46.4±12.8 years) were enrolled into the study. The expression of chemokine receptors (CCR-5, CXCR-3, CCR-4) and cytokines (IFN-γ, IL-2, IL-4, IL-10, TNF-α) on CD28+ and CD28- subtypes of T-cells were analysed by three colour FACS technique of peripheral blood samples. Serum ELISAs were performed to detect the CCR-5 ligands CCL-5, CCL-3; the CXCR-3 ligands CXCL-10, CXCL-9; and the CCR-4 ligand, CCL-17 before and after the administration of the TNF-α blocking agent infliximab.

Results: CD4+CD28- T-cells had higher ratios of CXCR-3 (as a typical type 1 receptor) to CCR-4 than CD4+CD28+ T-cells. Both, CD4+ and CD8+CD28- T-cells of AS patients produced more IFN-γ, TNF-α and IL-10 than their CD28+ counterpart (P<0.05), and lacked the production of IL-2 and IL-4. Serum levels of CXCL-9 were increased in AS patients with 59.2 pg/ml (34.1–730.5) compared to 32.5 pg/ml (20.0–79.5) in healthy controls (P=0.016). The levels of both type 1 (CCL-5, CXCL-9) and type 2 chemokines (CCL-17) decreased under blockade of TNF-α (P<0.05).

Conclusions: The profile of chemokine receptor expression and cytokine production by CD28- T cells suggests a type 1 immune reaction in AS, although IL-10 is frequently produced by CD28- T-cells. Treatment with TNF-α blocking antibodies decreased both types of chemokines in patients' sera.
INTRODUCTION

Polarisation and heterogeneity of T-cells is considered to be important in the initiation and perpetuation of synovial inflammation, and the model of type 1 and type 2 immune responses has attracted great interest in immune-mediated diseases.[1] In spondyloarthritis (SpA) including ankylosing spondylitis (AS), however, the results of cytokine profiles seem to be more complicated than in other diseases.[2] A few studies examined cytokines in SpA patients with divergent conclusions for the type of the immune reactions: Several investigators described an increment of pro-inflammatory cytokines (interleukin (IL)-6, interferon-\(\gamma\) (IFN-\(\gamma\))) and the anti-inflammatory cytokine IL-10 in active SpA and psoriatic arthritis.[3][4][5] Others suggest a Th2 cytokine profile in SpA with relatively little amounts of IFN-\(\gamma\) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and increased cytokine mRNA of IL-10 in synovial fluid compared to rheumatoid arthritis (RA) patients.[6] On the cellular level Th1 cytokine production appeared to be lower in patients with SpA than in healthy controls.[7][8]

The strong association between HLA-B27 and AS suggests an important role for CD8\(^+\) cytotoxic T-cells but also for CD4\(^+\) T-helper cells in this disease.[9][10] Indeed, immunohistological studies of sacroiliac biopsies revealed dense cellular infiltrations of T-cells and macrophages and massive expression of TNF-\(\alpha\) within the synovial part of the sacroiliac joints.[11] In SpA patients treated with TNF-\(\alpha\) blocking agents PBMCs stimulated with PMA/ionomycin produced either higher or lower levels of Th1 cytokines.[2][12][13] Recently our own group had described peripheral enrichment of pro-inflammatory IFN-\(\gamma\) producing CD4\(^+\) and CD8\(^+\) T-cells lacking the co-stimulatory molecule CD28 in AS patients, suggesting a role of these cells in a type 1 immune response, although other markers had not been tested so far.[14][15] Such CD28\(^-\) T-cells occur in many chronic inflammatory disorders including RA, Wegener’s granulomatosis and multiple sclerosis,[16][17][18] and are considered as markers for chronic inflammation and early aging.[19] Under these circumstances the CD28\(^-\) T-cells are part of the CD4\(^+\) as well as the CD8\(^+\) T-cell compartment, persist over years and include most of the oligoclonally expanded T-cells. Phenotypically, CD4\(^+\)CD28\(^-\) T-cells from RA and AS patients and CD8\(^+\)CD28\(^-\) T-cells from aged persons, and melanoma patients share the expression of various NK cell receptors, and lack the expression of the lymphocyte marker CD7.[20][21][22] Functionally these T-cells are capable to release large amounts of IFN-\(\gamma\), perforin and granzyme B, providing them with the possibility to lyse target cells.[23] In AS patients the prevalences of both CD4\(^+\) and CD8\(^+\)CD28\(^-\) T-cells depend on the disease status.[14][15]

Chemokines are directly involved in the extra-vasation of T-cells into inflamed tissue. Expression of a specific chemokine receptor profile on lymphocytes was described as an additional marker for the immune response in various immune-mediated diseases. Typical type 1 T-cells show strong surface expression of CCR-5 and CXCR-3 and produce high concentrations of IL-2 and IFN-\(\gamma\).[24][25] The lymphocytic type 2 response is characterized by high expression of CCR-3, CCR-4 and CCR-8, combined with increased production of IL-4, IL-5, IL-6, IL-10 and IL-13.[26][27] An overview of the examined chemokine receptors, their ligands and the corresponding immune response is provided in Table 1.
The aim of this study was to examine the expression of both type 1 and type 2 chemokine receptors on CD28+ and CD28− T-cells and to compare them with serum chemokine levels and the intracellular production of cytokines in T-cells from AS patients.

MATERIAL AND METHODS

Patients

Twelve consecutive patients (25% female, 44.9±14.7 years old) with definite AS according to the modified New York criteria [28] were recruited into the study. Patients with current pregnancy or lactation, a history of neoplasm, recent acute infection or history of any other chronic inflammatory disease were excluded from the study. The same exclusion criteria were applied for 27 age- and sex-matched healthy controls (22.2% female, 46.4±12.8 years old). After informed and written consent, clinical and laboratory parameters were routinely assessed and peripheral blood was drawn for further analyses as approved by the local ethics committee.

Patients’ characteristics including the Bath Ankylosing Spondylitis Metrology Index (BASMI),[29] the Bath Ankylosing Spondylitis Functional Index (BASFI) [30] and the Bath Ankylosing Disease Acticity Index (BASDAI) [31] are shown in Table 2. A subgroup of six patients with active AS disease were treated with chimeric anti-TNF-α monoclonal antibodies (infliximab, Remicade®, Schering Plough/Aesca, Vienna, Austria) at a dosage of 3 mg/ kg body weight. Under treatment the BASDAI improved from 6.2±1.9 to 3.2±2.0, erythrocyte sedimentation rate (ESR) from 36.8±19.5 mm/h to 11.2±3.0 mm/h and C-reactive protein (CRP) levels from 2.3±2.0 mg/dl to 0.4±0.2 mg/dl (shown as mean±SD).

Enzyme linked immunosorbent assays

Enzyme linked immunosorbent assays (ELISAs) were performed in duplicates to determine concentrations of CCL-5 (RANTES), CCL-3 (MIP-1α), CXCL-9 (MIG), CXCL-10 (IP-10) and CXCL-17 (TARC) according to the manufacturer's instructions (R&D Systems, Inc., USA, MN) in blinded sera.

Surface staining for chemokine receptors

To determine the peripheral level of CD3+CD4+CD28− and CD3+CD8+CD28− T-cells, the expression of chemokine receptors and intracellular cytokine production of CD4+ and CD8+CD28− T-cells, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. Surface staining of PBMCs was performed using flourescein-isothiocyanate (FITC) conjugated anti-CCR-5 (R&D Systems, Inc., Minneapolis, MN, USA), anti-CD28, anti-CD4, anti-CD8; phycoerythrin (PE)-conjugated anti-CD28, anti-CXCR-3 and anti-CCR-4; and peridinin chlorophyll protein-conjugated (PerCP) anti-CD4, anti-CD8 and anti-CD3 monoclonal antibodies (all from Becton Dickinson, San Diego, CA, USA).

Intracellular staining for cytokine production and flow cytometry

For intracellular staining, cells were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate and 1 µg/ml ionomycin in the presence of 10 µg/ml brefeldin A for 4 hours (Sigma, Munich, Germany). After cell surface staining for CD4 and CD28 with subsequent fixation and permeabilization, cells were stained with FITC-conjugated anti-
IFN-γ, anti-TNF-α, anti-IL-2, anti-IL-4, anti-IL-10 antibodies or control immunoglobulin (Becton Dickinson).

After final fixation with 1% cell fix (Becton Dickinson) cells were analyzed on a FACS-Calibur flow cytometer (Becton Dickinson). Data were analyzed using WinMDI software (Version 2.8, Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

Statistics

Results were tested for the distribution using the Kolmogorov-Smirnov test and expressed as median and range. The Mann-Whitney test was used to compare between independent groups, and the Wilcoxon ranking test to compare between paired data from the CD28+ and the CD28- T-cell compartments. The Spearman-Rho test was performed to test possible correlations. All statistical analyses were performed using the SPSS program, version 11.5 (Chicago, IL, USA).  \( P < 0.05 \) was considered to be statistically significant.

RESULTS

The prevalences of CD3+CD4+CD28- and CD3+CD8+CD28- T-cells in AS patients were increased to 2.3% (0.9 – 8.8%) and 38.6% (10.4 – 52.9%) in comparison to 0.6% (0.1 – 1.2%) and 24.5% (7.6 – 37.4%) in healthy controls (\( P < 0.001, P = 0.037 \), respectively).

Differential surface expression of chemokine receptors on CD28+ and CD28- T-cells

Surface expression of both type 1 (CCR-5, CXCR-3) and type 2 (CCR-4) chemokine receptors were examined by FACS analysis, and compared between the CD28+ and the CD28- T-cell subsets from AS patients and healthy controls. As shown in Figure 1, expression of chemokine receptors differed markedly between CD28+ and CD28- T-cells, both on CD4+ and CD8+ T-cells from AS patients and healthy controls. CD4+CD28- T-cells were not detectable in healthy controls. CCR-5 expression was low on all subtypes of T-cells. However, CXCR-3 was similarly expressed on CD4+CD28+ and CD28- T-cells, but more frequently on CD8+CD28+ than on CD28- T-cells \[ 22.7\% (5.7–28.7) \] vs. \[ 2.4\% (0.2–88.9) \] positive cells, \( P=0.010 \]. The CCR-4 receptors were more expressed on CD4+CD28+ than on CD28- T-cells \[ 41.1\% (25.0–62.5) \] vs. \[ 4.0\% (0.7–6.0) \] positive cells, \( P=0.001 \], and only on CD8+CD28+ from AS patients compared to CD28- T-cells \[ 12.3\% (3.2–76.8) \] vs. \[ 0.8\% (0.5–1.6) \] positive cells, \( P<0.001 \]. Taken together, CD4+CD28+ T-cells had a higher ratio of CXCR-3 / CCR-4 than CD4+CD28- T-cells \[ 12.3 (3.2–76.8) \] vs. \[ 0.8 (0.5–1.6) \], \( P<0.001 \) (Figure 1C). In healthy controls, however the CXCR-3 / CCR-4 ratio from controls was higher for the CD8+CD28+ than the CD8+CD28- T-cells \[ 6.9 (5.1–30.3) \] vs. \[ 0.4 (0.0–32.1) \], \( P=0.002 \). No correlation was detected between the expression of chemokine receptors on CD4+ and CD8+ T-cells and the clinical parameters (BASMI, BASFI, BASDAI) nor the serological parameters (ESR and CRP levels).

Synovial fluid samples were available from 4 patients, who underwent therapeutic joint puncture. In the synovial fluid, 1.6% (0.1–6.0) of the CD4+ T-cells and 12.2% (6.4–20.5) of the CD8+ T-cells lacked the costimulatory molecule CD28. As in the peripheral blood CD4+CD28+ and CD4+CD28- T-cells, but also CD8+CD28+ and CD8+CD28- T-cells showed equal expression of CXCR-3. The expression of CCR-4 was more frequent on CD28+ than on CD28- T-cells. The expression of CCR-5 was low on CD4+ and CD8+ T-cell
subsets. The CXCR-3 / CCR-4 ratio was calculated to be 13.3 (10.9–15.8) for CD4⁺CD28⁻ T-cells vs. 3.1 (0.7–5.9) for CD4⁺CD28⁺ (P=0.064) and 56.6 (48.8–64.4) for CD8⁺CD28⁻ T-cells vs. 12.2 (2.1–39.4) for CD8⁺CD28⁺ (P=0.021) (Figures 2A-H).

Intracellular cytokine profiles of CD28⁻ compared to CD28⁺ T-cells

As the observed chemokine receptor expression would argue for a type 1 immune response of fresh CD28⁻ T-cells in AS compared to their CD28⁺ counterparts, the T-cell subsets were further tested for their intracellular production of type 1 and type 2 cytokines after prior stimulation with PMA/ionomycin in the presence of brefeldin A.

From this functional perspective, production of the pro-inflammatory cytokines IFN-γ, TNF-α and the anti-inflammatory cytokine IL-10 were more frequent in both, the CD4⁺ and the CD8⁺CD28⁻ T-cells, compared to their CD28⁺ counterparts. IL-2 production was less frequent in CD4⁺ and CD8⁺CD28⁻ T-cells than in their CD28⁺ T-cell counterparts. IL-4 production was low in all CD4⁺ and CD8⁺ T-cells (Figures 3A-I). No differences in cytokine production were detected between CD28⁺ and CD28⁻ T-cells from controls and AS patients (data not shown).

Serum levels of chemokines

Expression of chemokine receptors on pro-inflammatory CD4⁺ and CD8⁺ T-cells of AS patients suggested a role for chemokines in AS disease. Therefore we tested the serum levels of typical type 1 and type 2 chemokines, and compared them between healthy controls and AS patients (Figures 4A-E). Serum levels of CXCL-9, a ligand of the type 1 CXCR-3 receptor, were increased in AS patients with 59.2 pg/ml (34.1–730.5) compared to 32.5 pg/ml (20.0–79.5) in healthy controls (P=0.016). In contrast the serum levels of the CCR-5 ligands, CCL-5 and CCL-3; the other CXCR-3 ligand tested, CXCL-10; as well as the CCR-4 ligand, CCL-17 showed no significant differences between AS patients and healthy controls. Although, a strong correlation was detected between serum levels of the type 1 chemokine CCL-5 and ESR levels of AS patients (R=0.725, P=0.025 using the Spearman - Rho test), correlations between the other serum chemokines, laboratory and clinical parameters tested negative.

Effects of TNF-α blocking treatment on chemokine receptor expression and serum chemokine levels

The expression of type 1 chemokine receptors on CD28⁻ T-cells and the increased serum levels of the CXCR-3 receptor ligand, CXCL-9 raised the question whether TNF-α blocking agents would affect the chemokine system. Retesting during successful TNF-α blocking treatment revealed that the expression of both the CXCR-3 and the CCR-4 receptors were unchanged on CD4⁺ and CD8⁺ independent from their CD28 subtype.

Concerning the chemokine levels under successful blockade of TNF-α the serum levels of CCL-5, CXCL-10 and CCL-17 decreased, whereas the serum levels of CCL-3 and CXCL-9 showed no differences before and after treatment. Thus both type 1 and type 2 chemokines were affected by blockade with TNF-α antibodies (Figures 4F-J).
DISCUSSION

According to this study lymphocytic expression of chemokine receptors and intracellular cytokine production preferentially show signs of a type 1 immune response on CD28\(^-\) T-cells from AS disease. Thus the assessment of the chemokine receptor profile for describing the type of immune response in AS has supported the cytokine production data available so far.[14][15] Especially the expression of the CXCR-3 receptor was increased on CD4\(^+\)CD28\(^-\) T-cells of AS patients, while the expression of the other type 1 chemokine receptor, CCR-5, was low on both CD28\(^+\) and CD28\(^-\) T-cells, even though CD28\(^+\) T-cells were described to frequently express CCR-5 for instance in patients with Wegener’s granulomatosis.[32][33] As the expression of CXCR3 on peripheral CD4\(^+\) T-cells is largely restricted to the memory population,[34] this is consistent with the described memory-effector phenotype of CD4\(^+\)CD28\(^-\) T-cells.

Others had already demonstrated an increased expression of CCR-4 on circulating CD4\(^+\) T-cells in AS patients compared to healthy controls and a correlation between the percentages of CD4\(^+\)CCR-4\(^+\) T-cells with AS disease status measured by the BASDAI score.[35] In our study, there was a preferential expression of CCR-4 on CD8\(^+\)CD28\(^-\) T-cells of AS patients compared to CD8\(^+\)CD28\(^+\) T-cells of healthy controls. CD4\(^+\) and CD8\(^+\)CD28\(^+\) T-cells of AS patients expressed CCR-4 more frequently than their CD28\(^-\) T-cell counterparts. Expression of both CXCR-3 and CCR-4 did not correlate with disease activity measured by the BASDAI score (data not shown).

A recent study demonstrated an increased expression of CXCR-3 on memory-effector T-lymphocytes in patients with inflammatory liver diseases compared to lymphocytes of healthy livers and the peripheral blood from healthy controls.[36] Also in our study, the expression of chemokine receptor CXCR-3 was increased on both, CD4\(^+\) and CD8\(^+\) subpopulations of T-cells from the synovial fluid. This expression pattern resulted in an increased CXCR-3 / CCR-4 ratio in CD4\(^+\)CD28\(^-\) and CD8\(^+\)CD28\(^-\) T-cells compared to CD4\(^+\)CD28\(^+\) and CD8\(^+\)CD28\(^+\) T-cells and may reflect a more activated immune status at the site of inflammation compared to the peripheral blood. Because of the increased prevalence of circulating CD4\(^+\) and CD8\(^+\)CD28\(^-\) T-cells in AS and their preferential expression of the type 1 chemokine receptor CXCR-3 in the peripheral blood as well as the synovial fluid, these memory-effector CD28 CXCR-3\(^+\) T-lymphocytes can be expected to play a role in AS. The presence of these cells supports the concept of a disease with a precise antigenic target as a stimulus for disease progression which, however, is still unknown for AS.

Interestingly stimulated fresh CD28\(^-\) T-cells produced more of the type 2 cytokine IL-10 than their CD28\(^+\) counterpart. This observation is in accordance with increased plasma levels of IL-10 in AS patients, which even correlated with disease activity. Also for rheumatoid arthritis, IL-10 has been discussed as a possible regulator of the immune dysfunction and correlated with progression of joint destruction.[37] On the other hand IL-10 is known to be an immunosuppressive cytokine and may be increased as an attempt to counterbalance the pathological pro-inflammatory immune status in AS disease. Thus both type 1 and type 2 cytokines are produced in stimulated CD28\(^-\) T-cells from AS patients despite a clear type 1 chemokine receptor profile of these cells.
TNF-α has been postulated to play a central role in many immune-mediated diseases including AS.[38] Under clinically successful blockade of TNF-α, we found no effect on the expression of CXCR-3 or CCR-4 on CD28- T-cells (data not shown), whereas a sustained accumulation of circulating CXCR-3 positive T lymphocytes had been reported under TNF-α blocking agents in RA patients.[39] It has been shown earlier that TNF-α blockade down-regulates both IFN-γ and TNF-α secreted by T-cells but does not induce a change in cytokines produced by monocytes during 3 months of treatment.[12] In our short-term study the chemokine levels of CCL-5, CXCL-10 and CCL-17 were reduced under blockade of TNF-α, whereas the serum levels of CCL-3 and CXCL-9 showed no differences before and after treatment. These results may reflect the complex mechanism of effect and counter-effect of chemokines involved in the pathogenesis of AS, but these data have to be confirmed in a larger cohort of AS patients.

The involvement of chemokines in the pathophysiology of AS opens a new perspective for possible therapeutic approaches. Both chemokines released from the inflamed synovial cells and chemokine receptors expressed on cells infiltrating the synovial tissue may serve as a new therapeutic target not only in RA, but also in AS.[40] We know already that antibody-mediated blockade of the CXCR3 chemokine receptor results in diminished recruitment of T helper 1 cells into sites of inflammation in an adjuvant-induced peritonitis model.[41] The in vivo neutralization of CXCL-9 or CXCL-10 reduced the severity of idiopathic pneumonia syndrome in a murine model compared with control-treated animals, an additive effect was observed when both ligands were blocked simultaneously.[42]

In conclusion, the chemokine CXCL-9 is increased in sera from patients with active AS compared to healthy controls, and CCL-5, CXCL-10 and CCL-17 decrease under successful treatment with a TNF-α blocking agent. The expression profile of chemokine receptors on T-cells reflects the pro-inflammatory, IFN-γ producing type of CD28- T-cells, whereas production of type 2 cytokines cannot be totally neglected.
ACKNOWLEDGEMENTS

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COMPETING INTERESTS

The author(s) declare that they have no competing interests.

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FIGURE LEGENDS

Figure 1. Expression of the chemokine receptors CCR-5 (A), CXCR-3 (B), CCR-4 (C) and the calculated ratio of CXCR-3 to CCR-4 (D) on CD4+ T-cells and CCR-5 (E), CXCR-3 (F), CCR-4 (G) and the calculated ratio of CXCR-3 to CCR-4 (H) on CD8+ T-cells from the peripheral blood of healthy controls (co) and AS patients (AS). Whiskers box plots show 50% of cases within the boxes and all data excluding mavericks between the endpoints of the whiskers (lines). P<0.05 is considered significant (using the paired Wilcoxon ranking test).

Figure 2. Expression of the chemokine receptors CCR-5 (A), CXCR-3 (B), CCR-4 (C) and the calculated ratio of CXCR-3 to CCR-4 (D) on CD4+ T-cells and CCR-5 (E), CXCR-3 (F), CCR-4 (G) and the calculated ratio of CXCR-3 to CCR-4 (H) on CD8+ T-cells from the synovial fluid of 4 AS patients (AS). The percentages of CD28+ (●) and CD28- (¤) T-cells positively stained for the chemokine receptor are shown for each patient. P<0.05 is considered significant (using the paired Wilcoxon ranking test).

Figure 3. Production of IL-2 (A), IFN-γ (B), TNF-α (C), IL-10 (D) in CD4+ T-cells and IL-2 (E), IFN-γ (F), TNF-α (G), IL-10 (H) in CD8+ T-cells after stimulation with PMA/ionomycin. Whiskers box plots show 50% of cases within the boxes and all data excluding mavericks between the endpoints of the whiskers (lines). P<0.05 is considered significant (using the paired Wilcoxon ranking test).

Figure 4. Chemokine levels of the CCR-5 ligands, CCL-5 (A) and CCL-3 (B), the CXCR-3 ligands, CXCL-10 (C) and CXCL-9 (D) and the CCR-4 ligand CXCL-17 (E) in healthy controls and AS patients. Whiskers box plots show 50% of cases within the boxes and all data excluding mavericks between the endpoints of the whiskers (lines). P<0.05 is considered significant (using the Mann-Whitney test).

After blockade of TNF-α, the serum levels of CCL-5 (F), CXCL-10 (H) and CCL-17 (J) decreased, whereas the serum levels of CCL-3 (G) and CXCL-9 (I) showed no differences before and after treatment. The serum levels before and after treatment with infliximab are marked by (●) for each single patient. P<0.05 is considered significant (using the paired Wilcoxon ranking test).
REFERENCES


### TABLES

**Table 1.** Overview of chemokine receptors, chemokines with alternative nomenclature in parentheses and the corresponding immune response.

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<th>chemokine</th>
<th>immune response</th>
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<td>CCL-5 (RANTES), CCL-3 (MIP-1α)</td>
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<tr>
<td>CXCR-3</td>
<td>CXCL-9 (MIG), CXCL-10 (IP-10)</td>
<td>type 1</td>
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<td>CCR-4</td>
<td>CCL-17 (TARC)</td>
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**Table 2.** Patients’ characteristics.

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<td>HLA-B27 positivity</td>
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<td>BASMI</td>
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<td></td>
</tr>
<tr>
<td>BASFI</td>
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<td>BASDAI</td>
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<td>C-reactive protein</td>
<td>2.0±1.6 [mg/dl]</td>
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Results are shown as mean±SD.
Figure 3

A. IL-2
B. IFN-γ
C. TNF-α
D. IL-4
E. L-10

CD4+ T-cells [%]

F. CD28 +
G. CD28 -
H. CD28 +
I. CD28 -
J. CD28 -

CD8+ T-cells [%]

0.001
0.028
0.039
0.001
0.001
0.040
0.020
0.011

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