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-1 β was dependent on TLR4. Overexpression of IL-1 β 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 NF κ B 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,^{5,6} whereas biglycan, hyaluronan fragments, high-mobility group box 1, and some endogenous heat-shock proteins activate both TLR2 and TLR4.⁷⁻⁹ 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.¹⁰⁻¹⁴ It has been reported that rheumatoid synovial fibroblast-like cells synthesize extra domain A-containing fibronectin.¹⁵ Furthermore, some inflammatory cytokines of high interest in the field of rheumatology such as TNF α , IL-1, and IL-6 can induce the expression of heat-shock protein 70 in cultured synovial fibroblast-like cells.¹² Another endogenous TLR4 ligand, the calcium-binding protein S100A8, has also been found in RA synovial membrane.^{16,17} Considering the highly inflammatory character of RA and the accompanying damage to the extracellular matrix, other endogenous TLR ligands are also very likely to be present in arthritic joints.

The idea of the involvement of TLR2 and TLR4 in RA is supported by their enhanced expression in blood and synovial cells of RA patients.¹⁸⁻²¹ In addition, monocyte-derived dendritic cells and synovial macrophages from RA patients are overresponsive to TLR2 and TLR4 stimulation compared with cells from healthy controls or cells from patients with other forms of inflammatory arthritis.^{22,23} Factors other than the level of TLR-2 and TLR-4 expression were suggested to contribute to the increased activation. As TLR-mediated inflammatory responses may induce further tissue damage and promote the generation of more endogenous ligands, it has been hypothesized that TLRs can engender a self-sustaining inflammatory loop responsible for chronic progression of inflammation.^{24,25} Nevertheless, the contribution of endogenous TLR ligands in the joint to local inflammatory and destructive processes has not thoroughly been studied yet. Therefore, we aimed to examine the involvement of endogenous TLR2 and TLR4 activation in joint inflammation, cartilage destruction, and bone erosion *in vivo*.

It is of interest that exogenous TLR ligands, including LPS, have extensively been used to aggravate or reactivate arthritis in distinct animal models²⁶⁻²⁹; however, the worldwide usage of microbial TLR ligands as adjuvants in arthritis models complicates the study of the contribution of endogenous ligands in arthritic process. In the present study, we used an adenoviral-based cytokine-overexpressing system instead of the commonly used arthritis models to circumvent the necessity of application of exogenous TLR2 and TLR4 ligands for evoking the immune response. TNF α and IL-1 β were the cytokines overexpressed locally in the joints. Years of research has implicated prominent roles of these cytokines in arthritis, and their prolonged overexpression in animal joints mimics the inflammatory and destructive processes observed in RA. Since the model used here does not involve systemic or adaptive immune responses, it enables us to specifically study the role of endogenous TLR2 and TLR4 ligands produced at the site of inflammation in the affected joints.

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-1 β virus was provided by Dr. C. D. Richards (McMaster University, Ontario, Canada) and was engineered as described previously.³⁰ AdTNF α 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.³¹ The empty viral vector Ad5del70-3 was used as negative control throughout the studies.

Induction of Arthritis Using Adenoviral Transfer of IL-1 β and TNF α 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 μ l of saline containing 3×10^5 plaque-forming units (PFU) AdIL-1 β or 1×10^7 PFU AdTNF α virus. A total of 1×10^7 PFU of the control virus Ad5del70-3 was injected into the contralateral knee joint. In the following studies, the dose of AdIL-1 β was enhanced to 3×10^6 PFU per joint to enforce IL-1 β -induced cartilage destruction. Previous reports have validated this adenoviral delivery system as an effective means of cytokine overexpression in synovial tissue.^{32,33}

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 μ m were stained using the H&E to study the

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, Wachttersbach, 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'-GGCAAATTCAACGGCACA-3' (forward) and 5'-GTTAGTGGGGTCTCGCTCTG-3' (reverse); and for cathepsin K, 5'-GAAGCAGTATAACAGCAAGGTGGAT-3' (forward) and 5'-TGCTCCCAAGTGGTTCATGG-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 (C_t) value of the gene of interest was corrected for the C_t of the reference gene glyceraldehyde-3-phosphate dehydrogenase to obtain the ΔC_t , then $\Delta\Delta C_t$ was calculated compared with Addel 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 xylol and rehydrated in serial dilutions of ethanol. Endogenous peroxidase was blocked using 1% hydrogen peroxide for 15 minutes. For NIMP-R14, 5 minutes treatment with 0.1% trypsin/0.1% CaCl_2 (pH 7.8) preceded this step. Sections were incubated with the following antibodies for 1 hour: rabbit anti-mouse cathepsin K antibodies (200 $\mu\text{g/ml}$; gift from Dr. E. Sakai, Nagasaki, Japan), rat anti-mouse NIMP-R14 (500 $\mu\text{g/ml}$; gift from Dr. M. Strath, London, UK), and rat anti-mouse F4/80 (500 $\mu\text{g/ml}$; AbD Serotec). Rabbit normal IgG (Santa Cruz Biotechnology) and rat IgG2b (BD Pharmingen) were used as negative controls. Subsequently, sec-

tions were incubated with biotinylated swine anti-rabbit or rabbit anti-rat antibodies, followed by peroxidase-labeled streptavidin. Color was developed with diaminobenzidine, and tissues were counterstained with hematoxylin. Staining was performed on two tissue sections per mouse ($n = 5$ wild-type and $n = 6$ $\text{TLR4}^{-/-}$ mice). Cathepsin K-positive cells were quantified along the bone surfaces in the patella-femur area using Leica Qwin software (Leica Microsystems, Rijswijk, The Netherlands). Mean number of positive cells per 1000 nm^2 for each mouse is depicted in the graph. Percentage of NIMP-R14- and F4/80-positive cells was scored arbitrarily in two sections per mouse in a blinded manner.

Assessment of Endogenous TLR4 Ligands in Patella Washouts on IL-1 Overexpression or Stimulation

HEK293 and HEK293-mTLR4/MD2/CD14 were purchased from InvivoGen and cultured according to the manufacturer's guidelines. For stimulations, 5×10^4 cells/well were used in flat-bottom 96-well plates. To validate the HEK293-mTLR4 cellular response, cells were stimulated with human IL-1 β and human TNF α (both 10 ng/ml; R&D Systems, Abingdon, UK), Pam3Cys (10 $\mu\text{g/ml}$; ECM Microcollections, Tuebingen, Germany), poly I:C (25 $\mu\text{g/ml}$; InvivoGen, Toulouse, France), LPS (10, 100, and 1000 ng/ml; Sigma-Aldrich, St. Louis, MO), and Gardiquimod (1 $\mu\text{g/ml}$) for 24 hours. To assess the induction of TLR4 agonists by IL-1 and TNF, 1-hour patella washouts obtained on several days of *in vivo* Adel or AdIL-1 β overexpression, or supernatants of patellae *ex vivo* cultured with IL-1 β or TNF α (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 Bio-plex cytokine assays. Where mentioned, 1 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ *Bartonella quintana* LPS as a TLR4 antagonist^{34,35} 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-1 β and TNF α

Local IL-1 β and TNF α production was determined after adenoviral gene transfer of these cytokines into the naïve

Figure 1. Induction of high intraarticular expression of IL-1 β and TNF α in wild-type (WT), *TLR2*^{-/-}, and *TLR4*^{-/-} mice. IL-1 β (A) or TNF α (B) overexpressing adenoviruses (3.5×10^5 and 1×10^7 PFU per joint, respectively) were injected into the knee joints of naive mice and concentrations of the respective cytokines were measured in the patella washouts collected 1 day after injection using Bioplex cytokine assays. Values represent the mean $\pm$ SEM of six mice per group. Injection of 1×10^7 PFU per joint of the control virus Ad5del did not induce any detectable levels of cytokines in wild-type or TLR-deficient mice (data not shown).

joints of C57BL/6 wild-type mice. Intra-articular injection of AdIL-1 β or AdTNF α 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-1 β for AdIL-1 and 120.0 ± 29.8 TNF α for AdTNF). 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-1 β or TNF α (data not shown).

Joint Pathology on Adenoviral Overexpression of IL-1 β and TNF α

Prolonged expression of IL-1 β or TNF α 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-1 β nor AdTNF α induced erosion of cartilage surface at these doses.

Although IL-1 β 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-1 β 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, TNF α -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-1 β 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-driven joint pathology, further studies focused on IL-1 β -induced arthritis. IL-1 inhibits chondrocyte PG and collagen synthesis at low concentrations; however, at high concentrations it also stimulates the synthesis of matrix degenerating proteases.³⁶ Therefore, we enhanced the dose of AdIL-1 β to enforce cartilage destruction. Joint inflammation on intraarticular injection of the enhanced dose of AdIL-1 β 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.

Figure 2. TLR4 dependency of local IL-1-induced joint pathology. **A:** Minor histological changes at day 4 of intraarticular injection of the Addel control virus (1×10^7 PFU per joint). **B–G** show the histological scores of arthritis in wild-type (WT) (black bars), $TLR2^{-/-}$ (gray bars), and $TLR4^{-/-}$ (white bars) mice at day 4 of intraarticular overexpression of AdIL-1 β (3.5×10^5 PFU per joint; **B–D**) or AdTNF α (1×10^7 PFU per joint; **E–G**). Synovial inflammation (**B** and **E**) was scored on H&E-stained tissue sections. PG depletion (**C** and **F**) and bone erosion (**D** and **G**) were scored on Safranin O-stained sections. Each parameter was scored on a 0- to 3-point scale in a blinded manner. Values are the mean $\pm$ SEM of six mice per group. n.s. = not significant; n.d. = not detectable. * $P < 0.05$ compared with wild-type by Mann-Whitney U -test.

Severe Bone Erosion and Osteoclast Formation on IL-1 β Overexpression Is Dependent on TLR4

Prolonged presence of high doses of IL-1 β 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 intra-

articular 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

	Infiltrate		Exudate		Synovial Inflammation
	NIMP-R14 ⁺ (%)	F4/80 ⁺ (%)	NIMP-R14 ⁺ (%)	F4/80 ⁺ (%)	
Wild-type	1.13 $\pm$ 0.18	30.0 $\pm$ 4.4	18.6 $\pm$ 2.6	92.1 $\pm$ 1.1	7.2 $\pm$ 1.9
$TLR2^{-/-}$	0.71 $\pm$ 0.18	34.2 $\pm$ 5.2	20.0 $\pm$ 1.8	91.7 $\pm$ 1.0	8.3 $\pm$ 1.4
$TLR4^{-/-}$	0.65 $\pm$ 0.09*	33.3 $\pm$ 3.6	20.8 $\pm$ 1.5	90.0 $\pm$ 2.3	10.0 $\pm$ 1.3

Synovial inflammation was scored on H&E-stained joint sections obtained at day 4 of intra-articular injection of AdIL-1 β (3.5×10^5 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.

Figure 3. Representative images of the knee joints at day 4 of AdIL-1 β overexpression. **Left panels** show synovial inflammation on H&E-stained sections, and **right panels** show PG depletion (loss of red staining) on Safranin O-stained sections. Both parameters were reduced in *TLR4*^{-/-} (**lower panels**) compared with wild-type (WT) (**upper panels**) and *TLR2*^{-/-} (**middle panels**) mice. Original magnification ($\times 100$) for H&E and ($\times 200$) for Safranin O staining. B = bone, C = cartilage, F = femur, JS = joint space, P = patella.

bone surface in wild-type mice (Figure 5, C and D). Quantitative analysis revealed that, consistent with PCR data, cathepsin K protein expression was significantly reduced in *TLR4*^{-/-} knee joints compared with the wild-type joints (Figure 5C). A representative picture of the multinucleated cathepsin K-expressing cells is shown in Figure 5D.

IL-1, but not TNF, Induces Release of Endogenous TLR4 Agonists from Patella

In vivo data indicated the involvement of TLR4 activation in the severity of joint inflammation and destruction on overexpression of IL-1 β . To assess the capability of IL-1 β and TNF α to induce endogenous TLR4 agonists, we used HEK293 cells stably expressing components of the murine TLR4 complex (TLR4, MD2, and CD14). Validation of the cytokine response of this cell line to a range of cyto-

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-1 β itself while being sensitive to TNF α (Figure 6A).

Incubation of HEK-TLR4 cells with patella washouts from AdIL-1 β -transduced wild-type joints resulted in robust TLR4 activation and high cytokine production, an effect not found on stimulation with washouts from the AdIL-1 β -transduced joints (Figure 6B). This indicates that AdIL-1 β overexpression in the joint induces the release of endogenous TLR4 ligands from patellae and the surrounding synovial tissue. Interestingly, these effects could be mimicked *ex vivo* when naive wild-type patellae were stimulated with recombinant IL-1 β . 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 TNF α and TNF might be present in the supernatants, high concentrations of the TNF blocker Enbrel, revealed to completely inhibit TNF α 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 response of the cells (Figure 6C). 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-1 $\text{rn}^{-/-}$ arthritis, in which the adaptive immune response represents a central part of the pathogenesis.^{34,37} The dominant role of TLR4

Figure 4. TLR4 dependency of local IL-1-induced cartilage destruction despite similar synovial inflammation. Data show joint pathology at day 4 of high AdIL-1 β overexpression (3.5×10^6 PFU per joint). Synovial inflammation (**A**) was scored on H&E-stained, and cartilage destruction (**B**) was scored on Safranin O-stained sections on a 0- to 3-point scale. Values are the mean $\pm$ SEM of at least five mice per group. n.s. = not significant. $**P < 0.01$ compared with wild-type (WT) by Mann-Whitney *U*-test. **C:** Representative images of Safranin O-stained tissue sections showing reduced joint pathology in *TLR4*^{-/-} mice compared with wild-type and *TLR2*^{-/-} mice. **Open** and **solid arrows** indicate cartilage destruction and bone erosion, respectively. Original magnification, $\times 100$.

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 β overexpression (3.5×10^6 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 Δ Ct, then $\Delta\Delta$ Ct was calculated compared with the Addel control. Cathepsin K protein expression (**C**) was detected by immunohistochemistry and quantified using Leica Qwin software. Values in **A** ($n \geq 5$) and **B** ($n = 4$) are the mean $\pm$ 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, $\times 200$. P = patella; F = femur.

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 β and TNF α 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 β and TNF α 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 $_2$,^{38–40} they exhibit

Figure 6. IL-1 β , but not TNF α , induces the release of endogenous TLR4 agonists from patellae. **A:** HEK293-mTLR4/MD2/CD14 cells were stimulated with IL-1 β , TNF α , and various TLR ligands. Stimulation was 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 β 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 β or TNF α (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 α inhibitor Enbrel (1 μ g/ml). **D:** HEK293-TLR4 cells were stimulated with *E. coli* LPS (100 ng/ml) or supernatants of *ex vivo*-cultured patella as in **C**, either or not following 30 minutes of preincubation with TLR4 antagonist (1 μ g/ml). IL-8 was measured in 24-hour culture supernatants using the Bioplex cytokine assay. Values are the mean $\pm$ 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 α 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 α particularly in joint inflammation.^{42,43} In this study, higher concentration of AdTNF α virus was used compared with AdIL-1 β 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 β was capable of inducing the release of endogenous TLR4 agonists from patella whereas TNF α 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 break-

down 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 neopeptide 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.⁴⁶

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.^{23,37} 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.⁵⁶ Importantly, the human homologues of serum amyloid A 3, ie, serum amyloid A 1 and serum amyloid A 2, are up-regulated in RA synovium, induced by IL-1 β , and contribute to the production of MMPs by primary chondrocytes,⁵⁷ 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.

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