Overexpression of Toll-like Receptors 3 and 4 in Synovial Tissue From Patients With Early Rheumatoid Arthritis

Toll-like Receptor Expression in Early and Longstanding Arthritis

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Objective. To analyze the expression, regulation, and biologic relevance of Toll-like receptors (TLRs) 1–10 in synovial and skin fibroblasts and to determine the expression levels of TLRs 2, 3, and 4 in synovial tissues from patients with early rheumatoid arthritis (RA), longstanding RA, and osteoarthritis (OA).

Methods. Expression of TLRs 1–10 in RA synovial fibroblasts (RASFs), OASFs, and skin fibroblasts was analyzed by real-time polymerase chain reaction (PCR). Fibroblasts were stimulated with tumor necrosis factor α, interleukin-1β (IL-1β), bacterial lipopeptide, poly(I-C), lipopolysaccharide, and flagellin. Production of IL-6 was determined by enzyme-linked immunosorbent assay and induction of TLRs 2–5, matrix metalloproteinases (MMPs) 3 and 13 messenger RNA by real-time PCR. Expression of TLRs 2–4 in synovial tissues was analyzed by immunohistochemistry.

Results. Synovial fibroblasts expressed TLRs 1–6, but not TLRs 7–10. Among the expressed TLRs, TLR-3 and TLR-4 were the most abundant in synovial fibroblasts, and stimulation of synovial fibroblasts with the TLR-3 ligand poly(I-C) led to the most pronounced increase in IL-6, MMP-3, and MMP-13. In contrast, skin fibroblasts did not up-regulate MMP-3 or MMP-13 after stimulation with any of the tested stimuli. In synovial tissues from patients with early RA, TLR-3 and TLR-4 were highly expressed and were comparable to the levels of patients with longstanding RA. These expression levels were elevated as compared with those in OA.

Conclusion. Our findings of high expression of TLRs, particularly TLRs 3 and 4, at an early stage of RA and the reactivity of synovial fibroblasts in vitro to TLR ligands suggest that TLR signaling pathways resulting in persistent inflammation and joint destruction are activated early in the disease process.

Rheumatoid arthritis (RA) is a systemic inflammatory disease that leads to joint destruction, deformity, and loss of function. Persistent synovial inflammation is one of the most characteristic features of RA and can be observed during early stages of the disease (1). In the majority of RA patients, erosions develop during the first 2 years after disease onset (2). Therefore, focusing on interventions during the very early phase of RA may increase our chances of stopping the disease and preventing irreversible disability.

In the inflammatory and matrix-degrading processes that characterize joint destruction in RA, RA synovial fibroblasts (RASFs) have been found to take an active part. RASFs, together with synovial macrophages, are the main cell types in the hyperplastic synovial lining layer and at sites of invasion into cartilage and bone.
Activated RASFs secrete a wide variety of proinflammatory cytokines, chemokines, and matrix-degrading enzymes, which perpetuate the chronic inflammation and lead to progressive, irreversible damage of the affected joint (3). Among the proinflammatory cytokines that are present at high levels in the inflamed joints, tumor necrosis factor α (TNFα) is considered to be the main activating stimulus for RASFs.

In recent years, it has been shown that RASFs can also be strongly stimulated by activation of Toll-like receptor (TLR) pathways (4). TLRs belong to the family of pattern-recognition receptors and play a crucial role in the activation of the innate immune system in response to invading microorganisms. According to their corresponding ligands, TLRs can be categorized in 2 main groups: TLRs 1, 2, 4, and 6 are receptors for lipid-based pathogen-associated molecular patterns (PAMPs), whereas TLRs 3, 7, 8, and 9 are receptors for nucleic acid–based PAMPs. Besides the main categories, TLR-5 represents the receptor for flagellin, the major structural protein of the flagella of gram-negative bacteria (4). The ligand for TLR-10 is not yet known. TLR-2 ligands are the most diverse group among all the TLR ligands due to the heterodimerization of TLR-2 with either TLR-1 or TLR-6.

TLR signaling leads to the up-regulation of co-stimulatory molecules by antigen-presenting cells and therefore facilitates the subsequent activation of the adaptive immune responses via the provision of the second signal to T cell stimulation. In the joints of patients with RA, exogenous and endogenous TLR ligands, such as peptidoglycan and necrotic cells, have been identified (5,6). The finding that activation of innate immunity via TLRs is connected to activation of the adaptive immune system, in addition to the identification of endogenous ligands for certain TLRs, strengthened the hypothesis that an early dysfunction of TLR pathways might result in autoimmune inflammation in joints (7).

In previous studies, we and other investigators have shown that TLRs 2, 3, and 4 are expressed in the synovium of patients with longstanding RA. Stimulation of the TLR-2 pathway in RASFs leads to translocation of NF-κB, secretion of proinflammatory cytokines and matrix metalloproteinases (MMPs), and expression of various chemokines (6,8–11). Likewise, stimulation of TLR-3 and TLR-4 pathways by synthetic or endogenous ligands induces the production of interferon-β, interleukin-6 (IL-6), and the chemokines CXCL10 and CCL5 (5).

In the present study, we comprehensively analyzed the expression, regulation, and functionality of TLRs in RASFs and RA synovial tissue at different stages of the disease as well as in skin fibroblasts. First, we established an expression profile of the currently known human TLRs, TLR-1 through TLR-10, in synovial and skin fibroblasts. Then, we compared the induced expression of the most abundantly expressed TLRs after various stimuli and their functionality in transmitting the production of disease-related molecules by RASFs. To complete our analysis, we quantified and compared the expression of TLRs in the synovium of RA patients at early and late stages of the disease as well as in patients with osteoarthritis (OA).

**PATIENTS AND METHODS**

**Patients and tissue preparation.** Skin fibroblasts from healthy volunteer donors were obtained by enzymatic digestion of skin biopsy samples as described previously (12). Synovial tissues were obtained from trauma patients and from RA and OA patients undergoing joint replacement surgery. Synovial samples from RA patients with a disease duration of <12 months (early RA) were obtained by blind needle-biopsy. All RA patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of RA (13). OA was diagnosed according to clinical features. The characteristics of the RA and OA patients are shown in Table 1.

For cell culture, tissues were minced and digested for 1 hour at 37°C in 150 mg/ml of Dispase II (Roche, Mannheim, Germany). Synovial fibroblasts were grown in Dulbecco’s modified Eagle’s medium (Gibco Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum, 50 IU/ml of penicillin/streptomycin, 2 mM l-glutamine, 10 mM HEPES, and 0.2% fungicide (all from Gibco Invitrogen). Cell cultures were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO2. Cultured synovial fibroblasts were used for experiments after 4–9 passages.

For immunohistochemistry, tissues were embedded in Tissue-Tek OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen, and stored at −80°C until further use.

**Immunohistochemistry.** Frozen sections (5 μm) of synovial tissues were thawed, fixed with acetone for 10 minutes, and air-dried. After washing in phosphate buffered saline (PBS), endogenous peroxidase was blocked with 0.3% H2O2 for 10 minutes. The slides were blocked with 1% bovine serum albumin/5% horse serum for 1 hour and incubated with the respective primary antibodies or isotype controls for 1 hour at room temperature or overnight at 4°C. After washing with PBS, slides were incubated for 30 minutes with the respective secondary antibodies. In slides incubated with biotinylated secondary antibodies, the signal was amplified with horseradish peroxidase (HRP)–conjugated streptavidin using a Vectastain Elite ABC kit (Vector, Burlingame, CA). All sections were developed with aminoethylcarbazole chromogen and counterstained with hematoxylin.
Primary antibodies used were polyclonal goat anti-human TLR-2 (2 μg/ml), polyclonal rabbit anti-human TLR-3 (1 μg/ml), polyclonal rabbit anti-human TLR-4 (1 μg/ml) (all from Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal mouse anti-human CD68 (2 μg/ml; Dako, Zug, Switzerland). Secondary antibodies were biotinylated mouse anti-goat and mouse anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG (all from Jackson ImmunoResearch, Soham, UK).

Two observers (CO and YR) who were blinded to the source of the tissues quantified the expression of each antigen semiquantitatively on a 5-point scale, where 0 = no staining; 1 = weak expression, single cells stained; 2 = mild expression, limited areas stained; 3 = moderate expression, weak overall expression or extended areas stained; and 4 = strong expression, strong overall staining.

Stimulation experiments. Synovial fibroblasts were seeded in 6-well plates at a density of 1 × 10^5/well. After 24 hours, cells were stimulated with 10 ng/ml of TNFα, 1 ng/ml of IL-1β (both from R&D Systems, Minneapolis, MN), 300 ng/ml of the bacterial lipopeptide (BLP) palmitoyl-3-cysteine-serine-

| Table 1. Characteristics of the study patients* |
|----------------|----------------|----------------|
|                | Longstanding RA patients (n = 11) | Early RA patients (n = 10) | OA patients (n = 11) |
| Age, mean (range) years | 64.55 (40–79) | 61.9 (41–76) | 75.64 (64–84)† |
| Sex, no. female/male   | 10/1           | 6/4           | NA             |
| Disease duration, mean (range) years | 21.8 (8–41)‡ | 0.4 (0.16–1) | NA             |
| Medications, no. taking/no. assessed |            |               |               |
| NSAIDs                | 3/11           | 9/10          | 1/11           |
| DMARDs                | 10/11          | 0/10          | 0/11           |
| Plus steroids         | 5/11           | 0/10          | 0/11           |
| Plus anti-TNF         | 2/11           | 0/10          | 0/11           |
| No. RF+ (>20 IU)/no. assessed | 8/11          | 7/9           | NA             |
| CRP, mean (range) mg/liter | 20.3 (2.4–121) | 32.3 (11–106)§ | NA             |

* NA = not assessed; NSAIDs = nonsteroidal antiinflammatory drugs; DMARDs = disease-modifying antirheumatic drugs; anti-TNF = anti–tumor necrosis factor; RF = rheumatoid factor.
† Patients with osteoarthritis (OA) were significantly older than patients with longstanding rheumatoid arthritis (RA) and patients with early RA.
‡ Disease duration was significantly longer in patients with longstanding RA than in patients with early RA.
§ C-reactive protein (CRP) levels were significantly higher in patients with early RA (n = 10) than in patients with longstanding RA (n = 8).

Primary antibodies used were polyclonal goat anti-human TLR-2 (2 μg/ml), polyclonal rabbit anti-human TLR-3 (1 μg/ml), polyclonal rabbit anti-human TLR-4 (1 μg/ml) (all from Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal mouse anti-human CD68 (2 μg/ml; Dako, Zug, Switzerland). Secondary antibodies were biotinylated mouse anti-goat and mouse anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG (all from Jackson ImmunoResearch, Soham, UK).

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Table 2. Primer and probe sequences used for real-time quantitative polymerase chain reaction analyses of TLRs and MMPs* |
<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
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<tbody>
<tr>
<td>TLR-1</td>
<td>CAGTGTCGTTGCTACACGC-AGTGT</td>
<td>TGGTTCATCCAAATTTAGCCCG-TTCC</td>
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<td>TLR-2</td>
<td>GCCGCAATACCTCGGTG</td>
<td>TCTATCCATGTCGGTGCC</td>
</tr>
<tr>
<td>TLR-3</td>
<td>CCTGTGTTGAATGCAATGGATCAG</td>
<td>ACCCTACTCACAAGGCCTAGCTAGC</td>
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<tr>
<td>TLR-4</td>
<td>CAGATGTCATCGAAGCTTCTCCTAG</td>
<td>CGATCTAGTATTAGCCCGTG</td>
</tr>
<tr>
<td>TLR-5</td>
<td>TGCCGGAAGGCTTCTCCTACTG</td>
<td>CGACTGCGGATGAGCTG</td>
</tr>
<tr>
<td>TLR-6</td>
<td>GAAGAAGAAGAAACCCCTTTGAGATAGC</td>
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<tr>
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<td>TTCATGCTGGAAGGCTTCTGGCTACCTA</td>
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<tr>
<td>TLR-8</td>
<td>TTATGGTGTCCAGGAACCTCAGCTA</td>
<td>TATACCAACGTTAGTACG</td>
</tr>
<tr>
<td>TLR-9</td>
<td>GGAGCTCTGCTACTGGTCTGGCTC</td>
<td>AAGCTGGTGTACACCAGGATC</td>
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<td>TLR-10†</td>
<td>CTTGCTGACCACTGCTTGCC</td>
<td>AGGTGCTGATGATGATGATGATG</td>
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<tr>
<td>MMP-13</td>
<td>TCTCAGAAATCTCGCAGGGAATTGAG</td>
<td>AGACTGAGATGAGATGAGATGAGATG</td>
</tr>
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* TLRs = Toll-like receptors; MMPs = matrix metalloproteinases.
† SYBR Green primer.
lysine-4, 10 μg/ml of poly(I-C) (both from InvivoGen, San Diego, CA), 100 ng/ml of lipopolysaccharide (LPS) from Escherichia coli J5 (List Biological Laboratories, Campbell, CA), or 100 ng/ml of flagellin from Salmonella typhimurium (InvivoGen). After 24 hours of stimulation, supernatants were collected and cells were lysed in RLT buffer. Total RNA was isolated using an RNeasy Mini Prep kit (Qiagen, Basel, Switzerland) with DNase treatment.

**Real-time polymerase chain reaction (PCR) analysis.** Complementary DNA (cDNA) was generated by reverse transcription of total RNA using random hexamers and MultiScribe reverse transcriptase (both from Applied Biosystems, Rotkreuz, Switzerland). As negative controls, non–reverse-transcribed samples were used. For quantification of messenger RNA (mRNA) levels, single-reporter real-time PCR was performed using an ABI Prism 7700 Sequence Detection system (Applied Biosystems). Eukaryotic 18S ribosomal RNA (rRNA) levels, which were measured with a predeveloped primer/probe system (Applied Biosystems), served as the endogenous control for relative quantification. The difference between the values of comparative threshold cycles (C_t) of the tested gene and that of 18S rRNA (ΔC_t) was used to calculate changes in the relative expression (ΔΔC_t) after stimulation, using the formula ΔΔC_t = ΔC_t (sample stimulated) – ΔC_t (sample unstimulated). The fold difference in regulation was calculated using the expression 2−ΔΔC_t. Only samples with a difference of at least 4 cycles between cDNA and non–reverse-transcribed RNA samples were considered for calculations.

Primers were designed with Primer 3 software (online at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were synthesized by Microsynth (Balgach, Switzerland). Dissociation curve analysis for each SYBR Green primer pair and reaction was performed to verify specific amplification. Primer sequences (forward primer, reverse primer, and probe) are shown in Table 2.

**Enzyme linked immunosorbent assay (ELISA).** Levels of IL-6 and TNFα were determined by ELISA, with the use of OptEIA kits from BD PharMingen (San Diego, CA).

**Statistical analysis.** Values are presented as the mean ± SEM. The Mann-Whitney U test or Wilcoxon’s signed rank test was used to determine significance. Spearman’s rank correlation test was used to determine correlations. Tests were performed with GraphPad Prism software (GraphPad Software, San Diego, CA). P values less than 0.05 were considered significant.
RESULTS

Expression of mRNA for TLRs 1–10 in synovial fibroblasts and skin fibroblasts. In previous studies, we and other investigators have shown that TLRs 2, 3, and 4 are expressed in RASFs. However, since no expression profile of all known human TLRs (TLRs 1–10) in synovial or skin fibroblasts has yet been established, we measured levels of mRNA for TLRs 1–10 using relative quantification real-time PCR in RASFs, OASFs, normal synovial fibroblasts, and skin fibroblasts (Figure 1A). The highest expression of mRNA was found for TLR-3, followed by TLR-4. In the TLR-2 subfamily, mRNA for TLRs 2, 1, and 6 were readily detected, whereas mRNA for TLR-10 could not be detected. Levels of mRNA for TLR-5 were only just above the detection limit, which was set at a minimum of 4 cycles difference between cDNA and control. Messenger RNA for members of the TLR-9 subfamily (TLRs 7–9) was not detectable in unstimulated fibroblasts, and it could not be induced by various stimuli (data not shown). TLR-2 mRNA levels showed the highest variability of all the TLRs. No significant differences in the expression of the various TLRs were found between RASFs, OASFs, normal synovial fibroblasts, and skin fibroblasts. However, there was a trend toward higher TLR-2 and TLR-3 mRNA levels in RASFs.

To determine whether the basal expression of TLRs 2, 3, 4, and 5 could be enhanced in RASFs, we stimulated the cells for 24 hours with the proinflammatory cytokines TNFα and IL-1β and with the TLR ligands BLP, poly(I-C), LPS, and flagellin, respectively. The expression of TLR-2 was significantly induced by all of the stimuli tested (Figure 1B), except for flagellin. In contrast, the expression of TLR-3 was significantly up-regulated only after stimulation with poly(I-C) or LPS, whereas neither
TNFα, IL-1β, BLP, nor flagellin had any effect. The expression of TLR-4 and TLR-5 mRNA was not significantly changed by any of the stimuli tested.

**Production of proinflammatory cytokines and matrix-degrading enzymes after stimulation with TLR ligands.** Since we found that TLRs 2, 3, 4, and 5 are expressed in synovial and skin fibroblasts, we analyzed whether they differ in their ability to transmit activation in these cells. First, we treated synovial and skin fibroblasts for 24 hours with various concentrations of ligands for TLRs 2, 3, 4, and 5 and measured the production of the proinflammatory cytokine IL-6. The TLR-3 ligand poly(I-C), as well as LPS, BLP, and flagellin, induced the production of IL-6 in RASFs (Figure 2A).

Saturation for TLR-2 and TLR-5 by BLP and flagellin, respectively, was almost reached at the lowest concentration of these ligands. Increasing TLR-2 stimulation up to a BLP dose of 300 ng/ml led to only a further slight increase in IL-6 production. In contrast, activation of TLR-3 and TLR-4 showed a clear dose-response, with maximum levels of IL-6 reached at a dose of 20 μg/ml and 200 ng/ml of poly(I-C) and LPS, respectively. When the concentrations at 50% of maximal stimulation were compared, poly(I-C) induced the
highest levels of IL-6 (Figure 2A). Consistent with the lack of expression of TLRs 7, 8, and 9, stimulation of synovial fibroblasts with ligands for TLRs 7, 8, and 9 did not induce IL-6 production (data not shown).

Skin fibroblasts also showed up-regulated production of IL-6 after stimulation with TLR ligands, but the amount was markedly less compared with that in RASFs for all of the stimuli tested (Figure 2B). No significant difference in the up-regulation of IL-6 after TLR stimulation between RASFs and OASFs was seen; nevertheless, a trend toward reduced levels of IL-6 in supernatants of OASF cultures was observed (data not shown). Measurements of TNFα showed no detectable levels of TNFα in the supernatants of RASFs stimulated with BLP, poly(I-C), LPS, or flagellin (data not shown).

Since a hallmark of RASF activation is the production of matrix-degrading enzymes, we next measured the expression of the MMPs 3 and 13 after stimulation with TLR ligands. Poly(I-C) induced the expression of MMP-3 mRNA at a mean $\pm$ SEM of 148 $\pm$ 58–fold and of MMP-13 mRNA by 191 $\pm$ 93–fold (Figure 2C). BLP, LPS, and flagellin also significantly induced MMP expression in RASFs. OASFs reacted to TLR stimulation in a manner similar to that of RASFs (data not shown). In contrast, the expression of mRNA for MMP-3 or MMP-13 in skin fibroblasts was not significantly changed after stimulation.

Expression of TLRs 2, 3, and 4 at different stages of RA as compared with expression in OA. To assess whether the activation of TLRs is an early event in the pathogenesis of RA, we compared the expression of TLRs in synovial tissues from patients with a very early stage of RA (<12 months) and from patients with longstanding RA. Snap-frozen synovial tissue sections from patients with early RA (n = 10), longstanding RA (n = 11), and OA (n = 11) were stained for TLRs 2, 3, and 4. For each antigen, the intensity of expression was assessed as described in Patients and Methods and assigned an expression score.

TLRs 2, 3, and 4 were present in the synovium of patients with early RA and were expressed to the same extent as in patients with longstanding RA (Figure 3). TLR-3 and TLR-4 were significantly more highly expressed in patients with early and longstanding RA than in patients with OA. The levels of TLR-2 did not differ between RA and OA samples. In addition, the expression of TLR-3 and TLR-4 was clearly more abundant than the expression of TLR-2. All 3 of the TLRs we analyzed were primarily expressed in the lining layer, but in samples with more abundant expression, this extended to cells in the sublining and perivascular areas.

Furthermore, comparison of TLR expression with the staining intensity for CD68 showed that the expression of TLRs was not connected with the number of macrophages in the synovium (data not shown).

**DISCUSSION**

The aim of this study was to comprehensively assess the expression and function of TLRs in synovial and skin fibroblasts. In addition, in an effort to define whether activation of TLR signaling may be an early event in the pathogenesis of RA, we comparatively analyzed the expression of TLRs in synovial tissues during early and late stages of RA.

Comparable to their expression in synovium, TLRs are expressed on a variety of cell types in the skin, including keratinocytes and Langerhans’ cells in the epidermis. Keratinocytes constitutively express mRNA for TLRs 1, 2, 3, 4, 5, 6, 9, and 10, but not TLR-7 or TLR-8 (14). We found that skin fibroblasts constitutively express mRNA for TLRs 1–6, but not TLRs 7–10. These data indicate that skin fibroblasts might also play a role in the defense against invading pathogens, since they express functional TLRs.

In a previous study, Kim et al (15) demonstrated significantly higher TLR-2 and TLR-4 mRNA levels in RASFs compared with OASFs. We found only a trend toward higher expression of TLR-2 and TLR-3 in RASFs as compared with OASFs, normal synovial fibroblasts, and skin fibroblasts, but the difference did not achieve statistical significance. However, even though the constitutive expression of TLR mRNA did not differ significantly between synovial fibroblasts and skin fibroblasts, activation of these cells with PAMPs resulted in differential patterns of expression of proinflammatory cytokines and matrix-degrading enzymes. In particular, RA synovial fibroblasts produced significantly higher amounts of IL-6, MMP-3, and MMP-13. Since it has been demonstrated that in pathologic conditions, skin fibroblasts appear to be more prone to produce matrix instead of matrix-degrading enzymes, it does not appear to be surprising that skin fibroblasts react differently to stimuli than do synovial fibroblasts (16,17). Taken together, these data illustrate the importance of fibroblasts as sentinel cells of the innate immune response as well as their ability to react to PAMPs with different effector molecules according to the needs of the tissues of which they are part.

Furthermore, we showed that synovial fibroblasts derived from trauma, RA, or OA patients constitutively expressed mRNA for TLRs 1–6, but not TLRs 7–10.
Among the TLR mRNA expressed in synovial fibroblasts, TLR-3 and TLR-4 mRNA was the most abundant, followed by members of the TLR-2 subfamily. Constitutive expression of TLR-9 has mainly been reported in plasmacytoid dendritic cells and B cells, but keratinocytes, pulmonary epithelial cells, and intestinal epithelial cells have also been shown to express TLR-9 (14,18–20). In contrast, we did not detect mRNA for TLR-9 in synovial fibroblasts or skin fibroblasts. The lack of TLR-9 mRNA expression excludes activation of synovial fibroblasts by DNA or single-stranded RNA via TLRs, as we have shown for CpG oligodeoxynucleotides in a previous study (6). Our finding is also consistent with those of several studies of other tissues, thus confirming TLR-3 as the main nucleic acid–specific TLR in nonimmune cells (5,21–23). Similarly, the expression of TLR-2 and TLR-4 in RA synovial fibroblasts has also been documented previously (6,8,24,25). Our study now adds TLR-5 to the TLR expression profile in synovial fibroblasts.

Consistent with the basal expression pattern in cultured synovial fibroblasts, TLR-3 and TLR-4 were also the most abundant of the measured TLRs in the synovium. Of particular interest is the finding that at an early stage of RA, the expression of TLR-3 and TLR-4 in synovium is elevated as compared with that in OA synovium. This result suggests that overexpression of TLRs is an early event and that their levels do not change over the course of the disease. Since TLRs can be induced within hours, as demonstrated in the in vitro experiments, we cannot rule out the possibility that up-regulation of TLRs is a secondary event in the course of the development of joint inflammation. However, the fact that the TLR levels in early RA synovial tissue were clearly higher than those in OA synovial tissue demonstrates that activation of TLR pathways is not a late event that is restricted only to the severe destructive stages of RA. Induction of TLRs may be due to unspecific triggering infections occurring at early stages of disease or may be due to the presence of endogenous ligands. In addition, genetic background, epigenetic changes, or environmental factors such as smoking might play a role in this overexpression.

Whether there is a functional reason for the increased expression of specific TLRs is still only a matter of speculation. Based on previously published data, it might be hypothesized that the availability of endogenous ligands might determine which TLRs are up-regulated (5,26).

In contrast to our findings, a previous study showed slightly higher expression of TLR-2 than TLR-4 in RA synovium (10). Reasons for this discrepancy might be differences in the patient cohorts examined or in the assay methods used. It must also be acknowledged that the expression of TLRs is not specific for RA, but has been found in a wide variety of inflammatory diseases, such as psoriasis, inflammatory bowel disease, and systemic lupus erythematosus (27–29). It is noteworthy that despite the differences in disease-modifying antirheumatic drug therapy between the patients with early and longstanding RA, the expression of TLR-3 and TLR-4 was not significantly different in these 2 patient populations.

Several exogenous pathogens have been proposed as triggers of RA; however, no specific microorganism associated with RA has yet been identified. Nonetheless, during the course of infection, bacterial or viral products may be deposited in the joints and lead to activation of synovial cells via TLRs (30). In addition, TLR activation by endogenous ligands has been reported; for example, hyaluronan, fibrin, heat-shock proteins have been shown to activate TLR-4 (26,31–33). We previously demonstrated that double-stranded RNA released from necrotic cells stimulates synovial fibroblasts in a TLR-3–dependent manner (5). Since these endogenous ligands are present in elevated amounts in inflamed joints, TLR expression levels and responsiveness of synovial cells to TLR ligands are critical factors. Here, we demonstrated that TLR-3 and TLR-4 are already highly expressed in the synovium at early stages of RA, as well as at later stages of disease. Together with the demonstration that RASFs stimulated in vitro with TLR-3 and TLR-4 ligands produce a wide range of proinflammatory cytokines, chemokines, and tissue-destructive enzymes, our data suggest that stimulation of TLR pathways occurs early in RA, resulting in the activation of synovial fibroblasts and contributing to the development of synovial inflammation and joint destruction.

AUTHOR CONTRIBUTIONS

Dr. Brentano had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Ospelt, Brentano, S. Gay, Kyburz.

Acquisition of data. Ospelt, Brentano, Kolling, Tak.

Analysis and interpretation of data. Ospelt, Brentano, Rengel, Stanczyk, Tak, S. Gay, Kyburz.


Statistical analysis. Ospelt, Brentano.
REFERENCES


