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Chemokine Secretion of Rheumatoid Arthritis Synovial Fibroblasts Stimulated by Toll-Like Receptor 2 Ligands

Matthias Pierer,* Janine Rethage,* Reinhart Seibl,* Roger Lauener,† Fabia Brentano,* Ulf Wagner,† Holm Hantzschel,‡ Beat A. Michel,* Renate E. Gay,* Steffen Gay,* and Diego Kyburz‡*

To analyze the role of Toll-like receptors (TLR) in the pathogenesis of rheumatoid arthritis, we have assessed the effects of stimulation of cultured synovial fibroblasts by the TLR-2 ligand bacterial peptidoglycan. By using high density oligonucleotide microarray analysis we identified 74 genes that were up-regulated >2.5-fold. Fourteen CC and CXC chemokine genes were among the genes with the highest up-regulation. Quantitative real-time PCR analysis confirmed up-regulation of granulocyte chemotactic protein (GCP)-2, RANTES, monocyte chemotactic protein (MCP)-2, IL-8, growth-related oncogene-2, and to a lesser extent, macrophage-inflammatory protein 1α, MCP-1, EXODUS, and CXCL-16. GCP-2, RANTES, and MCP-2 were detected in culture supernatants of synovial fibroblasts stimulated with peptidoglycan. Chemokine secretion induced by stimulation of rheumatoid arthritis synovial fibroblasts via TLR-2 was functionally relevant as demonstrated by chemotaxis assays. GCP-2 and MCP-2 expression, which have not been reported previously in rheumatoid arthritis, was demonstrated in synovial tissue sections of patients diagnosed with rheumatoid arthritis but not in those with osteoarthritis. Correspondingly, synovial fluid levels were significantly higher in patients diagnosed with rheumatoid arthritis as compared with osteoarthritis. Thus, we present evidence for an induction of chemokine secretion by activation of synovial fibroblasts via TLR-2, possibly contributing to the formation of inflammatory infiltrates characteristically found in rheumatoid arthritis joints.


The activation of the innate immune system via specialized pattern-recognition receptors by conserved bacterial structures like LPS, peptidoglycan (PGN) and bacterial lipopeptide (BLP) is thought to play a major role in host-pathogen interactions. Toll-like receptors (TLRs) have been shown to be important for the recognition of bacterial components (1). Among 10 members of the human TLR family described so far, TLR-2 was identified as the receptor for Gram-positive cell wall components like PGN and BLP (2–4). Although for most TLR the mode of activation is not fully understood, the direct binding of PGN to the extracellular domain of TLR-2 has been demonstrated recently (5).

The role of TLRs in the pathogenesis of arthritis is not well understood. However, the presence of bacterial PGN in joints of patients who have rheumatoid arthritis (RA) raises the possibility that TLRs may be involved in the pathogenesis of RA (6–8). In animal models of RA-like streptococcal cell wall arthritis (9) and adjuvant arthritis (10), bacterial components induce arthritis. Intriguingly, in rats a single i.p. injection of a Gram-positive bacterial cell wall preparation induces a chronic arthritis closely resembling human RA (9, 11, 12). In mice, a recent report has shown that severe destructive arthritis develops after intra-articular injection of bacterial PGN (13). These data suggest that PGN can give rise to an inflammatory reaction locally in the joint in the absence of live bacteria.

Notably, TLRs have been shown to bind endogenous molecules present in human tissue in the absence of bacterial infection, which implicates an additional role for TLR in autoimmune processes. TLR-2 has been identified to activate cells in response to heat shock protein 70 (14) and necrotic cells (15), both of which have been detected in RA synovial tissue (16).

Recently, we have reported the expression of functional TLR-2 in synovial fibroblasts of patients who have RA (17, 18). The aim of this study is to characterize TLR-2 inducible genes by using high-density oligonucleotide microarray techniques. The results indicate that RA synovial fibroblasts respond to TLR-2 ligands by a strong up-regulation of various CC and CXC chemokines. Moreover, supernatants of RA synovial fibroblasts stimulated with PGN were found to attract PBMCs. To infer whether this activation pathway might be operative in RA, we investigated synovial fluids of patients with different forms of arthritis and synovial tissue from RA and osteoarthritis (OA) patients. Granulocyte chemotactic protein (GCP)-2 and monocyte chemotactic protein (MCP)-2, two of the most strongly induced chemokines that have not been investigated in arthritis previously, were found in synovial fluids of RA patients in significantly higher levels as compared with OA or other forms of arthritis. Immunohistochemical studies of synovial biopsies from RA patients revealed synovial fibroblasts as major sources of both MCP-2 and GCP-2. Thus, RA synovial fibroblasts activated via TLR-2 produce chemokines that may contribute to arthritis induction and/or maintenance.

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Materials and Methods

Patients and controls

Synovial fluid samples were obtained during arthrocentesis from the knee joints of patients diagnosed as having either RA (n = 17; mean age 63 years, range 51 to 78 years) or OA (n = 10; mean age 65 years, range 48–84 years), and from patients who have other types of inflammatory arthritides (reactive arthritis, psoriatic arthritis, spondylarthropathies, or gout) (n = 12; mean age 48 years, range 29 to 58 years). Synovial fluid was collected and centrifuged at 2000 × g for 10 min to remove cells and debris. Aliquots were frozen at −70°C until analyzed. Synovial tissue was obtained from patients undergoing total joint replacement surgery. RA patients were diagnosed according to the American College of Rheumatology (formerly, the American Rheumatism Association) criteria (19). All patient samples were collected at the Department of Rheumatology, University of Zürich, Zürich, Switzerland, according to the guidelines of the Institutional Review Board.

Cell culture and medium

Synovial tissues were obtained from patients who had RA and OA undergoing synovectomy or joint replacement. Immediately after surgery, the synovial tissue was minced and digested with dispase at 37°C for 60 min. Gouty synovial tissue was minced and digested with dispase at 37°C for 120 min (20). Immediately after surgery, the synovial fluid was collected and centrifuged at 37°C for 60 min. After washing, the cells were grown in DMEM (Life Technologies, Basel, Switzerland) supplemented with 10% FCS, 50 IU/ml penicillin-streptomycin, 2 mM l-glutamine, 10 mM HEPES, and 0.2% fungizide (all Life Technologies). Cell cultures were maintained in a 5% CO₂-humidified atmosphere (data not shown).

Stimulation assays and oligonucleotide microarray experiments

Staphylococcus aureus PGN (Fluka, Buchs, Switzerland) and synthetic BLP (palmitoyl-Cys(RS)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly OH; Boehringer Mannheim, Rotkreuz, Switzerland) were used as TLR-2 ligands and were tested routinely for endotoxin using the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD). Endotoxin levels did not exceed 0.06 EU/ml in all tested samples. For the array experiments, two patient-derived RA synovial fibroblasts were grown in 75 cm² cell culture flasks and stimulated for 8 h with 10 μg/ml PGN. Total RNA was extracted and labeled with the Enzo BioArray HighYield RNA labeling kit (Enzo Diagnostics, Farmingdale, NY). The labeled RNA was hybridized on four patient-derived RA synovial fibroblast arrays used requiring multiplication of raw signals by a scaling factor to equalize concentrations of Bay 11-7082 used for the inhibition assays as demonstrated by NX. 10, 100, and 1000 μg/ml Bay 11-7082 used for the inhibition assays as demonstrated by NX.

Data analysis

Analysis was performed as previously described for Affymetrix microarray data (21). The normalization procedure recommended by Affymetrix was used requiring multiplication of raw signals by a scaling factor to equalize the trimmed mean (250, excluding 2% highest and 2% lowest) of the signals. Pair wise comparisons between data derived from PGN-stimulated versus unstimulated cell populations (n = 2) were performed, generating two comparisons by using the Affymetrix GeneChip Analysis Suite v5.0. An average change, achieved in both pair wise comparisons, of ≥2-fold was used as the cutoff for significant differences in gene expression. This criterion was chosen because changes of this magnitude were routinely validated in the American College of Rheumatology (22, 23). Identified genes of 12,000 named genes were analyzed using data mining and http://www.netaffx.com software tools (both Affymetrix).

Real-time RT-PCR

For the quantification of chemokine mRNA, total RNA was isolated using the RNeasy MiniPrep kit (Qiagen, Basel, Switzerland), reverse transcribed with random hexamers, and amplified by real-time PCR using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Rotkreuz, Switzerland). The primers for chemokines were designed using Primer Express software v1.0 (Perkin-Elmer Applied Biosystems) as are listed below. The endogenous control 18S cDNA was used for correcting the results with the comparative threshold cycle (Ct) method for relative quantification as described by the manufacturer. The differences of Ct values of sample and 18S cDNA were calculated (ΔCt). Relative expression levels were calculated following the formula ∆ΔCt = ∆Ct (sample − ΔCt (sample unstimulated)) the value used to plot relative expression levels was calculated using the expression 2−ΔΔCt. Dissociation analysis for each primer pair and reaction was performed to verify specific amplification.

Analysis of migrated cells was performed in companion cell culture experiments with cell culture inserts with microporous membrane by using flow cytometry as previously described (24). Supernatants of PGN-stimulated RA synovial fibroblast cultures were added to companion 24-well plates, and cell culture inserts were treated with 10 μg/ml PGN or left untreated for 24 or 48 h. Supernatants were collected and added to the lower chamber. For the unstimulated cultures PGN was added to the supernatants at a final concentration of 10 μg/ml. Aliquots (25 μl) of PBMCs from healthy volunteers isolated by Ficoll-Hypaque density gradient centrifugation resuspended in DMEM/10% FCS at a concentration of 10^6/ml were placed in the upper chamber. After incubation for 2 h at 37°C and 5% CO₂, the membrane was removed, washed with PBS, and nonmigrated cells on the upper surface of the membrane were removed. Cells were fixed in methanol for 5 min, stained with DiffQuick (Merck, Darmstadt, Germany), and mounted on glass slides. Cells on the lower surface were counted in four randomly selected high-power fields (magnification ×400) per group. V-FMLP (Sigma-Aldrich) at a concentration of 10 nM was used as a positive control. An insignificant number of cells was observed in the lower chamber at this time point and was not included in the analysis. Each experiment was performed in triplicate and repeated at least twice.

Analysis of migrated cells was performed in companion cell culture experiments with cell culture inserts with microporous membrane by using flow cytometry as previously described (24). Supernatants of PGN-stimulated RA synovial fibroblast cultures were added to companion 24-well plates, and 1 × 10^6 PBMC were placed in polyethylene terephthalate membrane cell culture inserts with high density 5.0 μm pores (both BD Biosciences, Basel Switzerland) as upper chamber. After incubation for 60 min, the transmigrated cells were harvested from the bottom well and labeled with PerCP-conjugated anti-CD4 and FITC-conjugated anti-CD14 Abs (BD
Biosciences) for flow cytometry. Cells were then analyzed on a FACS-Calibur using CellQuest software (BD Biosciences) and total cell counts were determined. For inhibition of the chemotactic effect, Abs against RANTES, GCP-2 (R&D Systems), or MCP-2 (Research Diagnostics, Flanders, NJ) were added at a final concentration of 2 μg/ml medium. Supernatants of four different RA synovial fibroblast and three different OA synovial fibroblast cultures were analyzed in duplicate.

Immunohistochemistry

The synovial tissue was collected from RA or OA patients undergoing joint replacement surgery. Immediately after surgery, the tissue was fixed in paraformaldehyde and embedded in paraffin. Subsequently, paraffin-embedded sections of 3 μm were made. Ag retrieval pretreatment was performed in a microwave oven using 10 mM citrate buffer, pH 6.0. Slides were then treated with 0.6% hydrogen peroxide in methanol to deplete endogenous peroxidase activity and avidin blocking according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). After pretreatment, sections were reacted with affinity-purified goat polyclonal Abs against human GCP-2 (sc-5813; Santa Cruz Biotechnology, Santa Cruz, CA) and affinity-purified goat polyclonal Abs against human MCP-2 (sc-1307; Santa Cruz Biotechnology) for 12 h at 4°C. After primary Ab reaction, the sections were incubated with biotinylated rabbit anti-goat IgG for 1 h, followed by incubation for 20 min with peroxidase-labeled streptavidin. To identify monocytes and macrophages, some slides were additionally incubated with mouse anti-human CD68 (clone PG-M1, IgG; DAKO, Carpinteria, CA) at 0.5 μg/ml for 1 h, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG Ab for 1 h and mouse alkaline phosphatase-conjugated anti-AP Abs (both from DAKO) for 30 min. Peroxidase-labeled cells were visualized using 3-ami-no-9-ethylcarbazole- and alkaline phosphatase-labeled cells using nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (both DAKO). As negative control goat anti-GCP-2 or anti-MCP-2 Abs were preincubated for 1 h with the peptide against which they were raised. Isotype-specific IgG was used as a negative control for the anti-CD68 staining. Various cell types were identified, including macrophages, lymphocytes, fibroblasts, endothelial cells, and dendritic cells, by immunohistochemical staining reactions and/or morphologic features. Synovial tissue components including lining cells and sublining cells including macrophages, lymphocytes, and fibroblasts were graded for immunostaining by a frequency of staining scale, scored 0–100%, in which 0% indicates no staining and 100% indicates that all cells were immunoreactive. Five fields (×400) were examined per section by two observers in a blinded fashion.

Statistical analysis

Values are presented as the mean ± SEM. The significance of the results was analyzed using Student’s two-tailed t test or Mann-Whitney U test where appropriate. Values for p < 0.05 were considered significant.

### Results

Gene array-based analysis of TLR-2-induced gene expression in RA synovial fibroblasts

To identify the gene expression changes in RA synovial fibroblasts in response to activation of TLR-2-dependent pathways we used high-density microarray-based analysis of gene expression. After 8 h of culture in the presence or absence of 10 μg/ml PGN in two different RA synovial fibroblast cultures, RNA was extracted, labeled, and hybridized onto four single Human Genome U95Av2 oligonucleotide probe arrays (Affymetrix). The concentration of PGN used has previously been described to efficiently stimulate RA synovial fibroblasts (18).

When a cutoff of a 2.5-fold change in RNA expression was used, 74 genes were up-regulated in both comparisons (±0.6% of the genes monitored). Among these genes up-regulated in the two biological replicates, 14 genes encoding chemokines were found (Fig. 1). In both comparisons, four chemokines were among the top-regulated chemokines.

### Table 1. Up-regulation of chemokine gene expression of RA synovial fibroblasts incubated with PGN

<table>
<thead>
<tr>
<th>Signal Log Ratio</th>
<th>Original Name</th>
<th>Systematic Name</th>
<th>Gene Name</th>
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<td>SCYB6</td>
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</tr>
<tr>
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<td>SCYB2</td>
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<tr>
<td>1.73</td>
<td>CXL16</td>
<td>CXL16</td>
<td>SRPSOX</td>
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</tbody>
</table>

**FIGURE 1.** Relative gene expression of PGN-treated and untreated RA synovial fibroblasts measured by microarrays. Of 12,000 named genes present on Hu95Av2 microarrays only the genes with at least one “present call” are shown. The average gene expression in the PGN-stimulated hybridizations is plotted on the x-axis, and the average change signal log ratio for each individual gene on the y-axis. The change signal log ratio was calculated with Microarray Suite software. Data for individual genes are averaged across four possible chip data comparisons. ▲, Significantly up-regulated chemokine genes.

**FIGURE 2.** Relative chemokine gene expression measured by real-time PCR. RA synovial fibroblasts were incubated for 12 h in the presence or absence of 10 μg/ml PGN. Expression of mRNA of the indicated chemokines was measured by real-time PCR. RNA yield differences were calculated using 18S RNA as endogenous control and the comparative C T method for relative quantification as described in Materials and Methods. Mean values of up-regulation of mRNA expression in PGN-treated as compared with untreated cultures and SEM are shown from five different RA synovial fibroblast cell lines.
five most strongly up-regulated genes (signal log ratio 10.2–7.6, Table I).

Besides the most strongly up-regulated group of chemokine genes, the remainder of genes whose expression levels differed in both experiments by ≥2.5-fold include enzymes (cyclooxygenase-2, matrix metalloproteinase 1) or enzyme inhibitors (α,α-antitrypsin), growth factors (insulin-like growth factor 1), signaling molecules (extracellular signal-regulated kinase 2), and various transcription factors (NF-κB, p50, STAT1, STAT4). Increased levels of these genes may affect a number of additional processes relevant to inflammation and tissue remodeling. Interestingly, only a modest down-regulation of genes occurred following PGN stimulation of RA synovial fibroblasts (Fig. 1). The marked up-regulation of several chemokine genes prompted us to study their expression in RA synovial fibroblasts stimulated with bacterial cell wall components.

A panel of nine chemokines was chosen for verification by real-time quantitative PCR. Total mRNA from primary cultures of synovial fibroblasts from six patients who have RA either stimulated with PGN for 12 h or left untreated was extracted and analyzed. The mRNAs for GCP-2, RANTES, and MCP-2 were found to be up-regulated >100-fold, growth-related oncogene-2 and IL-8 up-regulated 50–100-fold, whereas MCP-1, MIP-1α, EXODUS, and CXCL-16 were up-regulated in the range from 2- to 4-fold (Fig. 2), confirming the results of the oligonucleotide array experiments.

Different ligands of TLR-2 induce RA synovial fibroblasts to secrete GCP-2, RANTES, and MCP-2

To characterize the chemokine response of synovial fibroblasts to TLR-2 stimulation by PGN, levels of secreted chemokines were determined by ELISA. GCP-2, RANTES, and MCP-2 were chosen because they were up-regulated most strongly on the mRNA level and potentially attract granulocytes, monocytes, and T cells that are usually found in inflamed joints. The response of synovial fibroblasts derived from patients who have RA (n = 5) and OA (n = 4) was analyzed. Because there is evidence that TLR-2 forms heterodimers dependent on the stimulating ligand, PGN and BLP were used as TLR-2 engaging agonists because these ligands induce cellular responses via different cooperations of TLR-2 with TLR-1 or TLR-6 (25, 26). A total of 5 × 10^5 RA or OA synovial fibroblasts were stimulated with PGN or BLP for 24 h at a concentration of 10 μg/ml. Levels of GCP-2, which were up-regulated most strongly both in the microarray and real-time PCR experiments, were also up-regulated most strongly upon stimulation with PGN (4263 pg/ml vs 47 pg/ml untreated, p < 0.001) and BLP (3244 pg/ml, p < 0.001) (Fig. 3A). RANTES as well as MCP-2 were up-regulated upon TLR-2 stimulation with PGN 46-fold (1546 vs 33 pg/ml, p < 0.001, Fig. 3B) and 159-fold (893 vs 5.6 pg/ml, p < 0.001, Fig. 3C), respectively. Treatment of identical numbers of OA synovial fibroblasts with PGN or BLP resulted in a slightly lower up-regulation of all tested chemokines, however, reaching statistical significance only for the up-regulation of RANTES stimulated with PGN and BLP (Fig. 3B).

Inhibition of NF-κB activation reduces TLR-2-dependent up-regulation of RANTES, GCP-2, and MCP-2

Bay 11-7082, an inhibitor of IκB phosphorylation, and NF-κB activation (27) was used to determine whether chemokine induction by PGN is NF-κB dependent. Concentrations of Bay 11-7082 between 1.0 and 5 μM were used according to published studies (28). RA synovial fibroblasts were pretreated with Bay 11-7082 for 30 min, washed and subsequently stimulated with PGN for 4 h. Total RNA was then extracted and RANTES, GCP-2, and MCP-2 mRNA quantified by real-time PCR. As shown in Fig. 4, there was a dose-dependent decrease of mRNA levels of the tested chemokines ranging from marked reduction upon treatment with 1 μM Bay 11-7082 to almost complete inhibition in the RA synovial fibroblasts pretreated with 5 μM Bay 11-7082. These results indicate that NF-κB activation by IκB kinase is necessary for the PGN-induced up-regulation of chemokines in RA synovial fibroblasts.

**FIGURE 3.** Chemokine secretion of PGN- and BLP-stimulated RA synovial fibroblasts (GCP-2, RANTES, and MCP-2). RA and OA synovial fibroblasts were incubated with 10 μg/ml PGN, 10 μg/ml BLP, or medium alone. After 24 h, supernatants were collected and levels of GCP-2 (A), RANTES (B), and MCP-2 (C) were determined by sandwich ELISA. RA synovial fibroblasts (■) and OA synovial fibroblasts (□) differed significantly in their production of RANTES in response to PGN (B). The mean and SEM were calculated from three independent experiments with five different RA synovial fibroblast cultures and three different OA synovial fibroblast cultures. *, p < 0.001 vs control; #, p < 0.05.
Supernatants of PGN-stimulated RA synovial fibroblast cultures have a chemotactic effect

To analyze the biologic activity of the PGN-induced chemokine response of fibroblasts, PGN-conditioned supernatants of various RA synovial fibroblast cultures were tested for their chemotactic effect on PBMC in a modified Boyden Chamber assay. In assays with supernatants from untreated fibroblasts, a mean number of 16 cells (SD ± 6.2) per high-power field were found to have migrated through the membrane pores and adhered to the lower side. Addition of supernatants from PGN-stimulated RA fibroblasts for 24 h increased this number to a mean of 42.6 cells (SD ± 7.6, p < 0.001). This chemoattractive effect of PGN-elicited supernatants was even more pronounced after a 48-h incubation time of RA supernatants (mean 80.2 cells, SD ± 35.0) were significantly higher than concentrations in synovial fluids taken from knee joints of patients who have either RA (n = 17), OA (n = 10), or other types of inflammatory arthritides (total n = 12; psoriatic arthritis (n = 4), reactive arthritis (n = 3), spondylarthropathy (n = 3), and gout (n = 2)) were analyzed. GCP-2 concentrations as measured by sandwich ELISA showed highest concentrations in synovial fluid of RA patients (mean 1159.8 pg/ml, SEM ± 342) compared with OA patients (mean 26 pg/ml, SEM ± 6.25, p < 0.002 vs RA) and concentrations found in the group of patients diagnosed as having other inflammatory arthritides (mean 272.2 pg/ml, SEM ± 70.5, p < 0.05 vs RA, Fig. 6A). Also, the concentrations of MCP-2 detected in synovial fluids from patients who have RA (mean 207 pg/ml, SEM ± 35.0) were significantly higher than concentrations in synovial fluids from patients who have OA (mean 2.8 pg/ml, SEM ± 1.9, p < 0.001) and the concentrations found for other inflammatory arthritides (mean 72.1 pg/ml, SEM ± 25.9, p < 0.008, Fig. 6B).

GCP-2 and MCP-2 in synovial tissues of RA and OA patients

The presence and cellular distribution of GCP-2 and MCP-2 was analyzed in tissue sections from RA synovial membranes and control synovial tissues from OA patients by immunohistochemistry. GCP-2 and MCP-2 could be detected in synovial membranes of patients who have RA, whereas in tissue sections from patients

FIGURE 4. Inhibition of NF-κB activation reduces the expression of chemokine genes dose dependently. RA synovial fibroblasts were pretreated with increasing concentrations of IκB phosphorylation inhibitor Bay 11-7082 for 30 min and then stimulated with PGN (10 μg/ml) for 4 h at 37°C. The cells were lysed and RNA was extracted, followed by RT-PCR. The results for RANTES (A), GCP-2 (B), and MCP-2 (C) are shown. The data are mean ± SEM of four independent experiments. *, p < 0.001 vs control; #, p < 0.05.
who have OA, cells expressing either GCP-2 or MCP-2 were virtually absent (Fig. 7 and OA data not shown). In RA tissues, GCP-2-expressing cells were abundantly present in the lining layer, but also in cell-rich sublining regions and in close proximity to vessel walls. GCP-2 expression was found in all five investigated tissues derived from RA patients mainly in CD68-negative cells, identified as synovial fibroblasts by morphological criteria, but also CD68-positive macrophages showed GCP-2 expression to some extent (Fig. 7C). In the lining layer almost all cells (94%, SD ± 7.8) were immunopositive for GCP-2, whereas sublining areas had a mean of 56% (SD ± 22.4) positive cells (Table II, Fig. 7A). MCP-2 was also expressed in all tissues from RA patients and virtually absent in OA tissues. It was detected in 74% (SD ± 12.7) of the lining cells and 52.5% (SD ± 14.8) of the sublining cells and in the vascular endothelial cells of rheumatoid synovia (Table II and Fig. 7B). As seen for GCP-2 immunoreactive cells, a majority of MCP-2-positive cells both in the lining and sublining were not expressing CD68 (Fig. 7D), indicating that these cells are mainly fibroblasts.

**Discussion**

RA is characterized by chronic inflammation and destruction of joints. Infiltrating leukocytes can be found in the joint fluid as well as in the synovial tissue. Adhesion molecules and chemokines direct the migration of leukocytes from the peripheral blood to the joints. Various chemokines have been detected in synovial fluids and synovial tissue of RA patients and monocytes as well as fibroblasts have been identified as sources of chemokines (29–34). Chemokine expression of RA synovial fibroblasts is induced by the cytokines TNF-α, IL-1, and IL-18 (30, 35–37). Our studies provide evidence for an alternative pathway of induction of chemokine secretion via TLR-2.

The TLRs are a family of pattern recognition receptors, recognizing conserved microbial structures. TLRs expressed on APCs

![FIGURE 5. Chemotactic activity of PGN-stimulated RA synovial fibroblast supernatants.](http://www.jimmunol.org/)

![FIGURE 6. Concentrations of GCP-2 and MCP-2 in synovial fluid samples.](http://www.jimmunol.org/)
FIGURE 7. Expression patterns of MCP-2 and GCP-2 protein in synovial tissues from patients who have RA. Immunohistochemistry was performed with goat anti-human GCP-2 and goat anti-human MCP-2 polyclonal Abs. Cells immunopositive for GCP-2 or MCP-2 were stained with 3-amino-9-ethylcarbazole (red color). A and C, Synovial tissue from a patient diagnosed with RA showing positive staining for GCP-2. B and D, Synovial tissue from a patient diagnosed with RA showing positive staining for MCP-2. For the identification of macrophages, mouse anti-human CD68 Abs were used and CD68 immunopositive cells were counterstained with nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (dark-purple color) (C and D). Negative control staining in RA synovial tissue using polyclonal Abs against GCP-2 and MCP-2 preincubated for 1 h with the respective blocking peptides (E and F) is shown. Hematoxylin counterstaining is indicated in A and B and in E and F. Original magnification \( \times 100 \) (A, B, E, and F) and \( \times 200 \) (C and D).
have a determining influence on adaptive immune responses by
influencing Th cell differentiation (38). However, TLRs are also
expressed in a variety of nonimmune cells, where their function
is less well understood. We have recently shown that TLR-2 is ex-
pressed in the synovial tissue of RA patients and that RA synovial
fibroblasts express functional TLR-2 (17, 18). Stimulation of RA
synovial fibroblasts via TLR-2 resulted in up-regulation of proin-
flammatory cytokines and matrix metalloproteinases. To further
clarify the consequences of recognition of bacterial products by
synovial fibroblasts, we analyzed gene expression changes of RA
synovial fibroblasts after stimulation with the TLR-2 ligand PGN.
PGN was chosen because it was a potent stimulus in our previous
experiments and binds directly to TLR-2 (5). Moreover, bacterial
PGN has also been detected in synovial membranes of patients
who have RA in different studies (6–8). Using an oligonucleotide
array technique we demonstrate up-regulation of >2.5-fold of a
total of 74 genes. Fourteen of those were chemokine genes, of
which four showed the highest induction of all 12,000 analyzed
genes. Real-time PCR analysis validated the up-regulation of nine
chemokines upon PGN-mediated TLR-2 stimulation. This indi-
cates that TLR-2 stimulation of RA synovial fibroblasts results
predominantly in the production of chemokines, similarly to what
has previously been described for monocytes (39).

PGN induced chemokines of both major classes CXC and CC.
In our experiments, mRNA of the CXC chemokines GCP-2, IL-8,
GRO-1, GRO-2, GRO-3, and epithelial neutrophil-activating pep-
tide 78 were up-regulated by PGN stimulation of RA synovial
fibroblasts. IL-8, GRO-1, and epithelial neutrophil-activating pep-
tide 78 were previously detected in synovial fluids and tissue of
patients who have RA at increased levels as compared with OA
(30, 40, 41). As a consequence neutrophils expressing CXCR1
accumulate in large numbers in the synovial fluid, a characteristic
feature of RA (42). Moreover, CXC chemokines have angiogenic
as well as angiostatic properties. Activation of RA synovial fi-
broblasts via TLR-2 resulted in a strong up-regulation of angiogenic
CXC chemokines but not angiostatic ones, indicating that RA syn-
ovial fibroblasts may contribute to the angiogenic potential asso-
ciated with RA (43, 44). The importance of CXC chemokines in
RA is supported by studies of animal models of arthritis establish-
ing the efficacy of therapeutic strategies aimed at blocking the
binding of CXC chemokines to CXCR1/2 (45).

In addition to CXC chemokines, the CC chemokines MCP-2,
RANTES, EXODUS, MCP-4, MIP-1α, MCP-1, and EOTAXIN
were found to be up-regulated in response to stimulation via
TLR-2. CC chemokines attract a variety of different cells including
monocytes, basophils, eosinophils, and T cells (46, 47). Previous
studies of CC chemokine receptor expression in RA have detected
CC receptors 1–3 and 5 expressing monocytes/macrophages in the
peripheral blood and synovial compartment (48, 49). The mecha-
nisms involved in the T cell infiltration are less well understood
but several of the chemokines found to be up-regulated have been
shown to attract different populations of T cells (50–57). Similarly
as with the inhibition of CXC chemokines, CC chemokine inhibi-
tors had beneficial effects in animal models of RA (58). Minor
changes of the molecular structure of chemokines can severely
influence their biological activity (41, 42), because truncated forms
have been shown to be biologically inactive. To assess the biologic
relevance of the chemokine production of RA synovial fibroblasts,
supernatants were tested in Boyden chamber chemotaxis assays.
Stimulation of RA synovial fibroblasts with PGN resulted in a
significantly increased chemotactic activity for PBMC, suggesting
that the levels of chemokines are physiologically relevant. Analy-
isis of the migrated cells showed that both lymphocytic and mono-
cytes are attracted by PGN-stimulated RA synovial fibroblast
supernatants. A detailed investigation of the three most strongly
up-regulated chemokines indicated that both MCP-2 and RANTES
are of major importance for the attraction of CD14-positive mono-
cytes because addition of the respective blocking Abs to the cul-
tures resulted in a significant reduction of chemotaxis. However,
migration of CD4-positive T cells was only reduced by blocking
Abs against RANTES. Expression of RANTES in RA has been
described elsewhere, whereas the expression of GCP-2 and MCP-2
in RA has not been addressed so far. GCP-2 as well as MCP-2
were found in synovial fluids and synovial tissue of RA patients
at significantly higher levels as compared with patients diagnosed
with OA or with other arthritides. Immunohistochemistry estab-
lished CD68-negative fibroblast-like cells as well as CD68-positi-
ve macrophages as a source of GCP-2 and MCP-2.

Cultured RA and OA synovial fibroblasts both up-regulated chem-
omine expression on stimulation via TLR-2 to a similar extent for
GCP-2 and MCP-2 but RANTES concentrations were significantly
higher in RA synovial fibroblast culture supernatants. However,
when chemotactic activity of supernatants of stimulated RA and
OA synovial fibroblasts was compared, no significant differences
were seen, indicating that the reactivity of these cells to TLR-2
ligands in vitro is essentially the same. These in vitro data, how-
ever, contrast the significantly higher levels of GCP-2 and MCP-2
in RA synovial fluid as compared with OA indicating a role of
these chemokines in RA synovitis. The latter is supported by the
virtual absence of GCP-2 and MCP-2 staining in synovial tissues
derived from OA patients.

In monocytic cells TLR-2 signaling results in the activation of
NF-κB (59). We have investigated this pathway by pretreatment of
RA synovial fibroblasts with the highly specific inhibitor of IκB
kinase phosphorylation Bay 11-7082. Addition of Bay 11-7082
reduced dose dependently the levels of PGN-induced RANTES,
GCP-2, and MCP-2 mRNA. When a concentration of Bay 11-
7082 that has previously been shown to block completely NF-κB
activation (27) was used, also the induction of chemokines was
nearly abolished, indicating that NF-κB activation is indispensable
for TLR-2-induced chemokine production in RA synovial
fibroblasts.

The time course of chemokine expression with an early induc-
tion of chemokine mRNA within 4 h suggests that TLR-2 signal-
ing directly activated the chemokine genes. However, we cannot
rule out that chemokine secretion following TLR-2 stimulation of
RA synovial fibroblasts is dependent on the production of cyto-
kines by the same cells. Nevertheless, our results with in vitro
cultures clearly show that the chemokine production of synovial
fibroblasts is not dependent on the presence of other cells, such as
monocytes or lymphocytes, as a source of stimulating cytokines.

Taken together we demonstrate that RA synovial fibroblasts
produce various chemokines upon stimulation with the TLR-2 ag-
onist PGN. Although the potential of RA synovial fibroblasts to
synthesize chemokines is known, our results extend these previous

Table II. Percentages of GCP-2- and MCP-2-expressing cells in tissues
from patients diagnosed with RA and OA

<table>
<thead>
<tr>
<th></th>
<th>GCP-2-Positive Cells, %</th>
<th>MCP-2-Positive Cells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lining layer</td>
<td>92.6 ± 7.8</td>
<td>74 ± 12.7</td>
</tr>
<tr>
<td>Sublining layer</td>
<td>56.0 ± 22.4</td>
<td>52.5 ± 14.8</td>
</tr>
<tr>
<td>OA (n = 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lining layer</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Sublining layer</td>
<td>0.5 ± 0.58</td>
<td>0.32 ± 0.51</td>
</tr>
</tbody>
</table>

* Mean percentages ± SD.
results showing an astonishing variety of different chemokines in stimulated RA synovial fibroblasts. Cytokines such as IL-1β, TNF-α, and IL-18, derived from infiltrating monocytes or T cells stimulate synovial fibroblasts to secrete chemokines. However, the expression of chemokines at early stages of joint inflammation (60) argues for additional mechanisms of induction. In this study, we propose direct activation of RA synovial fibroblasts via TLR-2 resulting in chemokine secretion. Bacterial products or as yet undefined endogenous ligands of TLR-2 present in the joints might thereby initiate the accumulation of inflammatory cells in the joints, suggesting an involvement of RA synovial fibroblasts at a very early stage of the disease as part of the innate immune system. Our results support strategies targeting TLR-2 signaling pathways to inhibit joint inflammation in patients diagnosed with RA.

References


CORRECTIONS


The fourth author’s first name is incorrect. The correct first name is Christian.


In *Results*, the heading for the third column of Table I, “Mean Surface Marker Expression,” is incorrect. The heading for column three should read “LL-37-derived iDC/mDC.” All the data are correct in the table legend, *Materials and Methods*, and *Results* as originally published.


In *Results*, Figure 4 was printed in error in place of Figure 5. The figure legend is correct as originally published. The correct Figure 5 is shown below.