Local Interleukin-1-Driven Joint Pathology Is Dependent on Toll-Like Receptor 4 Activation

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Toll-like receptors (TLRs) may contribute to the pathogenesis of chronic inflammatory destructive diseases through the recognition of endogenous ligands produced on either inflammation or degeneration of the extracellular matrix. The presence of endogenous TLR agonists has been reported in rheumatoid joints. In the present study, we investigated the significance of TLR2 and TLR4 activation by locally-produced endogenous ligands in the severity of joint inflammation and destruction. Local joint pathology independent of systemic immune activation was induced by overexpression of interleukin (IL)-1 and TNF in naive joints using adenoviral gene transfer. Here, we report that at certain doses, IL-1-induced local joint inflammation, cartilage proteoglycan depletion, and bone erosion are dependent on TLR4 activation, whereas TLR2 activation is not significantly involved. In comparison, tumor necrosis factor α-driven joint pathology seemed to be less dependent on TLR2 and TLR4. The severity of IL-1-induced bone erosion and irreversible cartilage destruction was markedly reduced in TLR4−/− mice, even though the degree of inflammation was similar, suggesting uncoupled processes. Furthermore, the expression of cathepsin K, a marker for osteoclast activity, induced by IL-1β was dependent on TLR4. Overexpression of IL-1β in the joint as well as ex vivo IL-1 stimulation of patellae provoked the release of endogenous TLR4 agonists capable of inducing TLR4-mediated cytokine production. These data emphasize the potential relevance of TLR4 activation in rheumatoid arthritis, particularly with respect to IL-1-mediated joint pathology. (Am J Pathol 2009, 175:2004 –2013; DOI: 10.2353/ajpath.2009.090262)

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent joint inflammation and concomitant cartilage and bone destruction. Despite intensive research, many features of the immunopathology of RA are yet to be explored. The discovery of Toll-like receptors (TLRs) as essential components of the immune system has introduced new candidates to the field of research on the pathogenesis of arthritis. TLRs are a family of evolutionarily conserved transmembrane receptors, which are expressed by a variety of immune cells, including monocytes, macrophages, dendritic cells, neutrophils, B cells, and certain types of T cells; however, nonimmune cells such as fibroblasts and chondrocytes also express TLRs.1,2 The major function of TLRs is to recognize pathogen-associated molecular patterns, which are highly conserved in evolution and are shared by many microorganisms. At the same time, TLRs show considerable target specificity. For instance, diacylated and triacylated lipoproteins of Gram-positive bacteria are sensed by TLR2 in cooperation with TLR6 and TLR1, respectively, whereas lipopolysaccharides (LPSs) of Gram-negative bacteria are recognized by TLR4.2

Signal transduction through TLRs leads to the activation of several transcription factors among which are NFκB and activator protein 1. Thereby, TLR activation controls the expression of a number of proinflammatory cytokines such as Interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF), chemokines such as IL-8 and macrophage inflammatory protein-1, and matrix metalloproteinases (MMPs) all of which are relevant to the pathogenesis of RA.3,4 Besides pathogen-associated molecular patterns, TLRs are capable of recognizing endogenous ligands produced or released on cell stress, inflammation or degradation of extracellular matrix. In this context, TLR4 can

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recognize some matrix components such as heparan sulfate and extra domain A of fibronectin, whereas biglycan, hyaluronan fragments, high-mobility group box 1, and some endogenous heat-shock proteins activate both TLR2 and TLR4. The presence of endogenous TLR ligands such as fibronectin fragments, high-mobility group box 1, and heat-shock proteins has been shown in rheumatoid synovium. It has been reported that rheumatoid synovial fibroblast-like cells synthesize extra domain A-containing fibronectin. Furthermore, some inflammatory cytokines of high interest in the field of rheumatoid synovium. It has been reported that rheumatoid synovial fibroblast-like cells synthesize extra domain A-containing fibronectin.15

Induction of Arthritis Using Adenoviral Transfer of IL-1β and TNFα Genes

Local joint inflammation and destruction was induced in C57BL/6 wild-type, TLR2−/− and TLR4−/− mice (n = 6 mice/group) by intra-articular injection of 6 μl of saline containing 3 × 10⁶ plaque-forming units (PFU) AdIL-1β or 1 × 10⁷ PFU AdTNFα virus. A total of 1 × 10⁷ PFU of the control virus Ad5del70-3 was injected into the contralateral knee joint. In the following studies, the dose of AdIL-1β was enhanced to 3 × 10⁶ PFU per joint to enforce IL-1β-induced cartilage destruction. Previous reports have validated this adenoviral delivery system as an effective means of cytokine overexpression in synovial tissue.32

Preparation of Patella Washouts and Measurement of Cytokines

Patellae with surrounding tissue were isolated after intra-articular injection of the viruses. Patella washouts were prepared by culturing the patellae in RPMI 1640 containing 0.1% bovine serum albumin for 1 hour at room temperature. Cytokine concentrations were determined using the Bioplex cytokine assays from Bio-Rad (Hercules, CA) following the manufacturer’s instructions.

Histology

For histological assessment of arthritis, total knee joints were isolated at day 4 of viral transduction and fixed during 4 days in 4% formaldehyde, then decalcified in 5% formic acid and embedded in paraffin. Tissue sections of 7 μm were stained using the H&E to study the

Materials and Methods

Animals

Male C57BL/6 mice were purchased from Janvier, France. TLR2−/− and TLR4−/− mice in C57BL/6 background were provided by Prof. S. Akira (Osaka, Japan). The mice were housed in filter-top cages, and water and food were provided ad libitum. Gender-matched animals (10 to 12 weeks of age) were used in all experiments. Animal studies were approved by the Institutional Review Board and were performed according to the related codes of practice.

Adenoviral Vectors

AdIL-1β virus was provided by Dr. C. D. Richards (McMaster University, Ontario, Canada) and was engineered as described previously. AdTNFα virus was a gift from Dr. J. K. Kolls (Children’s Hospital of Pittsburgh, Pittsburgh, PA). Virus construction and production was as reported in previous studies. The empty viral vector Ad5del70-3 was used as negative control throughout the studies.
inflammatory cell influx or using the Safranin O staining to determine proteoglycan (PG) depletion and cartilage and bone destruction. Histopathological changes were scored on a scale from 0 to 3 by two observers in a blinded manner as described previously.26

**Isolation of RNA from Synovial Biopsies**

Synovial biopsies from knee joints were isolated from lateral and medial sides of patellae using a 3-mm punch (Stiefel, Wachtersbach, Germany). Total RNA was isolated in 1 ml of TRIzol reagent (Sigma-Aldrich, St. Louis, MO), then precipitated with isopropanol, washed with 70% ethanol, and dissolved in water. RNA was treated with DNase and subsequently reverse transcribed into complementary DNA using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase.

**Quantitative Real-Time PCR**

Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) for quantification with SYBR Green and melting curve analysis. Primer sequences (forward and reverse, respectively) were as follows: for glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene), 5’-GGCAATTCAAAGGGACA-3’ (forward) and 5’-GTTAGTTTGTCGTCCTGCTG-3’ (reverse); and for cathepsin K, 5’-GAAGCAGTATAACAGCAAGGTGGAT-3’ (forward) and 5’-TGCTCCTCCAAGTGTTCATG-3’ (reverse). PCR conditions were as follows: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with data collection during the last 30 seconds. For all PCRs, SYBR Green Master Mix was used in the reaction. Primer concentrations were 300 nmol/L. The threshold cycle (Ct) value of the gene of interest was corrected for the Ct of the reference gene glyceraldehyde-3-phosphate dehydrogenase to obtain the ΔCt, then ΔΔCt was calculated compared with Adel control of mice with the same genotype.

**Immunohistochemistry**

The presence of active osteoclasts was evaluated by immunohistochemical staining for cathepsin K on paraffin sections of the knee joints 4 days after viral transduction. The percentage of polymorphonuclear and mononuclear cells was evaluated using staining for NIMP-R14 and F4/80 markers, respectively. Sections were deparaffinized in xylene and rehydrated in serial dilutions of ethanol. Endogenous peroxidase was blocked using 1% hydrogen peroxide for 30 minutes to specifically inhibit the TLR4-dependent response. HEK293-mTLR4 cells were preincubated with 1 μg/ml TNF blocker Enbrel (Amgen, Thousand Oaks, CA) and Gardiquimod (1 μg/ml) for 24 hours. To assess the induction of TLR4 agonists by IL-1 and TNF, 1-hour patella washouts were obtained on several days of in vivo Adel or AdIL-1β overexpression, or supernatants of patellae ex vivo cultured with IL-1β or TNFα (10 ng/ml each, n = 6 patellae per group) for 24 hours were added to HEK293 and HEK293-TLR4 cells in a volume ratio of 1:10. Assays were performed in triplicate and human IL-8 was measured in culture supernatants as readout using the Bioplex cytokine assays. Where mentioned, 1 μg/ml TNF blocker Enbrel (Amgen, Thousand Oaks, CA) was added to the cultures to inhibit the TLR4-independent TNF-mediated response. HEK293-TLR4 cells were preincubated with 1 μg/ml Bartonella quintana LPS as a TLR4 antagonist34,35 for 30 minutes to specifically inhibit the TLR4-mediated response.

**Statistical Analysis**

Group measures are expressed as the mean ± SEM. Statistical significance was assessed using the Mann-Whitney U-test performed on GraphPad Prism 4.0 software (GraphPad Software). P values lower than 0.05 were considered significant.

**Results**

**Local Cytokine Production on Adenoviral Gene Transfer of IL-1β and TNFα**

Local IL-1β and TNFα production was determined after adenoviral gene transfer of these cytokines into the naïve
joints of C57BL/6 wild-type mice. Intra-articular injection of AdIL-1β or AdTNFα virus in the wild-type mice resulted in the production of high levels of the respective cytokine detectable in the patella washouts one day after gene transfer (Figure 1, A and B). Concentrations of both cytokines decreased in time, with low levels being still detectable at day 11 (153.1 ± 20.8 pg/ml IL-1β for AdIL-1β and 120.0 ± 29.8 TNFα for AdTNFα). Comparison of the local cytokine concentration in wild-type, TLR2−/− and TLR4−/− mice revealed that TLR2 and TLR4 gene deficiency did not affect the viral transduction or cytokine production, as the cytokine expression was similar in the three groups (Figure 1). Furthermore, intra-articular injection of a similar dose of the control virus Ad5del70-3 into the joint did not induce detectable levels of IL-1β or TNFα (data not shown).

Joint Pathology on Adenoviral Overexpression of IL-1β and TNFα

Prolonged expression of IL-1β or TNFα in the knee joints of wild-type mice induced pathological changes in the joint resembling those observed in RA. These included joint inflammation, ie, synovial hyperplasia and inflammatory cell influx, bone erosion and depletion of matrix PGs in articular cartilage (Figure 2). The Ad5del70-3 virus used as negative control throughout the experiments sporadically induced very low degree of synovial inflammation, but was unable to cause any sign of cartilage or bone damage in wild-type or TLR−/− animals (Figure 2A). The severity of synovial inflammation, PG depletion and bone erosion in the wild-type mice was similar for IL-1 and TNF overexpression at the virus doses chosen here. Neither AdIL-1β nor AdTNFα induced erosion of cartilage surface at these doses.

Although IL-1β production on its adenoviral overexpression was similar in wild-type and TLR−/− mice (Figure 1), the severity of joint inflammation was significantly reduced in TLR4−/− mice compared with wild-type mice at the AdIL-1β dose used here (Figure 2B). Immunohistochemical staining for NIMP-R14 and F4/80, markers for polymorphonuclear and mononuclear cells, respectively, revealed the presence of polymorphonuclear cells and relatively lower numbers of mononuclear cells (Table 1). Despite significant reduction in the extent of synovial inflammation in TLR4−/− mice (Figure 2B), the composition of exudate as well as infiltrate cells remained un-

changed (Table 1), suggesting a general effect on various cell types.

In addition to synovial inflammation, joints of TLR4−/− mice exhibited also less severe PG depletion and bone erosion compared with wild-type mice (Figure 2, C and D). TLR2−/− animals tended to have reduced joint inflammation and bone erosion; however, this reduction did not reach statistical significance (Figure 2, B and D). PG loss in the cartilage of TLR2−/− mice was not affected as well (Figure 2C).

In comparison with IL-1, TNFα-induced joint inflammation remained unaffected in the knockout mice, and PG depletion and bone erosion seemed less dependent on TLR2 and TLR4, because no significant differences were found between wild-type and TLR−/− mice (Figures 2, E–G). Representative images of the joint inflammation and damage on IL-1β overexpression in wild-type mice in comparison with TLR2−/− and TLR4−/− mice are shown in Figure 3.

IL-1-Mediated Cartilage Destruction Partially Depends on TLR4 Activation

Considering the more striking TLR4 dependency of IL-1-induced joint pathology, further studies focused on IL-1β-induced arthritis. IL-1 inhibits chondrocyte PG and collagen synthesis at low concentrations; however, at high concentrations it also stimulates the synthesis of matrix degrading proteases. Therefore, we enhanced the dose of AdIL-1β to enforce cartilage destruction. Joint inflammation on intraarticular injection of the enhanced dose of AdIL-1β was increased to near maximum score and was similar in wild-type, TLR2−/−, and TLR4−/− animals (Figure 4A). At this dose, a marked erosion of articular cartilage was observed in the wild-type mice (Figures 4, B and C). Interestingly, the severity of cartilage destruction was clearly diminished in TLR4−/− mice compared with wild-type animals despite similar degree of joint inflammation (Figures 4, B and C). TLR4−/− mice remained significantly protected at later time point (day 11 of virus injection) when severe erosion of articular cartilage was apparent (data not shown). TLR2−/− animals had a similar extent of cartilage erosion as the wild-type mice, again emphasizing the specific TLR4 dependency of IL-1-mediated cartilage destruction.
Severe Bone Erosion and Osteoclast Formation on IL-1β Overexpression Is Dependent on TLR4

Prolonged presence of high doses of IL-1β caused severe bone erosion in patella as well as femur of wild-type mice (Figure 5A). Although TLR2−/− mice had similar degree of bone erosion as the wild-type mice, TLR4−/− animals expressed substantially less bone erosion (Figure 5A). Remarkably, a large number of multinucleated cells with osteoclast-like morphology were observed along the outer bone surfaces as well as in the intratra-ecular space in H&E-stained tissue sections of the wild-type mice. Therefore, we examined the expression of the osteoclast marker cathepsin K using quantitative PCR and immunohistochemistry and compared wild-type and TLR4−/− mice in this respect. Expression of cathepsin K mRNA in synovial tissue of wild-type mice was up-regulated by IL-1 overexpression compared with Addel control (Figure 5B). While TLR2−/− mice showed similar up-regulation, TLR4−/− mice had ~40% lower expression (Figure 5B). On immunohistochemistry, cathepsin K protein was highly expressed in osteoclast-like cells adjacent to the

Table 1. TLR4 Deficiency Does Not Affect the Composition of Inflammatory Cells in the Joint Despite Significant Reduction in the Extent of Inflammation

<table>
<thead>
<tr>
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<th>Infiltrate</th>
<th>Exudate</th>
<th>Synovial Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIMP-R14 (%)</td>
<td>F4/80 (%)</td>
<td>NIMP-R14 (%)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.13 ± 0.18</td>
<td>30.0 ± 4.4</td>
<td>18.6 ± 2.6</td>
</tr>
<tr>
<td>TLR2−/−</td>
<td>0.71 ± 0.18</td>
<td>34.2 ± 5.2</td>
<td>20.0 ± 1.8</td>
</tr>
<tr>
<td>TLR4−/−</td>
<td>0.65 ± 0.09</td>
<td>33.3 ± 3.6</td>
<td>20.8 ± 1.5</td>
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Synovial inflammation was scored on H&E-stained joint sections obtained at day 4 of intra-articular injection of AdIL-1β (3.5 × 10⁵ PFU per joint). Percentages of cells expressing NIMP-R14 and F4/80, markers for polymorphonuclear and mononuclear cells, respectively, in infiltrate and exudate cells were scored on immunohistochemically stained sections. *P < 0.05 compared with wild-type by Mann-Whitney U-test.
kines and TLR ligands revealed that HEK-TLR4 cells produce IL-8 in a dose-dependent manner on stimulation with LPS (Figure 6A). Although no response to TLR2 and TLR7 ligands (Pam3Cys and Gardiquimod, respectively) were observed, low response to TLR3 stimulation (poly I:C) was detected. Importantly, HEK-TLR4 cells did not respond to IL-1β itself while being sensitive to TNFα (Figure 6A).

Incubation of HEK-TLR4 cells with patella washouts from AdIL-1β-transduced wild-type joints resulted in robust TLR4 activation and high cytokine production, an effect not found on stimulation with washouts from the Addel-transduced joints (Figure 6B). This indicates that AdIL-1β overexpression in the joint induces the release of endogenous TLR4 ligands from patellae and the surrounding synovial tissue. Interestingly, these effects could be mimicked in vivo when naive wild-type patellae were stimulated with recombinant IL-1β. Patellae with minimal surrounding tissue were incubated with IL-1 or TNF for 24 hours, and the culture supernatants were used to detect TLR4 agonists using the HEK-TLR4 cell line. Since the latter cells respond to TNFα and TNF might be present in the supernatants, high concentrations of the TNF blocker Enbrel, revealed to completely inhibit TNFα effects in the same assay, were added to inhibit nonspecific responses. As expected, supernatants of IL-1-stimulated patellae induced TLR4 activation; however, supernatants from TNF-stimulated patellae did not (Figure 6C). The negative control cell line HEK293 did not respond to any of these stimuli, excluding any non-TLR4-mediated effects in the same assay. The cytokine response of HEK-TLR4 cells to IL-1-stimulated patella supernatants was abolished in the presence of TLR4 antagonist, confirming the TLR4 specificity of the response (Figure 6D).

**Discussion**

We have recently demonstrated the involvement of TLR4 activation in two chronic destructive models of arthritis, ie, collagen-induced and spontaneous IL-1rn−/− arthritis, in which the adaptive immune response represents a central part of the pathogenesis.34,37 The dominant role of TLR4...
in the established phase of arthritis rather than the onset suggested the contribution of endogenous rather than exogenous TLR4 agonists in arthritic process. In the present study, we addressed the question whether locally produced endogenous TLR2 or TLR4 ligands contribute to the severity of inflammatory and destructive processes in the joint. Here, we used sustained local overexpression of IL-1\beta and TNF\alpha as model cytokines for RA and excluded any effect of TLR2 or TLR4 gene deficiency on the viral transduction and the induced cytokine production (Figure 1). The failure to distinguish between the immunomodulatory roles of TLRs and their sole innate activation by local endogenous ligands has been overcome in this model, because no systemic and adaptive immune activation is involved.

Although both IL-1\beta and TNF\alpha are produced in high concentrations by inflamed RA synovium and similarly stimulate the production of other inflammatory mediators such as IL-6, IL-8, and prostaglandin E\textsubscript{2}-40 they exhibit

![Figure 5](image_url)

**Figure 5.** TLR4 dependency of local IL-1-induced bone erosion (A) and cathepsin K expression (B–D) despite similar synovial inflammation at day 4 of high AdIL-1\beta overexpression (3.5 x 10⁶ PFU per joint). Cathepsin K mRNA expression (B) was measured by quantitative real-time PCR. The threshold cycle (Ct) value of cathepsin K was corrected for the Ct of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to obtain the \(\Delta\text{Ct}\), then \(\Delta\Delta\text{Ct}\) was calculated compared with the AdEl control. Cathepsin K protein expression (C) was detected by immunohistochemistry and quantified using Leica Qwin software. Values in A (n > 5) and B (n = 4) are the mean ± SEM. The horizontal bars in C represent the mean. n.s. = not significant. *P < 0.05 compared with wild-type (WT) by Mann-Whitney U-test.

**D:** Representative images of immunohistochemical staining of cathepsin K, the osteoclast marker involved in bone resorption. Positive cells at the outer surface of the bone, mineralized cartilage and the intratrabecular space are depicted in the figure. Original magnification, \(\times200\). P = patella; F = femur.

![Figure 6](image_url)

**Figure 6.** IL-1\beta, but not TNF\alpha, induces the release of endogenous TLR4 agonists from patellae. A: HEK293-mTLR4/MD2/CD14 cells were stimulated with IL-1\beta, TNF\alpha, and various TLR ligands. Stimulations were performed at least in triplicate. B: HEK293-mTLR4 cells were stimulated with patella washouts obtained at day 4 or 7 of in vivo Ad5del or AdIL-1\beta overexpression. A volume ratio of 1:10 of patella washouts:culture medium was used. C: HEK293 and HEK293-TLR4 cells were stimulated with supernatants of patellae ex vivo cultured with IL-1\beta or TNF\alpha (10 ng/ml each) for 24 hours. A volume ratio of 1:10 of the patella supernatants was used in the presence of the TNF\alpha inhibitor Enbrel (1 μg/ml). D: HEK293-TLR4 cells were stimulated with supernatants of patellae ex vivo cultured with IL-1\beta or TNF\alpha (10 ng/ml each) for 24 hours. A volume ratio of 1:10 of the patella supernatants was used in the presence of the TNF\alpha inhibitor Enbrel (1 μg/ml). Media was measured in 24-hour culture supernatants using the Bioplex cytokine assay. Values are the mean ± SEM n = 6 patellae per group in B–D. n.s. = not significant, n.d. = not detectable. *P < 0.05 by Mann-Whitney U-test.
specific characteristics as well. IL-1 inhibits chondrocyte anabolic functions and mediates breakdown of PGs in cartilage.\(^{36}\) Furthermore, it promotes the production of nitric oxide and tissue destructive enzymes and the activation of osteoclasts and bone resorption.\(^{39}\) TNF\(_x\) is mainly involved in synovial inflammation through activation of endothelial cells and amplification of chemokines; however, it also contributes to osteoclast differentiation and activation via up-regulation of RANKL expression on mesenchymal cells and T cells.\(^{41}\) Several animal models of arthritis support a central role for IL-1 in driving cartilage destruction, as opposed to the role of TNF\(_x\) particularly in joint inflammation.\(^{42,43}\) In this study, higher concentration of AdIL-1\(_{\beta}\) virus was used compared with AdIL-1\(_{\beta}\) virus to achieve similar joint inflammation and destruction and permit an equitable comparison of the two cytokines. Comparable degree of synovial inflammation and tissue damage induced by IL-1 and TNF would allow the production and release of similar amounts of endogenous TLR ligands in case both cytokines would possess this capability.

Histological examination of the joints revealed TLR4 dependency of IL-1-induced local joint pathology. TLR4\(^{-/-}\) animals were protected against multiple pathological effects mediated by IL-1, including synovial inflammation, cartilage PG depletion, and bone damage (Figures 2 and 3), whereas the effects of TNF seemed less dependent on TLR4. Indeed, the subsequent ex vivo assays confirmed that IL-1\(_{\beta}\) was capable of inducing the release of endogenous TLR4 agonists from patella whereas TNF\(_x\) was not (Figure 6). The difference between IL-1 and TNF in this respect might have resulted from differential regulation of matrix degrading enzymes or differential induction of intracellularly expressed endogenous ligands; however, further studies in this respect are warranted. Involvement of TLR4 in systemic TNF-driven arthritis models such as TNF transgenic model requires further investigation as well as other immune processes might be involved there. Furthermore, a role for TLR4 seems plausible in later phases of TNF transgenic arthritis model where the disease pathogenesis becomes IL-1 dependent.\(^{44}\) The presence of endogenous TLR2 ligands in the tested conditions cannot be excluded and might explain the tendency of reduced joint pathology in TLR2\(^{-/-}\) animals. Nevertheless, TLR2 deficiency did not exert any considerable influence on local joint pathology in this model where adaptive immunity is not involved. Of high relevance, IL-1-driven cartilage and bone destruction was still TLR4 dependent under the condition of similar degree of inflammation, as occurred when AdIL-1 dose was enhanced (Figures 4 and 5). This indicates that the role of TLR4 in inflammation may be uncoupled from its role in joint destruction and suggests that reduced cartilage and bone destruction in TLR4\(^{-/-}\) mice does not necessarily rely on diminished inflammation.

Given the poor regenerative capacity of cartilage and conceding the central role of IL-1 in cartilage destruction, the substantial role of TLR4 in IL-1-mediated cartilage destruction is of crucial importance. Besides enzymes released from synovial cells and the consequent breakdown of cartilage matrix, breakdown may have resulted from the direct TLR4 activation of chondrocytes and the induction of MMPs from the latter. A direct effect of TLR4 activation on chondrocyte anabolic function, eg, collagen type II and aggrecan synthesis, has been reported before.\(^{1}\) In addition, TLR4 activation of primary osteoarthritic chondrocytes strongly induces MMP and NO release from these cells.\(^{45}\) In vivo evidence supporting the contribution of TLR4 to MMP-mediated cartilage destruction comes from our previous findings indicating TLR4-dependent expression of the MMP-specific aggrecan neoepitope VDIPEN in murine arthritic joints.\(^{46}\) Involvement of TLR4 in cartilage destruction makes it an intriguing candidate to target in combination with TNF to provide protection against cartilage destruction, an area where TNF blockers seem to fail.\(^{47}\)

A role for TLR4 in driving cathepsin K expression and the concomitant bone erosion (Figure 5) is in line with a previous report on promotion of osteoclastogenesis in monocyte cultures by TLR4 stimulation of the co-cultured fibroblast-like synoviocytes.\(^{48}\) Moreover, this observation is consistent with recent findings in another in vivo model of arthritis in which TLR4 activation was found to be partially responsible for cathepsin K expression in the joint.\(^{49}\)

The present data point toward a role for TLR4 in the "danger model" of immunity and, hence, may have implications for other inflammatory and tissue-destructive diseases beyond RA. Evidence from noninfectious disease conditions such as myocardial and hepatic ischemia-reperfusion injury and nonbacterial lung injury supports the involvement of TLR4-activating self-molecules in "sterile" inflammation.\(^{49-52}\) A role for TLR4 in atherosclerosis, where it might interact with endogenous ligands in atherosclerotic plaque, has also been indicated.\(^{53}\)

In the context of RA, several reports support the presence of TLR4 agonists in RA synovial fluid and serum, and indicate that activation of TLR4 by endogenous ligands partially defines the inflammatory character of RA synovial tissue.\(^{53,57}\) Indeed, the spontaneous production of proinflammatory cytokines, and some MMPs by RA synovial membrane cells can be inhibited by overexpression of dominant-negative forms of MyD88 and Mal, two essential adaptor molecules in signaling through TLR2 and TLR4.\(^{54}\)

Endogenous TLR4 agonists may either be derived from the inflammatory or necrotic cells, or become released on degradation of the extracellular matrix. Detection of endogenous TLR4 agonists in supernatants of patellae ex vivo cultured with IL-1 where inflammatory cells are absent (Figure 6) suggests resident components of the joint such as extracellular matrix as one of the sources of TLR4 agonists. Arthritic joints most presumably contain multiple TLR4 ligands, some of which might have greater clinical impact than others. For instance, concentration of extra domain A\(^{+}\) fibronectin in RA synovial fluid, but not plasma, is revealed to be a valuable predictor of radiographical joint destruction in RA patients.\(^{55}\) An important marker of inflammation, serum amyloid A\(_3\) has, recently been reported to activate TLR4 using a higher affinity for the TLR4/MD2 receptor complex than the classical TLR4 ligand of microbial origin.
lipid A. Importantly, the human homologues of serum amyloid A, i.e., serum amyloid A 1 and serum amyloid A 2, are up-regulated in RA synovium, induced by IL-1β, and contribute to the production of MMPs by primary chondrocytes, hence representing good candidates to activate TLR4. The exact source and nature of endogenous TLR4 agonists in our system remain, however, to be determined. The relative contribution of various ligands and the insight in the mechanisms of TLR4 activation in RA will provide opportunities to develop novel RA-specific therapeutic interventions without interfering with innate immune function in antimicrobial defense.

Acknowledgments

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References