Interleukin 16 Expression in Relation to Disease Activity in Rheumatoid Arthritis

SABINE BLASCHKE, HARALD SCHULZ, GERHARD SCHWARZ, VOLKER BLASCHKE, GERHARD ANTON MÜLLER, and MONIKA REUSS-BORST

ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology characterized by an infiltration of CD4+ T lymphocytes within the rheumatoid synovium. Cytokines have been shown to play a modulatory role in the pathogenesis of RA. We analyzed the expression of a T cell derived cytokine, interleukin 16 (IL-16), in relation to disease activity to characterize its biologic function in RA.

Methods. Secreted IL-16 was measured by enzyme immunoassay in sera and synovial fluids (SF) from 25 patients with RA in comparison to 20 control samples from patients with osteoarthritis (OA). IL-16 expression in peripheral blood mononuclear cells (PBMC) was characterized by flow cytometric analysis after intracellular cytokine staining for IL-16. In synovial tissue specimens, IL-16 mRNA expression was analyzed by real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR). In parallel, expression of IL-16 was localized in synovial tissues by *in situ* hybridization and immunohistochemistry. Results were analyzed in relation to disease activity.

Results. IL-16 was detected at significantly higher levels in sera and SF of patients with RA in comparison to OA ($p < 0.001$). Flow cytometry of PBMC showed that a great proportion of both CD4+ and CD8+ T cells constitutively expressed the IL-16 protein. In synovial tissues, IL-16 mRNA levels were significantly elevated in comparison to OA controls ($p < 0.001$). *In situ* hybridization for IL-16 producing cells revealed a predominant accumulation of IL-16 positive cells within the inflammatory infiltrates. A significant correlation between IL-16 expression and local inflammatory activity could not be established ($r = 0.27$, $p = 0.19$) by microscopic analysis of the synovial cell infiltrate. In addition, no significant association was observed between serum, SF, and synovial tissue expression of IL-16 and clinical disease activity in RA.

Conclusion. These data suggest IL-16 might play a role in the pathogenesis of chronic inflammation in RA. The lack of significant correlation between IL-16 expression, clinical disease activity, and local inflammatory activity suggests a regulatory rather than a proinflammatory function for IL-16 in the pathogenesis of chronic synovial inflammation in RA. (J Rheumatol 2001;28:12–21)

Key Indexing Terms:
INTERLEUKIN 16 DISEASE ACTIVITY
RHEUMATOID ARTHRITIS REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

Rheumatoid arthritis (RA) is a systemic inflammatory disease of unknown etiology. The rheumatoid synovium is characterized by an infiltration of CD4+ T cells, macrophages, B cells, and proliferating fibroblasts[1], and aggressively invades cartilage and bone, thus destroying joints' ability to function.

The role of T cells in the pathogenesis of RA remains controversial — association of disease susceptibility with HLA-DR4 antigens[2] and prominent accumulation of CD4+ T cells expressing several activation markers within the rheumatoid synovium[3] provide evidence for the importance of T cells in RA. However, synovial T cell hyporesponsiveness[4] reflected by a reduced response to mitogenic stimulation[5] and impaired T cell receptor (TCR) mediated signaling[6] and the limited beneficial effects of T cell directed therapies[7] argue against a central role of T cells in the pathogenesis of RA. This hypothesis is supported by the finding that T cell derived cytokines have only been detected at low levels within synovial fluids (SF) and synovial tissue cells of RA, whereas cytokine products from macrophages and synovial fibroblasts [interleukin 1ß (IL-1ß), tumor necrosis factor-α (TNF-α)] were present in abundance[8]. Since antiinflammatory cytokines were also detected within the rheumatoid synovium, activation of synovial T cells was thought to be confined by the local production of inhibitory factors such as transforming growth factor-ß[9] and IL-10[10].

IL-16 is a T cell derived cytokine originally identified and purified as lymphocyte chemoattractant factor from con-

From the Department of Nephrology and Rheumatology, Department of Pathology, Department of Orthopaedics, and Department of Dermatology, University of Göttingen, Göttingen, Germany.

S. Blaschke, MD; M. Reuss-Borst, MD, Professor of Medicine; G.A. Müller, MD, Professor of Medicine, Head, Department of Nephrology and Rheumatology; H. Schulz, MD, Department of Pathology; G. Schwarz, MD, Department of Orthopaedics; V. Blaschke, Department of Dermatology.

Address reprint requests to Dr. S. Blaschke, Department of Nephrology and Rheumatology, Georg-August-University, Robert-Koch Str. 40, 37075 Göttingen, Germany. E-mail: sblasch@gwdg.de

Submitted May 9, 2000 revision accepted August 28, 2000.

canavalin A stimulated human PBMC[11]. It was shown that CD8+ T cells synthesize and store the bioactive protein[12], while CD4+ T cells store the precursor molecule (pro-IL-16)[13]. IL-16 acts via the CD4 molecule as its receptor[14] and exerts a broad spectrum of both pro- and antiinflammatory biologic activities: these functions include the selective chemotactic and competence growth factor activity for CD4+ T cells[11], stimulation of IL-2 receptor (CD25) and MHC class II expression on resting T cells[15], and activation of tyrosine kinase p56[1ck 16]. However, IL-16 has also been shown to suppress CD3/TCR mediated T cell activation in mixed lymphocyte reactions (transient induction of T cell anergy)[17,18] and to inhibit mitogen induced IL-2 production by CD4+ T cells[19].

In RA, the presence of IL-16 has been described in synovial tissues from patients with RA[20]. However, the functional activity of this T cell derived cytokine in the pathogenesis of RA is not completely understood. Results of adoptive transfer experiments in a human SCID mouse model of RA[21] suggest an immunosuppressive role for IL-16 in the pathogenesis of RA, since adoptive transfer of synovial tissue derived syngeneic CD8+ T cells induced a marked reduction of T cell and macrophage activity that was blocked by antibodies against IL-16.

To characterize the functional activity of IL-16 in the pathogenesis of RA, we investigated IL-16 expression in sera, SF, PBMC, and synovial tissues from patients with RA in relation to disease activity.

MATERIALS AND METHODS

Patients and samples. Heparinized blood samples (20 ml) and sera (10 ml) were obtained from 25 patients with RA and 20 with OA as controls. All patients with RA met the criteria established by the American College of Rheumatology[22]. Most of them were receiving disease modifying drugs and corticosteroids. Disease activity was evaluated by clinical and laboratory findings as described[23]. In brief, the clinical judgment of disease activity in RA was based upon the Ritchie score index, number of swollen joints, and erythrocyte sedimentation rate (ESR). The disease activity score (DAS) was determined according to the following equation:

$$DAS = 0.53938 \times \sqrt{(\text{Ritchie score})} + 0.06465 \times (\text{no. of swollen joints}) + 0.330 \times \ln(\text{ESR}) + 0.224$$

Patients' characteristics are summarized in Table 1.

SF specimens were collected from 15 patients with RA and 15 patients with nonrheumatoid joint disease (13 OA, 2 psoriatic arthritis). Synovial tissue was obtained at the time of surgery from 25 patients with RA and 10 with OA undergoing synovectomy or arthroplasty. Synovial tissue samples were split in half for cryopreservation prior to RNA isolation and paraffin embedding for *in situ* hybridization and immunohistochemical analysis.

Immunoassay for IL-16. IL-16 concentrations in serum and SF specimens were measured by a solid phase sandwich ELISA (Biosource, Ratingen, Germany) according to the manufacturer's instructions: in brief, 50 µl of samples and human recombinant IL-16 standards were applied to the wells of an anti-IL-16 monoclonal antibody (Mab) coated ELISA plate. After addition of 100 µl of a biotinylated rabbit anti-human IL-16 polyclonal antibody, plates were incubated 3 h at room temperature. Plates were vigorously washed 3 times with phosphate buffered saline (PBS)-0.05% Tween 20 and 100 µl streptavidin peroxidase solution was added to each well. After another washing step the chromogen (3,3',5,5'-tetramethylbenzidine dihydrochloride) was applied for 30 min at room temperature. Optical densities of the samples were then measured in duplicate at 450 nm. Using the MikroWin software the standard curve of known IL-16 concentrations was established and employed to determine IL-16 concentrations within the samples. The sensitivity of the assay was 5 pg/ml.

Table 1. Baseline characteristics of patients with RA.

Patients	
Sex, m:f	7:18
Age, mean (range), yrs	55.7 (34–77)
IgM, RF, % positive	61.3
DAS, mean (range)	3.21 (1.52–5.46)
No. of swollen joints, mean (range)	4 (2–11)
ESR, mean (range), mm/h	28.3 (5–91)
CRP, mean (range), mg/l	24.2 (5.6–198.3)
Disease duration, median (range), yrs	4 (1–15)
Therapy	
NSAID	24
Corticosteroids	21
DMARD	20
Arthroplasty	15
Synovectomy	10

RF: rheumatoid factor, DAS: disease activity score, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, NSAID: nonsteroidal antiinflammatory drugs, DMARD: disease modifying antirheumatic drugs.

Flow cytometric analysis of PBMC. PBMC were isolated from heparinized blood samples by Ficoll-Hypaque density centrifugation (Biochrom, Berlin, Germany). Cells were then characterized by flow cytometric analysis after intracellular cytokine staining as described[24]: in brief, isolated PBMC were resuspended at a density of 2×10^6 cells/ml in PBS/0.1% bovine serum albumin (BSA) and stained for cell surface antigens with fluorochrome labeled antibodies. Anti-CD4, anti-CD8, anti-CD3 antibodies and isotype control (IgG_1/IgG_{2a}) were obtained from Becton-Dickinson (Heidelberg, Germany). Cells were fixed in 4% paraformaldehyde for 15 min at 4°C, washed twice in PBS/0.1% BSA, and permeabilized for 1 h at 4°C with 0.1% saponin (w/v) solution (Sigma, Deisenhofen, Germany) containing 5% nonfat dry milk to block nonspecific binding. For intracellular cytokine staining PBMC were incubated with 0.25 µg of an R-phycoerythrin (PE) conjugated mouse anti-human IL-16 antibody (PharMingen, Hamburg, Germany) for 30 min at 4°C, washed again, and resuspended in PBS for flow cytometric analysis. To reveal specificity of staining target cells were incubated with an isotype matched immunoglobulin of irrelevant specificity at the same concentration (PE conjugated IgG_1 isotype control, PharMingen). List mode data were acquired on a FACSCalibur (Becton-Dickinson) flow cytometer. Dead cells and monocytes were excluded by forward and side scatter gating. Typically, 10,000 events were acquired and analyzed using the CellQuest software. Bivariate dot plots were generated upon data reanalysis to display the frequencies of individual cells coexpressing certain levels of cell surface antigens and intracellular IL-16.

Reverse transcriptase-polymerase chain reaction. To detect IL-16 gene transcripts in synovial tissue samples, total cellular RNA was extracted using the RNeasy Kit (Qiagen, Hilden, Germany). The concentration and purity of RNA were determined spectrophotometrically. For synthesis of first-strand complementary DNA (cDNA) 1 µg isolated RNA was digested with RNase-free DNase I (Pharmacia, Freiburg, Germany) and incubated with 1.5 µM $oligo(dT)_{12\text{-}18}$ primer (GibcoBRL, Eggenstein, Germany), 5× reverse transcriptase buffer, 0.4 µM of each dNTP (MBI Fermentas, St. Leon-Rot, Germany) and 200 U of reverse transcriptase (Superscript II, GibcoBRL) in a total volume of 50 µl at 42°C for 1 h. For detection of ß-actin and IL-16 transcripts, polymerase chain reaction (PCR) was performed with 100 ng cDNA, 1 µl of each dNTP (10 mM), 1.5 mM $MgCl_2$, 5 µl of PCR buffer, 0.5 µl of each primer (10 nmol/µl), and 2.5 U of Taq polymerase (GibcoBRL) in a final vol-

ume of 50 µl. Primer sequences were as follows: ß-actin (forward primer) 5'→3'CCCAGCCATGTACGTTGCTAT, (reverse primer) 5'→3'GGGTGGCTTTTAGGATGGCAA (product size 1047 bp); IL-16 (forward primer) 5'→3'ATGCCCGACCTCAACTCCTCCAC, (reverse primer) 5'→3'GCCACCCAGCTGCAAGATTTC (product size 261 bp). cDNA samples were amplified for 35 cycles of 45 s at 95°C, 45 s at 60°C (57°C, respectively), 90 s at 72°C, followed by a final elongation step of 10 min at 72°C. PCR products were separated by agarose gel electrophoresis (1.5% agarose gel) and visualized by ethidium bromide staining. PCR products were cloned into the *Sma I* restriction site of the pBluescript vector (Stratagene, Heidelberg, Germany) to generate internal controls. Each PCR analysis included the internal standard as positive control and distilled water instead of cDNA as negative control. To control the specificity of the PCR assay PCR products were sequenced in both directions by cycle sequencing with dye-dideoxy terminator dNTPs on an automated DNA sequencer (Applied Biosystems, Weiterstadt, Germany).

Real-time quantitative RT-PCR. To quantitate IL-16 mRNA expression a method of real-time quantitative RT-PCR was established. PCR was performed in a total volume of 20 µl containing 100 ng of cDNA, 2 µl SYBR Green master mix (Roche Diagnostics, Mannheim, Germany), 4 mM $MgCl_2$, and 11.25 pmol of each oligonucleotide primer. Primer sequences were as follows: ß-actin forward primer LF: 3'→5'CCCAAGGCCAACCGCGAGAAGAT; ß-actin reverse primer LR: 3'→5'GTCCCGGCCAGCCAGGTCCAG, product size 219 bp; IL-16 forward primer LF: 3'→5'AAGGGGCATCTCCAACATCATCAT; IL-16 reverse primer LR: 3'→5'CTCCTGCCAAGCTGAACCCAAGAC, product size 332 bp. Samples were loaded into glass capillaries, centrifuged, and placed in a LightCycler® apparatus (Roche Diagnostics). Amplification was performed in duplicate for each sample in a 3 step cycle procedure. Cycle conditions for IL-16 (ß-actin) included denaturation of the template DNA for 1 cycle of 95°C, 30 s, programmed temperature transition rate of 20°C/s, amplification of the target DNA for 35 cycles of 95°C, 0 s, 62°C (66°C), 5 s, and extension at 72°C, 13 s, each with a temperature transition rate of 20°C/s. Melting curve analysis was performed for 1 cycle at 95°C, 0 s, and 70°C (78°C), 15 s, to determine melting temperatures of the primer-dimer product and the specific PCR product. Fluorescence was measured at a separate detection step (2 s) at a temperature between the melting points of primer-dimers and the specific PCR product (ß-actin: 87°C; IL-16: 83°C) to exclude fluorescence attributable to primer-dimers. Each run included external standards as positive controls and water as negative control. Standards were generated by densitometric quantification of specific PCR products after agarose gel electrophoresis and preparation of 10-fold dilution series ranging from 10 amol/µl to 0.0001 amol/µl. External standards were used to construct a standard curve. The cDNA concentration in each sample was then calculated automatically by reference to the standard curve. To standardize IL-16 mRNA concentrations transcript levels of the housekeeping gene ß-actin were determined in parallel for each sample. Results were then expressed as the ratio of the molar amounts of IL-16 mRNA and ß-actin mRNA.

In situ hybridization. For *in situ* detection of IL-16, mRNA transcripts digoxigenin-11-UTP (Boehringer Mannheim, Germany) labelled anticomplementary (sense) and complementary (anti-sense) probes were generated by *in vitro* transcription with oligonucleotide primers specific for the T3 and T7 promotor regions of the transcription vector pBluescript. *In situ* hybridization was performed as described[25]. Hybridization to a sense probe and hybridization after nuclease digestion of the target RNA (RNase H, 20 µg/ml) served as negative control.

Immunohistochemistry. Serial sections of synovial tissue samples were stained for cell surface markers CD4, CD8, and CD68 by an indirect alkaline phosphatase technique[26]. The following primary mouse Mab were used: anti-CD4 (clone 1F6, dilution 1:20; Novocastra, Dossenheim, Germany), anti-CD8 (clone C8/144B, dilution 1:20; Dako, Hamburg, Germany), and anti-CD68 (clone PG-M1, dilution 1:50; Dako). Secondary antibodies (alkaline phosphatase conjugated rabbit anti-mouse antibodies) and substrate (naphthol-AS-MX-phosphate/fast red) were obtained from Sigma. Slides were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and subjected to microscopic analysis.

Immunohistochemical detection of IL-16 expression in synovial tissues was performed with a mouse anti-human IL-16 IgG_1 antibody (hybridoma supernatant, kindly provided by W. Schlüsener, Tübingen, Germany) as described[27]. In brief, paraffin sections were pretreated by microwave and endogenous peroxidase activity was blocked with 3% hydrogen peroxide. After blocking with normal rabbit serum (Dako), sections were incubated at 4°C overnight with anti-IL-16 IgG_1 antibodies. An isotype control serum was always included as negative control. Slides were washed 3 times in PBS and a biotinylated rabbit anti-mouse IgG polyclonal antibody (Dako) was applied. After incubation for 30 min at room temperature slides were washed again and the strepavidin-peroxidase complex (streptAB complex, HRP; Dako) was added for 10 min at room temperature. Peroxidase activity was then visualized by addition of 3-amino-9-ethylcarbazol substrate solution.

Microscopic analysis. Microscopic analysis was performed as described[26]: hematoxylin and eosin stained sections were randomly analyzed at a magnification of 250× by 2 independent observers without knowledge of disease activity. Tissues were scored semiquantitatively for synovial lining hyperplasia (score 0–3: 0 = 1–2, 1 = 3–4, 2 = 5–6, 3 = > 7 cell layers), and the degree of infiltration with lymphocytes and polymorphonuclear granulocytes (PMN) using a 5 point scale: 0 = 0 cells, 1 = 1–5 cells, 2 = 5–25 cells, 3 = 25–50 cells, 4 = > 50 cells per high power field (HPF, magnification 250×). The inflammatory score was determined by the sum of the scores for the synovial lining hyperplasia and the infiltration with lymphocytes and PMN. Similarly, the presence of CD4, CD8, CD68, and IL-16 positive cells was scored on a 5 point scale. Interobserver differences were resolved by mutual agreement. At least 5 separate areas were examined for each biopsy specimen to minimize sampling error. Results represent the mean of all area scores and both examiners' scores.

Statistical analysis. Data were expressed as the mean ± SEM. Samples with values below the detection limit of the assay were regarded as negative. Statistical differences between groups were evaluated by Student's t test. P values were determined by 2 sided calculation. Correlations between IL-16 levels and the disease activity score were quantified applying Spearman's rank correlation test.

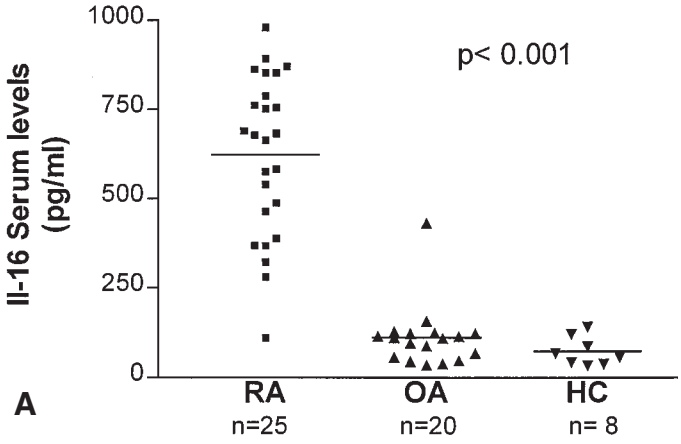
RESULTS

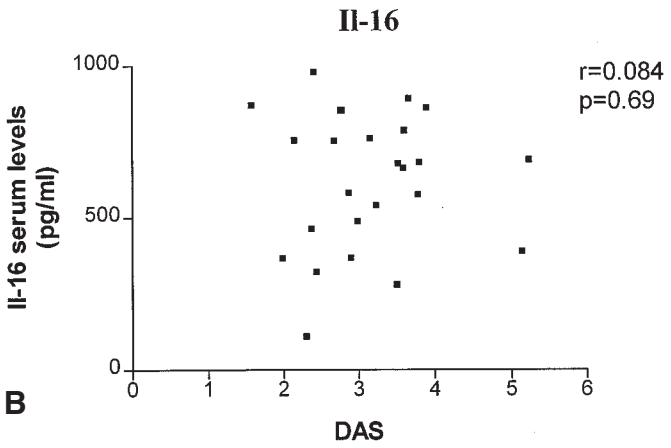
IL-16 concentrations in RA sera. IL-16 levels were measured by enzyme immunoassay (EIA) in sera from 25 patients with RA. Results were compared with IL-16 concentrations in sera from 20 patients with OA and 8 healthy blood donor controls. The sensitivity of the assay was 5 pg/ml.

As illustrated in Figure 1A, IL-16 was detected at significantly higher levels in RA sera (623.2 ± 45.26 pg/ml) compared to OA (112.0 ± 20.67 pg/ml; $p < 0.001$). In control sera values ranged between 34 and 142 pg/ml (mean 78 pg/ml). In contrast to the C-reactive protein (CRP) included as a positive control, analysis of IL-16 serum levels in relation to disease activity scores for RA patients revealed no significant correlation ($r = 0.084$, $p = 0.69$) (Figure 1B).

IL-16 expression in unstimulated PBMC from RA patients. IL-16 expression in PBMC from 15 RA patients and 6 healthy controls was analyzed by flow cytometric analysis after intracellular staining with a fluorochrome conjugated anti-IL-16 antibody. To characterize the immunophenotype of IL-16 positive cells PBMC were also stained for CD4– and CD8– surface antigens. Frequencies of IL-16 positive PBMC were obtained by gating on CD4+ and CD8+ cells, respectively.

Two color flow cytometry for CD4 (or CD8) versus IL-16





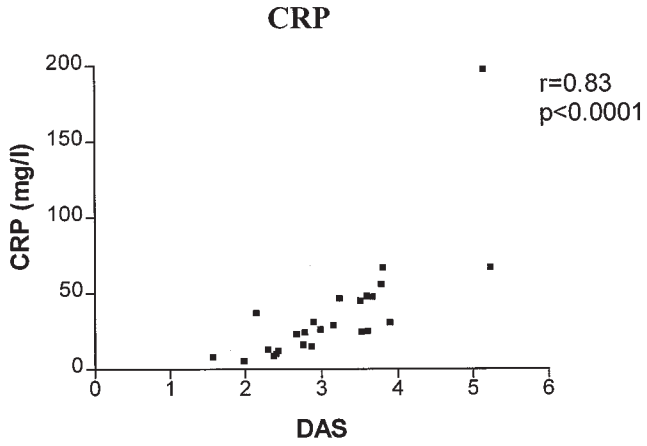


Figure 1. A. Expression of IL-16 in sera from patients with RA and healthy controls (HC). Sensitivity of the ELISA was 5 pg/ml. Horizontal line represents the mean value of each group. IL-16 concentrations in RA sera were significantly elevated in comparison to OA ($p < 0.001$) and controls ($p < 0.001$). B. Correlation between serum IL-16 concentrations and CRP levels and disease activity in patients with RA (Spearman rank correlation test).

of unstimulated PBMC from RA patients revealed that a great proportion of both CD4+ (64.9 ± 2.57%) and CD8+ cells (80.25 ± 1.66%) expressed IL-16 protein compared with isotype control Mab. Similar percentages of CD4+ and CD8+ cells staining positive for IL-16 were found in subsequent experiments with controls (Figure 2). These data indicated that the majority of resting CD4+ and CD8+ T cells express IL-16 in both patient and control groups.

In correspondence with IL-16 serum concentrations from patients with RA, a significant correlation between the fraction of IL-16 positive PBMC and the DAS of RA patients could not be established (data not shown).

IL-16 concentrations in SF of patients with RA. IL-16 levels were also analyzed by EIA in SF of 15 patients with RA and 15 OA controls. Elevated IL-16 concentrations were found in SF specimens of RA patients (10.34 ± 1.59 ng/ml). In correspondence with serum data, significantly lower IL-16 concentrations were detected in patients with OA (0.96 ± 0.146 ng/ml; $p < 0.001$) (Figure 3A). Notably, in both patient and control groups IL-16 levels were found at 10-fold higher concentrations within the SF specimens in comparison to mean serum levels. A significant correlation between SF IL-16 levels and the DAS in patients with RA was not observed ($r = 0.19$, $p = 0.49$) (Figure 3B).

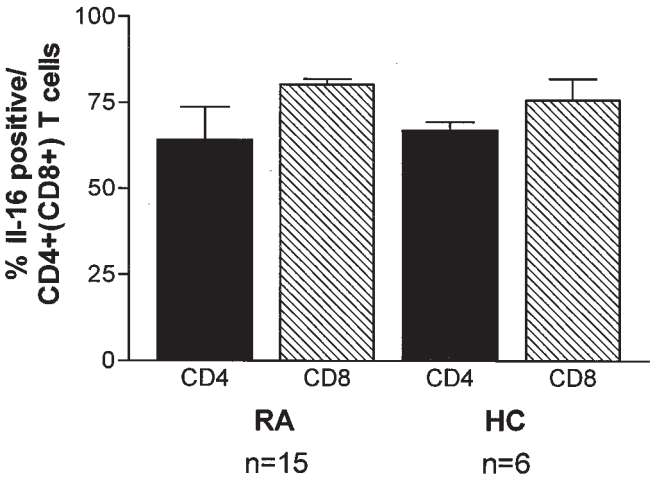


Figure 2. Analysis of IL-16 positive PBMC by 2 color flow cytometry in patients with RA and controls. PBMC were characterized after intracellular cytokine staining with a phycoerythrin conjugated anti-IL-16 antibody and CD4 (CD8) surface antigen staining. A great proportion of CD4+ and CD8+ T lymphocytes express IL-16 in both patients and controls.

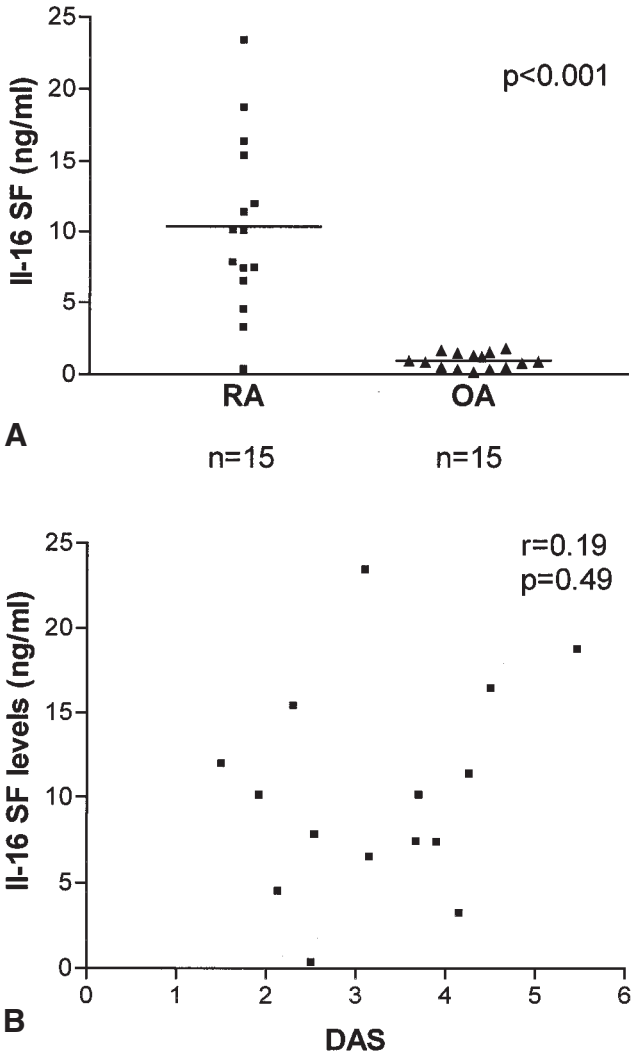


Figure 3. A. IL-16 levels in SF from patients with RA and OA measured by ELISA. Horizontal line represents the mean value of each group. B. Correlation between SF IL-16 concentrations and disease activity in patients with RA.

IL-16 expression in RA synovium. To analyze IL-16 mRNA expression within the synovium as the primary focus of disease activity, total cellular RNA was extracted from synovial tissues of 18 patients with RA and 10 with OA. cDNA was synthesized and subjected to RT-PCR analysis with specific primers for IL-16. IL-16 mRNA was detected in 15 of 18 RA tissue samples, but in only 2 of 10 OA samples, as illustrated in Figure 4A.

The amount of IL-16 mRNA expression was determined by real-time quantitative RT-PCR using LightCycler® technology (Figure 4B). To compare IL-16 mRNA expression between different samples IL-16 mRNA levels were standardized for ß-actin. Results revealed that levels of IL-16 mRNA expression were significantly elevated in synovial tissue samples from patients with RA compared to OA ($p < 0.001$). However, there was no significant correlation between IL-16 mRNA expression and the DAS for patients with RA ($r = 0.13$, $p = 0.63$) (Figure 4C).

To localize IL-16 mRNA producing cells within the synovial membrane a method of nonradioactive *in situ* hybridization was established. As shown in Figure 5A, IL-16 positive cells exhibited strong hybridization signals and were predominantly detected in the sublining area within lymphocytic infiltrates. In addition, IL-16 positive cells were detected scattered throughout the synovial lining layer. Only a few hybridization signals were recognized within the OA synovium. Negative controls included hybridization to a sense probe (Figure 5B) and hybridization after nuclease digestion of the target RNA.

Results could be confirmed by immunohistochemistry of synovial tissue sections with a polyclonal anti-IL-16 antibody. As illustrated in Figure 6A, IL-16 positive cells were detected at high frequency within the inflamed rheumatoid synovium, whereas rare immunoreactive cells stained positive for IL-16 in the control group. No staining was observed when an isotype control was used instead of primary antibody (Figure 6B).

To determine which cell types are predominantly responsible for synovial IL-16 production, serial synovial tissue sections were analyzed by *in situ* hybridization for IL-16 mRNA and immunohistochemical staining for CD4, CD8, and CD68 surface antigens. Results indicated that a great proportion of infiltrating CD4+, but also CD8+ T lymphocytes within the subsynovial area (Figure 7A-7C) and CD68 negative cells within the synovial lining layer (data not shown) stained strongly positive for IL-16.

Analysis of IL-16 expression within the synovial cell infiltrate in relation to disease activity. To characterize IL-16 expression in relation to local disease activity, synovial tissue samples from 25 patients with RA were semiquantitatively scored for histologic and immunohistologic features by microscopic analysis. Results were compared between 2 patient subgroups with low and high disease activity ($DAS \leq 2.5$ vs $DAS \geq 3.5$) to analyze the potential dependence of IL-16 expression on scores for local inflammatory activity and clinical disease activity (Table 2).

Histologic analysis revealed a difference between inflammatory scores: local inflammatory activity tended to be higher in patients with high DAS, but this difference did not reach statistical significance ($p = 0.34$).

Immunohistologic scores for surface antigens CD4, CD8, and CD68 showed strong variations between the patients: semiquantitative assessment of CD4 and CD8 positive cells revealed similar scores for synovial tissues of patients with low and high disease activity ($p = 0.47$, $p = 0.32$, respectively). A significant difference between the 2 patient subgroups was observed only for the immunohistologic scores of CD68 expression ($p = 0.02$). No significant correlation was found between local inflammatory activity and IL-16 expression ($r =$

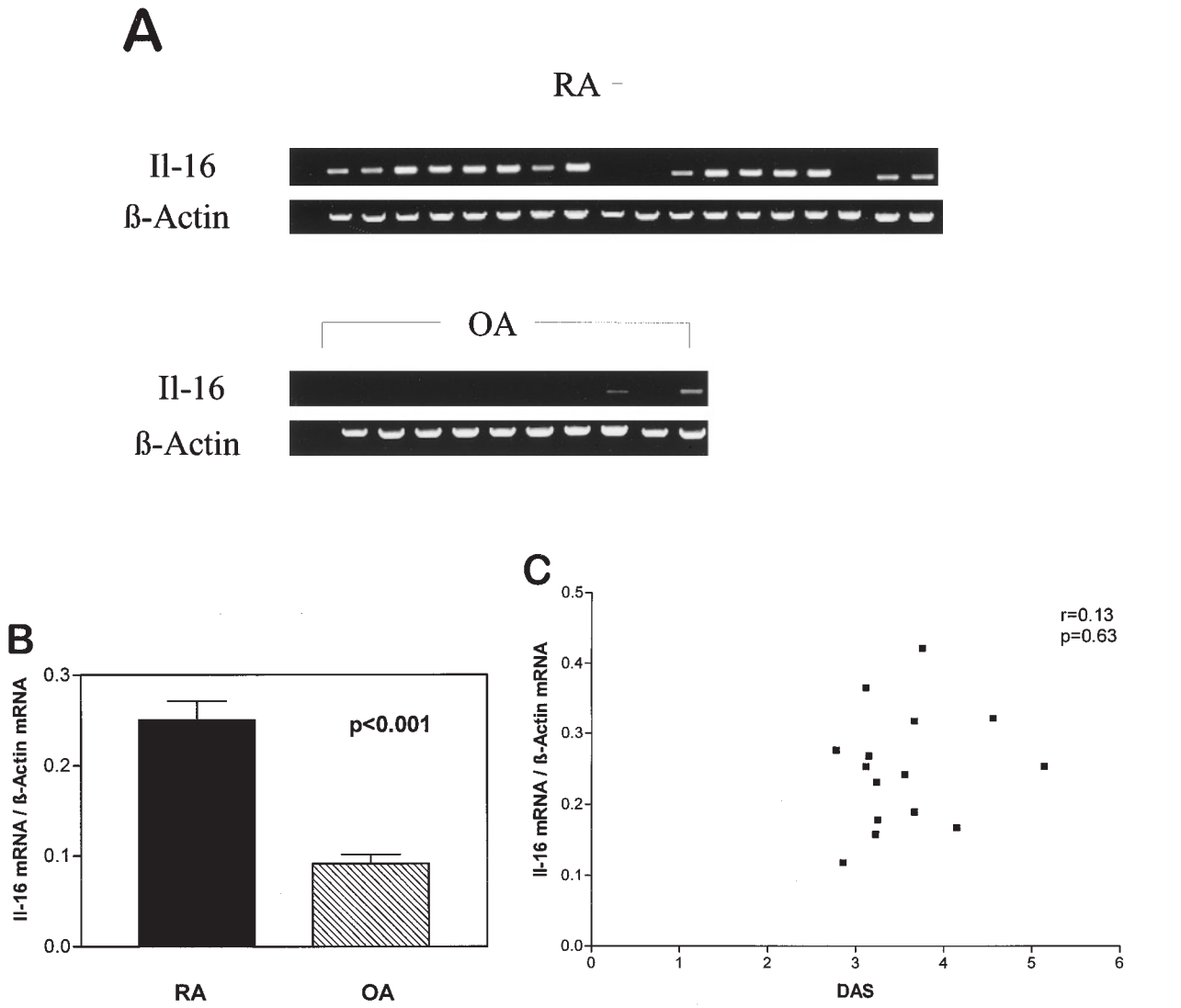


Figure 4. IL-16 mRNA expression in synovial tissues from 18 patients with RA and 10 with OA by real-time quantitative RT-PCR. A. Total RNA was extracted from synovial tissue specimens; cDNA samples were normalized to yield comparable amounts of ß-actin products and then amplified with specific primers for IL-16. B. Analysis of IL-16 mRNA expression; values were determined in duplicate (mean ± SEM). IL-16 levels were standardized for ß-actin to compare IL-16 mRNA expression between different samples. IL-16 gene expression related to ß-actin expression was significantly elevated in RA compared to OA ($p < 0.001$). C. Correlation between IL-16 mRNA expression in RA synovial tissues and disease activity.

0.27, p = 0.19) (Figure 8). In addition, there was no association between IL-16 expression and clinical disease activity (p = 0.36).

DISCUSSION

We investigated the relationship between expression of IL-16, a novel T cell derived cytokine, and disease activity in RA to further characterize its biologic function during the pathogenesis of RA.

In agreement with previous results[20], analysis of serum IL-16 concentrations revealed significantly higher IL-16 levels in sera from patients with RA in comparison to OA patients and healthy controls ($p < 0.001$). However, in contrast to the CRP as an established marker for inflammation, a close correlation

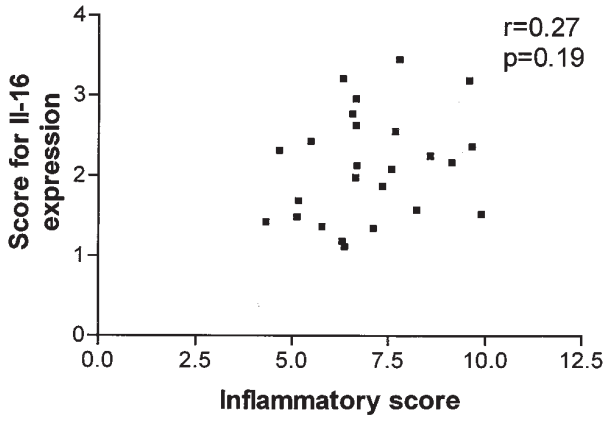


Figure 8. Correlation between IL-16 expression and local inflammatory activity in RA synovial tissue.

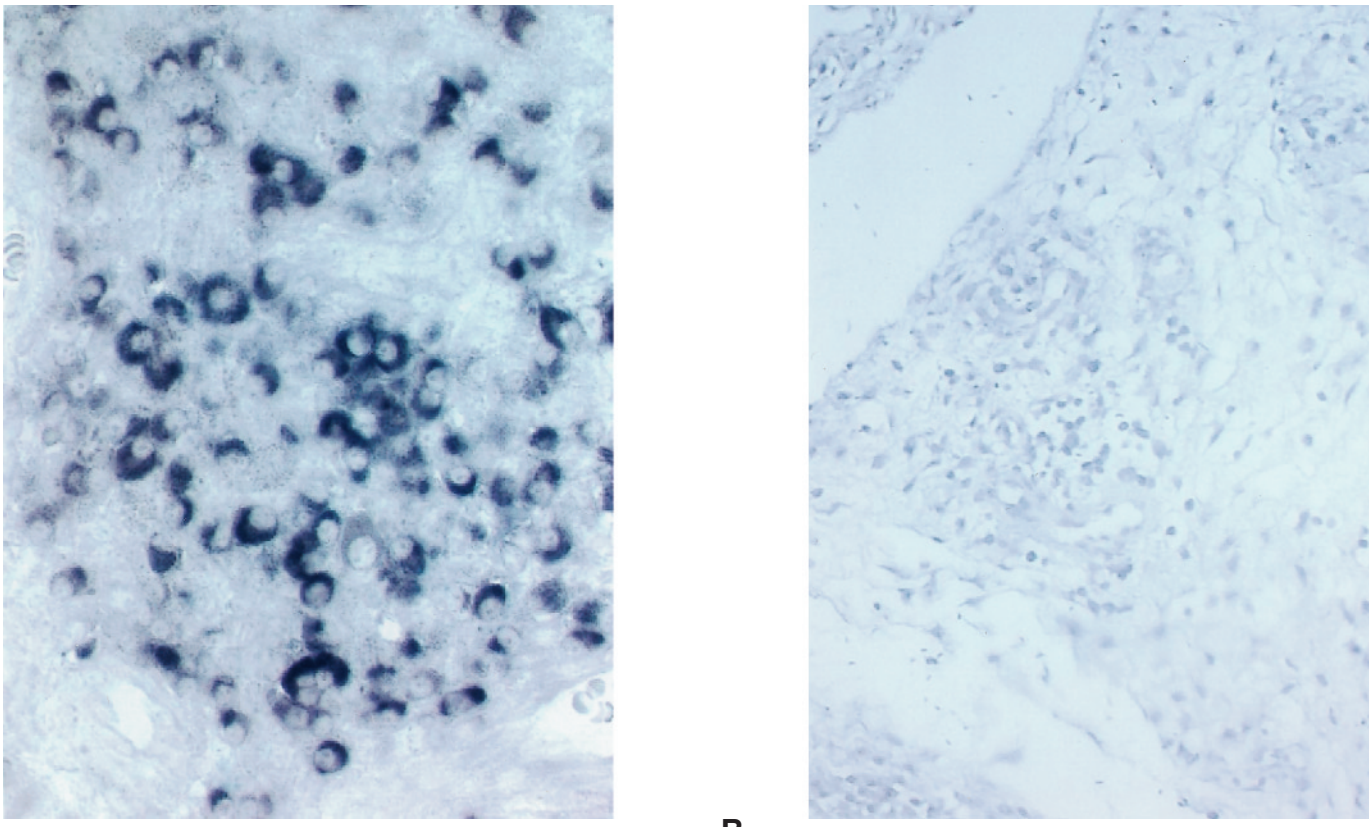


Figure 5. Paraffin sections of synovial tissues from patients with RA (A) and OA were analyzed by *in situ* hybridization for IL-16 mRNA expression. In RA, IL-16 was strongly expressed within infiltrating lymphocytes, while in OA only rare IL-16 positive cells were observed (data not shown). B. As negative control, *in situ* hybridization studies with a sense probe were always included. (Magnification A ×400, B ×200; hematoxylin counterstain.)

Table 2. Histologic and immunohistologic features of synovial tissues from patients with RA with low (DAS ≤ 2.5) and high (DAS ≥ 3.5) disease activity.

	Disease Activity		
	DAS ≤ 2.5, n = 12	DAS ≥ 3.5, n = 13	p
Inflammatory score*	6.74 ± 0.43 (1–10)	7.34 ± 0.45 (1–12)	0.34
CD4†	2.08 ± 0.23 (1–4)	2.31 ± 0.21 (1–4)	0.47
CD8†	1.17 ± 0.11 (1–4)	1.39 ± 0.18 (1–4)	0.32
CD68†	1.67 ± 0.19 (1–4)	2.31 ± 0.17 (1–4)	0.02
IL-16†	1.99 ± 0.18 (1–4)	2.24 ± 0.19 (1–4)	0.36

*Values represent mean ± SEM (range) scores for inflammation identified on hematoxylin/eosin stained tissue sections.
†Values represent mean ± SEM (range) scores for infiltration by CD4+ T cells, CD8+ T cells, CD68+ macrophages, and cells positive for IL-16.
DAS: disease activity score[30].

between disease activity and serum IL-16 levels in RA could not be established (p = 0.69). These findings for IL-16 differ from cytokines with predominant proinflammatory activity in RA, such as TNF-α, IL-1ß, and IL-6. Several studies have described increased serum levels of these macrophage derived cytokines and significant correlations between serum cytokine concentrations and disease activity in RA[28-30].

Since IL-16 represents a T cell derived cytokine, expression of IL-16 in RA was next characterized in PBMC by FACS analysis after intracellular cytokine staining. Results revealed that IL-16 is produced by a great proportion of both CD4+ and CD8+ T lymphocytes. These data correspond with previous results: IL-16 mRNA was shown to be synthesized constitutively in CD8+ T cells as well as in CD4+ T cells[31]. In addition, similar quantities of the 68 kDA precursor protein pro-IL-16 have been observed in both unstimulated CD4+ and CD8+ T cells[24]. However, resting CD8+ T lymphocytes express active caspase-3 for cleavage of pro-IL-16 into its bioactive 13 kDa form[32], whereas CD4+ T cells require antigenic stimulation for processing of pro-caspase-3 into its 20 kDa active form and subsequent release of bioactive IL-16[33]. Since we detected no significant differences in percentages of IL-16 positive lymphocytes between RA and healthy control samples in this study, it is proposed that activation of CD4+ T lymphocytes may account for elevated IL-16 serum levels in RA.

To study local IL-16 expression within affected joints IL-16 levels were analyzed in SF from patients with RA in com-

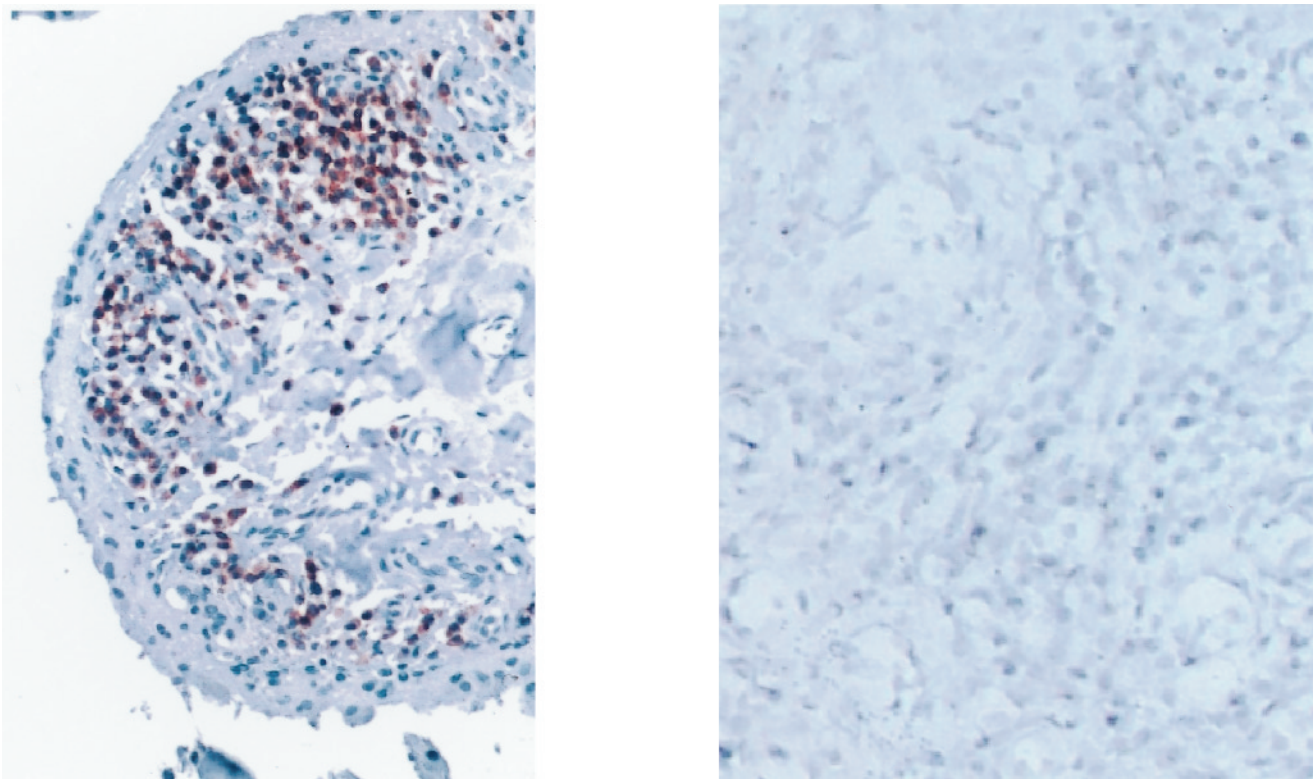


Figure 6. Immunohistochemical detection of IL-16 in synovial tissues from patients with RA (A) and OA; tissues were incubated with a mouse anti-IL-16 polyclonal antibody and stained by the immunoperoxidase method. In RA synovial tissue, IL-16 positive cells were detected at high frequency within lymphocytic infiltrates. In OA synovial tissues only rare IL-16 positive cells were observed (data not shown). An isotype control (B) was always included as negative control. (Magnification ×200; hematoxylin counterstain.)

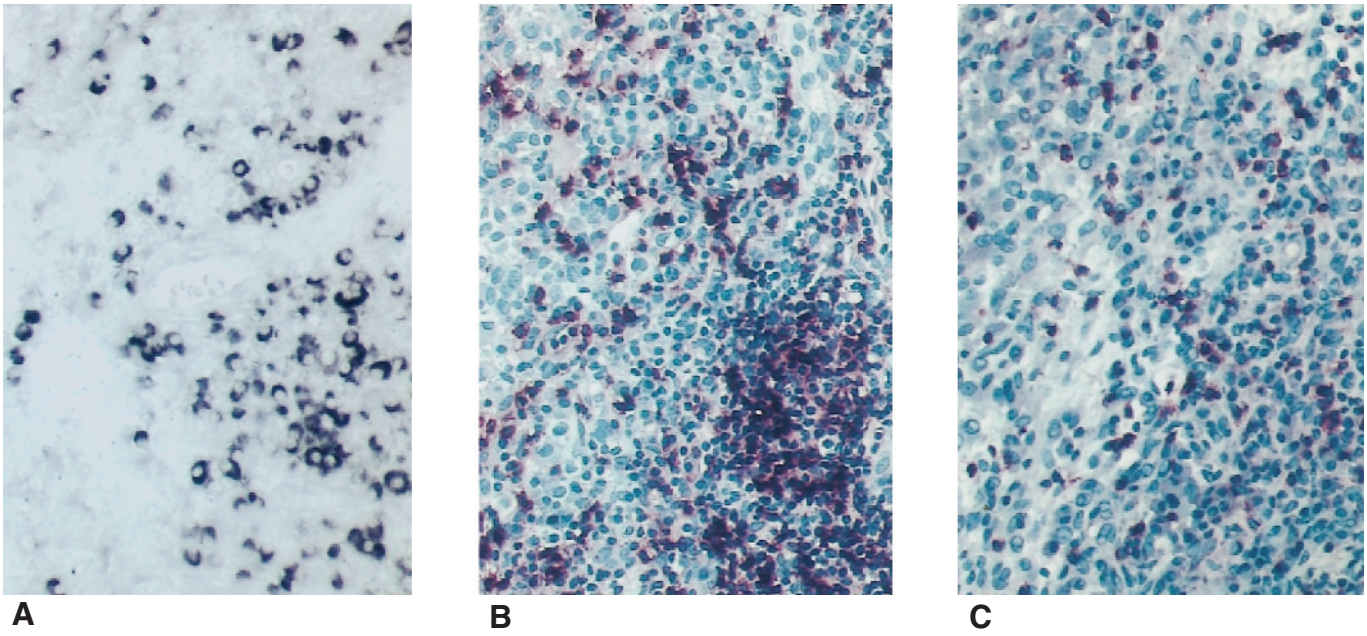


Figure 7. Combination of *in situ* hybridization for IL-16 mRNA and immunohistochemical staining for CD4 and CD8 surface antigens on serial sections. Deparaffinized tissue sections were sequentially stained for IL-16 mRNA by *in situ* hybridization (blue) and CD4 or CD8 surface antigens by immunohistochemical staining (red), followed by hematoxylin counterstain. A. IL-16 is strongly expressed within infiltrating lymphocytes. A great proportion of CD4+ cells (B) and also CD8+ cells (C) within the subsynovial tissue stained positive for IL-16 (original magnification ×200).

parison to OA patients. Results revealed a marked elevation of SF IL-16 in patients with RA ($p < 0.001$). In contrast, other T cell derived cytokines such as IL-2, IL-4, and interferon-γ (IFN-γ) have been detected only in small quantities within SF, whereas highly elevated levels were reported for macrophage derived proinflammatory cytokines (TNF-α and IL-1ß)[8]. Comparison of mean IL-16 levels in serum and SF revealed almost 10-fold higher concentrations in SF from both patient and control groups, as described[20]. This finding suggests local production of IL-16 within the joint. To analyze local IL-16 expression total RNA was isolated from synovial tissue samples and subjected to RT-PCR. For quantification of detectable IL-16 mRNA levels a highly sensitive method of real-time quantitative PCR was established. In accord with SF data, IL-16 mRNA was detected at significantly higher levels within synovial tissue samples from RA patients compared to the control group of OA patients ($p < 0.001$). However, neither SF IL-16 concentrations nor IL-16 mRNA levels correlated significantly with clinical disease activity in patients with RA ($p = 0.49$, $p = 0.63$, respectively).

These results could be confirmed at the single cell level by *in situ* hybridization and immunohistochemistry: IL-16 expression was detected at both the mRNA and protein level in high abundance within the rheumatoid synovium. By combining *in situ* hybridization and immunohistochemistry for cell surface antigens on serial synovial tissue sections, IL-16 production was identified within the lining layer, as described[20], and within the inflammatory infiltrates of the sublining layer in a great proportion of CD4+ T cells, but also in CD8+ T cells. These findings correspond well with results of FACS analysis in PBMC after intracellular IL-16 staining, as described above. Given its function as a CD4+ cell-specific chemoattractant and competence growth factor[11,12], local production of IL-16 by infiltrating T lymphocytes within the rheumatoid synovium was thus considered to play a critical role in the pathogenesis of RA by chemoattraction of circulating lymphocytes. In the context of IL-16 mediated activation of lymphocytes and induction of IL-2 receptor (CD25) expression[12], local IL-16 expression could provide a proliferative stimulus further increasing lymphocyte accumulation. Assuming predominant proinflammatory functions for IL-16 in RA, a significant correlation between local IL-16 production and local inflammatory activity was hypothesized. To address this issue IL-16 expression was analyzed within the synovial cellular infiltrate by microscopic analysis. However, a positive correlation between the scores for local inflammation and IL-16 expression could not be proved ($r = 0.27$, $p = 0.19$).

Previous histopathological studies have reported different results for the relationship between histopathological changes and clinical disease activity: a close association between histopathologic features of rheumatoid synovial tissue and disease activity has been reported[34,35], but a significant reduction of synovial inflammation without clinical improvement[36] has also been described. In contrast, longitudinal studies have consistently shown a close association between the degree of synovial tissue macrophage infiltration, the expression of monokines (IL-6, TNF-α), and local disease activity[37]. These findings support the hypothesis that synovial tissue macrophages and monocyte derived cytokines play a central role in the pathogenesis of chronic inflammation and joint erosion in RA.

The failure to prove a significant relationship between IL-16 expression and both clinical and local inflammatory activity, in contrast with findings for monocyte derived proinflammatory cytokines, argues against a predominant proinflammatory function for IL-16 in the pathogenesis of RA. Due to the lack of correlation between disease activity and IL-16 expression it is proposed that IL-16 production might be controlled by local regulatory mechanisms or that IL-16 itself might exert additional regulatory function in chronic synovial inflammation. Recent studies document that IL-16 mediates pleiotropic functions: chemoattraction of CD4+ cells (T cells, eosinophils, monocytes)[11], activation of resting CD4+ T cells, as indicated by upregulation of HLA-DR antigens and IL-2R expression[15], and *in vitro* modulation of surface receptor expression and cytokine release in CD4+ monocytes[38] characterize proinflammatory activities of IL-16. However, there is emerging evidence for additional antiinflammatory functions of IL-16 as reflected by transient induction of CD4+ T cell anergy[18], inhibition of TCR/CD3 mediated T cell stimulation in mixed lymphocyte reactions[17], and suppression of mitogen induced IL-2 production[19]. In addition, adoptive transfer experiments in a SCID mouse model for human RA have recently revealed further immunosuppressive effects of IL-16[21]: synovial tissue derived autologous CD8+ T cells adoptively transferred in human synovium-SCID mouse chimeras were shown to induce a marked reduction of local IFN-γ, IL-1ß, and TNF-α production, an effect that could be blocked by antibodies against IL-16. Treatment of SCID mouse chimeras with human recombinant IL-16 led to a significant inhibition of proinflammatory cytokine production within the synovium. It is thus proposed that IL-16 may exert a regulatory function in chronic synovial inflammation by downmodulating further T cell activation. Negative feedback regulation of T cell activation in RA could explain impaired synovial T cell proliferation[5,6] and low level expression of other T cell derived cytokines[8] previously described in RA.

Evidence for antiinflammatory effects of IL-16 in SCID mice has prompted consideration of IL-16 as a novel therapeutic agent in RA. This would be based on the premise that the high levels of IL-16 in rheumatoid synovium are not quite sufficient to achieve its antiinflammatory effect. Further studies are required to define the role of IL-16 in the pathogenesis of RA and to predict its therapeutic potential in chronic RA.

Our data reveal that IL-16 is produced in abundance within the synovial tissue of patients with RA. Considering its proinflammatory properties IL-16 may promote recruitment

of CD4+ T cells into the rheumatoid synovium. However, the lack of correlation between IL-16 expression, local inflammatory activity, and clinical disease activity indicates that IL-16 is not a useful marker of inflammation in RA and might play a regulatory rather than a predominant proinflammatory role in the pathogenesis of RA.

REFERENCES

1. Harris ED. Rheumatoid arthritis. Pathophysiology and implications for therapy. N Engl J Med 1990;322:1277-89.
2. van Zeben D, Hazes JM, Zwinderman AH, et al. Association of HLA-DR4 with a more progressive disease course in patients with rheumatoid arthritis. Results of a follow-up study. Arthritis Rheum 1991;34:822-30.
3. Panayi GS, Lanchbury JS, Kingsley GH. The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. Arthritis Rheum 1992;35:729-35.
4. Firestein GS, Zvaifler NJ. How important are T cells in chronic rheumatoid synovitis? Arthritis Rheum 1990;33:768-73.
5. Allen ME, Young SP, Michell RH, Bacon PA. Altered T lymphocyte signaling in rheumatoid arthritis. Eur J Immunol 1995;25:1547-54.
6. Maurice MM, Lankester AC, Bezemer AC, et al. Defective TCR-mediated signaling in synovial T cells in rheumatoid arthritis. J Immunol 1997;159:2973-8.
7. Moreland LW, Pratt PW, Mayes MD, et al. Double-blind, placebo-controlled multicenter trial using chimeric monoclonal anti-CD4 antibodies, cM-T412, in rheumatoid arthritis patients receiving concomitant methotrexate. Arthritis Rheum 1995;38:1581-8.
8. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Ann Rev Immunol 1996;14:397-440.
9. Bucht A, Larsson P, Weisbrot L, et al. Expression of interferon-gamma, IL-10, IL-12 and transforming growth factor beta mRNA in synovial fluid cells from patients in the early and late phases of rheumatoid arthritis. Clin Exp Immunol 1996;103:357-67.
10. Isomäki P, Luukkainen R, Saario R, Toivanen P, Punnonen J. Interleukin-10 functions as an antiinflammatory cytokine in rheumatoid synovium. Arthritis Rheum 1996;39:386-95.
11. Cruikshank WW, Center DM. Modulation of lymphocyte migration by human lymphokines. II. Purification of a lymphotactic factor. J Immunol 1982;128:2569-74.
12. Cruikshank WW, Center DM, Nisar N, et al. Molecular and functional analysis of a lymphocyte chemoattractant factor: Association of biologic function with CD4 expression. Proc Natl Acad Sci USA 1994;91:5109-13.
13. Center DM, Kornfeld H, Cruikshank WW. Interleukin-16 and its function as CD4 ligand. Immunol Today 1996;17:476-81.
14. Cruikshank WW, Greenstein JL, Theodore AC, Center DM. Lymphocyte chemoattractant factor induces CD4-dependent intracytoplasmatic signaling in lymphocytes. J Immunol 1991;146:2928-34.
15. Parada NA, Center DM, Kornfeld H, et al. Synergistic activation of CD4+ T cells by IL-16 and IL-2. J Immunol 1998;160:2115-20.
16. Ryan TC, Cruikshank WW, Kornfeld H, Collins TL, Center DM. The CD4-associated tyrosine kinase $p56^{lck}$ is required for lymphocyte chemoattractant factor-induced T lymphocyte migration. J Biol Chem 1995;270:17081-6.
17. Theodore AC, Center DM, Nicoll J, Fine G, Kornfeld H, Cruikshank WW. CD4 ligand IL-16 inhibits the mixed lymphocyte reaction. J Immunol 1996;157:1958-64.
18. Cruikshank WW, Lim K, Theodore AC, et al. IL-16 inhibition of CD3-dependent lymphocyte activation and proliferation. J Immunol 1996;157:5240-8.
19. Ogasawara H, Takeda-Hirokawa N, Sekigawa J, Hashimoto H, Kaneko Y, Hirose S. Inhibitory effect of interleukin-16 on interleukin-2 production by CD4+ T cells. Immunology 1999;96:215-9.
20. Franz JK, Kolb SA, Hummel KM, et al. Interleukin-16, produced by synovial fibroblasts, mediates chemoattraction for CD4+ T lymphocytes in rheumatoid arthritis. Eur J Immunol 1998;28:2661-71.
21. Klimiuk PA, Goronzy JJ, Weyand CM. IL-16 as an anti-inflammatory cytokine in rheumatoid synovitis. J Immunol 1999;162:4293-9.
22. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31: 315-24.
23. Van der Heijde DM, van't Hof MA, van Riel PL, et al. Judging disease activity in clinical practice in rheumatoid arthritis: first step in the development of a disease activity score. Ann Rheum Dis 1990; 49:916-20.
24. Chupp GL, Wright EA, Wu D, et al. Tissue and T cell distribution of precursor and mature IL-16. J Immunol 1998;161:3114-9.
25. Blaschke S, Schwarz G, Moneke D, Binder L, Müller GA, Reuss-Borst MA. Epstein-Barr virus infection in peripheral blood mononuclear cells, synovial fluid cells, and synovial membranes of patients with rheumatoid arthritis. J Rheumatol 2000;27:866-73.
26. Tak PP, Kummer JA, Hack CE, et al. Granzyme-positive cytotoxic cells are specifically increased in early rheumatoid synovial tissue. Arthritis Rheum 1994;37:1735-43.
27. Blaschke V, Reich K, Middel P, et al. Expression of the CD4+ cell-specific chemoattractant interleukin-16 in mycosis fungoides. J Invest Dermatol 1999;113:658-63.
28. Eastgate JA, Symons JA, Wood NC, Grinlinton TM, di Giovine FS, Duff GW. Correlation of plasma interleukin-1 levels with disease activity in rheumatoid arthritis. Lancet 1988;2:706-9.
29. Vreugdenhil G, Lowenberg B, Van Eijk HG, Swaak AJ. Tumor necrosis factor alpha is associated with disease activity and the degree of anemia in patients with rheumatoid arthritis. Eur J Clin Invest 1992;22:488-93.
30. Okamoto H, Yamamura M, Morita Y, Harada S, Makino H, Ota Z. The synovial expression and serum levels of interleukin-6, interleukin-11, leukemia inhibitory factor, and oncostatin M in rheumatoid arthritis. Arthritis Rheum 1997;40:1096-105.
31. Laberge S, Cruikshank WW, Kornfeld H, Center DM. Histamine-induced secretion of lymphocyte chemoattractant factor from CD8+ T cells is independent of transcription and translation: evidence for constitutive protein synthesis and storage. J Immunol 1995;155:2902-10.
32. Zhang Y, Center DM, Wu DM, et al. Processing and activation of pro-interleukin-16 by caspase-3. J Biol Chem 1998;273:1144-9.
33. Wu DM, Zhang Y, Parada NA, et al. Processing and release of IL-16 from CD4+ but not CD8+ T cells is activation dependent. J Immunol 1999;162:1287-93.
34. Waxman BA, Sledge CB. Correlation of histochemical, histologic and biochemical evaluations of human synovium with clinical activity. Arthritis Rheum 1973;16:376-82.
35. Rooney M, Whelan A, Feighery C, Bresnihan B. The immunohistologic features of synovitis, disease activity and in vitro IgM rheumatoid factor synthesis by blood mononuclear cells in rheumatoid arthritis. J Rheumatol 1989;16:459-67.
36. Tak PP, van der Lubbe PA, Cauli A, et al. Reduction of synovial inflammation after anti-CD4 monoclonal antibody treatment in early rheumatoid arthritis. Arthritis Rheum 1995;38:1457-65.
37. Tak PP, Smeets TJM, Daha MR, et al. Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity. Arthritis Rheum 1997;40:217-25.
38. Hermann E, Darcissac E, Idziorek T, Capron A, Bahr GM. Recombinant interleukin-16 selectively modulates surface receptor expression and cytokine release in macrophages and dendritic cells. Immunology 1999;97:241-8.