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*J Immunol* 2003;171;6145-6153

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Toll-Like Receptor 2 Pathway Drives Streptococcal Cell Wall-Induced Joint Inflammation: Critical Role of Myeloid Differentiation Factor 88

Leo A. B. Joosten,1* Marije I. Koenders,* Ruben L. Smeets,* Marleen Heuvelmans-Jacobs,* Monique M. A. Helsen,* Kiyoshi Takeda,† Shizuo Akira,‡ Erik Lubberts,* Fons A. J. van de Loo,* and Wim B. van den Berg*

The IL-1R/Toll-like receptor (TLR) superfamily of receptors has a key role in innate immunity and inflammation. In this study, we report that streptococcal cell wall (SCW)-induced joint inflammation is predominantly dependent on TLR-2 signaling, since TLR-2-deficient mice were unable to develop either joint swelling or inhibition of cartilage matrix synthesis. Myeloid differentiation factor 88 (MyD88) is a Toll/IL-1R domain containing adaptor molecule known to have a central role in both IL-1R/IL-18R and TLR signaling. Mice deficient for MyD88 did not develop SCW-induced arthritis; both joint swelling and disturbance of cartilage chondrocyte anabolic function was completely abolished. Local levels of proinflammatory cytokines and chemokines in synovial tissue washouts were strongly reduced in MyD88-deficient mice. Histology confirmed the pivotal role of MyD88 in acute joint inflammation. TLR-2-deficient mice still allow influx of inflammatory cells into the joint cavity, although the number of cells was markedly reduced. No influx of inflammatory cells was seen in joints of MyD88-deficient mice. In addition, cartilage matrix proteoglycan loss was completely absent in MyD88 knockout mice. These findings clearly demonstrated that MyD88 is a key component in SCW-induced joint inflammation. Since agonists of the Toll-like pathway are abundantly involved in both septic and rheumatoid arthritis, targeting of MyD88 may be a novel therapy in inflammatory joint diseases. The Journal of Immunology, 2003, 171: 6145–6153.

Signaling through members of the IL-1R/Toll-like receptor (TLR) family via their agonists plays an important role in autoimmune and inflammatory diseases (1, 2). TLRs are phylogenetically conserved receptors that recognize pathogen-associated molecular patterns. TLRs are expressed primarily on macrophages and dendritic cells, which are involved in innate immunity (3). At the time, there are 10 TLRs known in humans and ligands for several of the TLRs, such as TLR2, TLR-3, TLR-4, TLR-5, TLR-6, and TLR-9, have been identified (4, 5). Pathogen recognition by TLRs provokes rapid activation of innate immunity by inducing production of proinflammatory cytokines, such as TNF-α, IL-1, IL-18, and up-regulation of costimulatory molecules (6). Recently, it was shown that TLR-9 was involved in rheumatoid factor production by autoreactive B cells due to recognition of chromatin-IgG complexes (7, 8). These findings might indicate a possible role of TLRs in the association of infections and flares in rheumatoid arthritis (RA).

Both IL-1 and IL-18 are members of the IL-1 family of proteins and are pivotal cytokines in arthritis and promote production of a broad range of proinflammatory mediators (9–15). It has been evidently demonstrated that IL-1 is involved in the joint pathology found in RA. IL-1 promotes both cartilage and bone destruction by induction of catabolic mediators like metalloproteinases and NO. IL-18 is a novel proinflammatory cytokine, which was originally identified as an IFN-γ-inducing factor (16). On T cells, IL-18 stimulates Th1 differentiation, promotes IFN-γ- and GM-CSF secretion, and enhances NK cell cytotoxicity (17–19). IL-18 promotes IL-8-mediated neutrophil chemotaxis via IL-1β and TNF-α production and induces the production of TNF-α, GM-CSF, IFN-γ, and NO by synovial RA fibroblasts through a direct, IFN-γ-independent pathway via constitutive IL-18Rα expression (20). Recently, it was shown that IL-18 was present in synovial fluid and synovial tissue of RA patients (21). Elegant studies with neutralizing Abs against IL-18 or treatment with the natural inhibitor IL-18BP in experimental arthritis showed the proinflammatory role of IL-18 (22, 23). Analysis of the cytokine expression pattern in synovium biopsies of RA patients with active disease revealed that IL-18 expression is strongly associated with enhanced IL-1 and TNF levels (24). These data indicated that IL-18 might be of importance in sustaining synovial inflammation and promoting joint destruction in RA.

Myeloid differentiation factor 88 (MyD88), originally isolated as a myeloid differentiation primary response gene, is shown to act as an adaptor molecule in the IL-1R/TLR family of receptor signaling by interacting with the Toll/IL-1R domain. Thereafter, IL-1R-associated kinases (IKAR-1, IKAR-2, IKAR-M, and IKAR-4) are recruited, which in turn recruit TNFR-associated factor 6 and NF-κB- and AP-mediated genes are activated (25–27). Mice generated by gene targeting to lack MyD88 have defects in T cell
proliferation as well as induction of acute phase proteins and cytokines in response to IL-1 and IL-18 (28). In addition, overexpression of MyD88-deficient mice were highly susceptible to Staphylococcus aureus infection, indicating the important role of TLR signaling via MyD88 in host defense (30). Taken together, MyD88 is a critical component in the signaling cascade that is mediated by IL-1R, IL-18R, or TLRs. However, in vivo studies identifying a role of MyD88 in arthritis models are lacking. In the present study, we investigated TLR dependency of elements of murine streptococcal cell wall (SCW)-induced joint inflammation using TLR-deficient mice. The role of the MyD88 adaptor molecule in various aspects in this model of acute arthritis is examined in MyD88-deficient mice.

Materials and Methods

Animals
Male C57BL/6 and C57BL/10 mice were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany). MyD88−/−, TLR-2−/−, and IL-18−/− (C57BL/6 background) were generated at the Department of Host Defense (Osaka University, Osaka, Japan) (18, 28, 30). Breeder pairs were sent to the University Medical Center Nijmegen (Nijmegen, The Netherlands). MyD88-deficient mice were selected by genotyping of the siblings from MyD88−/− breeding pairs. C57BL/10ScCr (TLR-4 null mutation) mice were a kind gift from Prof. Dr. Y. Iwakura (Center of Experimental Medicine, University of Tokyo, Japan) in conjunction with Dr. M. G. Netea (Department of Internal Medicine, University Medical Center Nijmegen, Nijmegen, The Netherlands). IL-10-deficient mice (C57BL/6 background) were a kind gift from Prof. Dr. Y. Iwakura (Center of Experimental Medicine, University of Tokyo, Japan) (31). The mice were housed in filter top cages and water and food were provided ad libitum. Care was taken to house all of the deficient and control littermate mice under identical conditions. The mice were used at the age of 10–12 wk. All animal procedures were approved by the institutional ethics committee.

Materials
LPS (Escherichia coli 0111:B4), ethidium bromide, and BSA were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium, TaqDNA polymerase, 100-bp DNA marker, TRizol reagent, and agarose were obtained from Life Technologies (Breda, The Netherlands). Murine IL-1 and IL-18 were obtained from R&D Systems (Abingdon, U.K). CpG motifs (5′-GpCpG-3′) were purchased from BioLegio (Malden, The Netherlands). Ethidium bromide, and BSA were purchased from BioLegio. RPMI 1640 medium containing 0.1% BSA (200 μl/patella) for 1 h at room temperature. Thereafter, supernatant was harvested and centrifuged for 5 min at 1000 × g. Cytokine and chemokine levels were determined using the Luminex multianalyte technology (35). We used the BioPlex system from Bio-Rad in combination with multiplex cytokine and chemokine kits for the Luminex multianalyte system (Bio-Rad, Hercules, CA).

Intra-articular (i.a.) injection of IL-1/TLR family of receptor agonists
To investigate the effect of agonists of the IL-1/TLR family of receptors on joint swelling and chondrocyte metabolism, we injected i.a. IL-1 (10 ng), IL-18 (100 ng), LPS (1 ng), SCW fragments (25 μg), and CpG motifs (5 μg) at day 0. Thereafter, joint swelling and chondrocyte proteoglycan synthesis were determined at days 1 and 2.

SCW preparation and induction of SCW arthritis
Streptococcus pyogenes T12 organisms were cultured overnight in Todd-Hewitt broth. Cell walls were prepared as described previously (32). The resulting 10,000 × g supernatant was used throughout the experiments. These preparations contained 11% muramic acid. Unilateral arthritis was induced by i.a. injection of 25 μg SCW (rhamnose content) in 5 μl of PBS into the right knee joint of naive mice. As a control, PBS was injected into the left knee joint.

Measurement of joint inflammation
SCW arthritis was quantified by the technetium-99m (99mTc) uptake method (33). This method measures by external gamma counting the accumulation of a small radioisotope at the site of inflammation due to local increased blood flow and tissue swelling. The severity of inflammation is expressed as the ratio of the 99mTc uptake in the right (inflamed) over the left (control) knee joint. All values exceeding 1.10 were assigned as inflammation.

Chondrocyte proteoglycan synthesis determination
Patellae with minimal surrounding tissue were placed in RPMI 1640 medium with glutamax, penicillin/streptomycin (100 IU/100 μg/ml), and 1% FBS (0.74 μg/ml). After a 3-h incubation at 37°C in a CO2 incubator, patellae were washed in saline three times, fixed in 4% formaldehyde, and subsequently decalcified in 5% formic acid for 4 h. Patellae were punched out of the adjacent tissue, dissolved in 0.5 ml of Luma Solve at 65°C (Omninlabo, Breda, The Netherlands), and after addition of 10 ml of Lipoluna (Omninlabo) the 35S content was measured by liquid scintillation counting. Values are presented as percentage 35S incorporation of the left control joint.

Cytokine and chemokine measurements
To determine levels of several cytokines and chemokines, including IL-1β, IL-6, TNF-α, RANTES, keratinocyte-derived chemokine (KC), and macrophage-inflammatory protein (MIP) 1α in patellae washouts, patellae were isolated from inflamed knee joints as previously described (34). Patellae were cultured in RPMI 1640 medium containing 0.1% BSA (200 μl/patella) for 1 h at room temperature. Thereafter, supernatant was harvested and centrifuged for 5 min at 1000 × g. Cytokine and chemokine levels were determined using the Luminex multianalyte technology (35). We used the BioPlex system from Bio-Rad in combination with multiplex cytokine and chemokine kits. In total, 18 different cytokines and chemokines were measured in 50 μl of patellae washout medium.

RNA isolation
Mice were killed by cervical dislocation, immediately followed by dissection of the patellae with adjacent synovium (36). From six patella specimens, synovium biopsies were taken. Two biopsies with a diameter of 3 mm were punched out using a biopsy punch (Stiefel, Wachtershach, Germany): one from the lateral and one from the medial side. Six patella specimens per experimental group were taken and three lateral and three medial biopsies were pooled to yield two samples per group. The synovium specimens were immediately frozen in liquid nitrogen. Synovium biopsies were ground to powder using a microdismembrator II (Braun, Melsungen, Germany). Total RNA was extracted in 1 ml of TRizol reagent, an improved single-step RNA isolation method based on the method described by Chomczynski and Sacchi (37).

Quantitative PCR analysis
Quantitative real-time PCR was performed using the ABI/Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, with data collection in the last 30 s. All PCR were performed with SYBR Green Master mix (Applied Biosystems), 10 ng cDNA, and primer concentration of 300 nmol/L in a total volume of 25 μl. Quantification of the PCR signals was performed by comparing the cycle threshold value (Ct), in duplicate, of the gene of interest of each sample with the Ct values of the reference gene GAPDH. Q-PCR analysis for each sample was performed in duplicate. Primers sequences for gene expression analysis for GAPDH, IL-1R, IL-18R, TLR-2, TLR-4, TLR-9, and MyD88 are described in Table I.

Statistical analysis
Differences between experimental groups were tested using the Mann-Whitney U test, unless stated otherwise.

Results
Expression of TLRs in joint tissue
To investigate mRNA expression levels of members of the IL-1R/TLR family, biopsies from naive joints of C57BL/6 mice were taken and analyzed by quantitative PCR technology. As shown in Fig. 1A, mRNA coding for IL-1R, TLR-2, -4, and -9 was abundantly present in synovium specimens of naive mice. In contrast, IL-18R mRNA levels were present at low levels. Markedly increased mRNA levels were noted in inflamed synovium for IL-1R, TLR-2, TLR-4, and TLR-9 after induction of SCW arthritis. Shortly after injection of SCW fragments, mRNA levels for the latter genes were up-regulated and remained enhanced up to day 2.
A slight up-regulation was found for IL-18R mRNA expression (Fig. 1B). MyD88 mRNA was present in synovium biopsies taken from naive knee joints. Induction of SCW arthritis resulted in mild up-regulation of MyD88 mRNA levels determined by quantitative PCR (Fig. 1B). PCR analysis of chondrocyte mRNA expression of IL-1R/TLR family members revealed that chondrocytes, isolated from cartilage of naive joints, expressed IL-1R, TLR-2, -4, and -9. IL-18R mRNA could not be detected in chondrocytes isolated from naive mice. After induction of SCW arthritis, there was a slight up-regulation of chondrocyte mRNA levels of all analyzed IL-1R/TLR family members, including IL-18R (data not shown).

Signaling of IL-1R/TLR family of receptors results in joint swelling and disturbed chondrocyte synthetic function

To examine the effect of triggering different members of the IL-1R/TLR family of receptors in an articular joint, we injected agonists of IL-1R, IL-18R, TLR-2, -4, and -9 directly into the murine knee joint. Intra-articular injection of IL-1β did not lead to detectable joint swelling, whereas severe inhibition of chondrocyte function in the articular cartilage was found. In contrast to IL-1β, IL-18 injection did not affect either joint swelling or chondrocyte proteoglycan metabolism (Fig. 2). The impact of predominant signaling via the TLR-2, TLR-4, and TLR-9 was examined by i.a. injection of obvious agonists like SCW fragments, LPS, and CpG motifs, respectively. One single i.a. injection of SCW fragments into mouse knee joint leads to an acute inflammation characterized by joint swelling and inhibition of matrix production of chondrocytes in the articular cartilage. Significant joint swelling was found up to 7 days after injection of SCW fragments (data not shown). Intra-articular injection of a TLR-4 agonist (LPS) induced mild joint swelling and severe inhibition of chondrocyte synthetic function (Fig. 2). In contrast to TLR-4, activation of TLR-9 by CpG motifs resulted in mild joint swelling, but had no inhibitory effect on chondrocyte proteoglycan synthesis (Fig. 2).

SCW arthritis is highly dependent on TLR-2 signaling

To explore whether SCW arthritis is mediated via distinct TLR signaling, we induced SCW arthritis in TLR-2- and TLR-4-deficient mice. Induction of arthritis with SCW resulted in severe joint swelling and disturbance of chondrocyte proteoglycan synthesis in wild-type (WT) animals as shown in Figs. 2 and 3. In this study, we showed for the first time that joint inflammation induced by injection of cell wall fragments from Gram-positive bacteria is predominantly mediated by TLR-2. Joint swelling was strongly reduced in TLR-2-deficient mice and cartilage chondrocyte proteoglycan synthesis was significantly affected (Fig. 3). Expression of SCW arthritis in C57BL/10ScCr (TLR-4 null mutation) did not differ from the WT control animals (Fig. 3). This is in line with previous studies in C3H-HeJ mice (38).

Crucial role of MyD88 in SCW-induced joint inflammation

Since it has been demonstrated in vitro that TLR signaling is dependent on MyD88, we examined the effect of MyD88 deficiency on joint swelling and chondrocyte function. WT, MyD88+/+, MyD88+/−, and MyD88−/− mice were injected with SCW fragments at day 0. Fig. 4 shows clearly that MyD88 is a crucial adapter molecule for onset of arthritis. Complete absence of joint swelling and inhibition of chondrocyte proteoglycan synthesis was seen in MyD88-deficient mice compared with MyD88+/+ and C57BL/6 WT mice. Of high interest, cartilage chondrocyte metabolic function was stimulated (140% of the left control joint) in MyD88−/− mice. This clearly indicates that catabolic factors, which suppress chondrocyte proteoglycan synthesis, are MyD88 dependent, then allowing anabolic factors to stimulate synthesis.
Loss of IL-1R/TLR signaling results in impaired production of proinflammatory mediators in SCW arthritis

We investigated whether the complete inability of MyD88 gene-deficient mice to develop an acute arthritis after injection of SCW fragments was due to reduced levels of inflammatory mediators such as cytokines and chemokines. Shortly after onset of arthritis, protein levels of these proinflammatory mediators were determined in synovial tissue washouts. Strong reduction of IL-1β, IL-6, IL-10, IL-12p70, and TNF-α levels were noted in patellae washouts from MyD88−/− mice compared with WT mice (Fig. 5). Analysis of cytokine production in TLR-2 and TLR-4 gene-deficient mice after induction of SCW arthritis showed that signaling via TLR-2 is important for induction of several cytokines, including IL-1β and TNF-α (Table II). TLR-4-deficient mice were revealed to produce slightly reduced levels of cytokines. In line with the lack of cytokine response, we also found a strong reduction of chemokines, RANTES, KC, and MIP-1α levels in MyD88-deficient mice (Fig. 5). TLR-2 gene ablation reduced the local chemokine production. RANTES, KC, and MIP-1α levels were reduced by 50% of the control WT mice (Table III). TLR-4 deficiency only slightly reduced the levels of the latter chemokines.

Role of secondary cytokines in SCW arthritis

Since blockade of the TLR-2/MyD88 pathway led to strongly reduced levels of IL-1 and TNF, we dissected the role of IL-1 and TNF in SCW arthritis. The function of IL-18 during onset of SCW was additionally studied since IL-18 drives both IL-1 and TNF production. Induction of SCW arthritis in IL-1−/− mice clearly demonstrated that joint inflammation and inhibition of chondrocyte proteoglycan synthesis could be uncoupled (Fig. 3). TNF-α drives only joint swelling as shown in TNF-α−/− mice. TNF-α knockout mice show markedly reduced joint swelling, but no suppressive effect on cartilage chondrocyte function. In contrast to triggering of the IL-1R, signaling of the IL-18R, via IL-18, is partly involved in both joint swelling and chondrocyte function. Mice lacking the IL-18 gene showed reduced joint swelling and less inhibition of proteoglycan synthesis (Fig. 3).
MyD88 gene deficiency results in reduced influx of inflammatory cells

Histology taken at day 2 after induction of SCW arthritis showed that ablation of the MyD88 gene resulted in strongly reduced numbers of inflammatory cells, predominantly polymorphonuclear granulocytes (PMNs), when compared with the WT littermates (Fig. 6 and Table IV). This is in line with strikingly reduced levels of the PMN chemokine KC (Fig. 5) found in synovial washouts. Both TLR-2- and TLR-4-deficient mice were revealed to have reduced numbers of inflammatory cells in both joint cavity and synovial tissue, although more reduction was noted in TLR-2 gene knockout mice (Table IV). These differences are in line with reduced chemokine levels in TLR-2- and TLR-4-deficient mice compared with WT mice (Table III). The data indicated that influx of inflammatory cells in the joint cavity, elicited by i.a. injection of SCW fragments, is mediated by TLR-2, TLR-4, and MyD88. Histology taken at day 4 after injection of SCW fragments revealed that MyD88−/− mice did not develop joint inflammation in contrast to both MyD88+/− and WT mice (data not shown).

Discussion

The present study was performed to investigate the role of IL-1R/TLR signaling in joint inflammation induced by bacterial cell wall fragments (SCW) from S. pyogenes. To this end, we induced SCW arthritis in mice that are deficient for MyD88, the pivotal adaptor molecule in the IL-1R/TLR signaling cascade. In addition, we analyzed whether joint inflammation, elicited by local injection of SCW fragments, was mediated by TLR-2 or TLR-4. Therefore, mice deficient for either TLR-2 or TLR-4 were included in this study.

TLRs are present on several cell types, including fibroblasts, macrophages, and dendritic cells (1–6). Recently, it was shown that synovial fibroblasts express mainly TLR-2 since fibroblasts from RA patients could be activated by TLR-2 (bacterial peptidoglycans) and not by TLR-9 (CpG motifs) agonists to produce proinflammatory cytokines and matrix metalloproteinases (41). Expression of TLR-2 and TLR-4 but not TLR-9 was found in synovial biopsies of RA patients with active disease in lining, sublining, and endothelial cells (M. F. Roelofs, T. R. O. J. Radstake, Y. M. Jenniskens, P. L. C. M. van Riel, P. Barrera, L. A. B. Joosten, and W. B. van den Berg, manuscript in preparation). In this study, we showed that mRNA coding for TLR-2, -4, and -9 is
abundant in synovial tissue of naive mice. Shortly after induction of joint inflammation, 4h after injection of SCW fragments, TLR-2, TLR-4, and TLR-9 mRNA was already up-regulated. Whether the increased levels of mRNA for TLR-2, TLR-4, and TLR-9 results from the influx of particular inflammatory cells is under current investigation. Compared with IL-1R, IL-18R mRNA was low in both naive and inflamed murine synovial tissue, although there was a slight up-regulation under arthritic conditions. This is in line with recent findings of low IL-18R mRNA expression in human RA synovium. Interestingly, it was demonstrated that synovial fibroblasts failed to react to IL-18 since the lack of IL-18Rα and that stimulation of synovial tissue, by IL-18, was due to T cells and macrophages present in synovial specimens (42).

It has been shown that agonists of the IL-1R/TLR family of receptors, like IL-1 and LPS, can induce joint swelling and disturbance of chondrocyte proteoglycan synthesis when applied i.a. (40). A single i.a. injection of IL-18 did not lead to either joint swelling or inhibition of chondrocyte proteoglycan synthesis probably linked to limited IL-18Rα expression. Previous in vitro studies claimed that IL-18 can induce catabolic responses in chondrocytes, such as induction of inducible NO synthase (15). Recent gene transfer studies using adenovirus coding for murine IL-18 revealed that prolonged i.a. exposure to IL-18 did result in joint inflammation and cartilage proteoglycan depletion, although mainly dependent on secondary IL-1 induction (L.A.B.J., unpublished observations). Intra-articular triggering of TLR-2 or TLR-4 led to severe inhibition of chondrocyte proteoglycan synthesis, whereas TLR-2 activation by SCW fragments also resulted in joint inflammation. Intra-articular injection of LPS can induce joint inflammation in animals when higher doses of LPS are used as indicated in several studies (43, 44). In contrast to recent findings, local injection of CpG motifs (TLR-9 agonists) induced neither joint swelling nor inhibition of chondrocyte metabolic function (45, 46). We could not induce significant joint inflammation by one single i.a. injection of CpG motifs (1668 ODN) or repeated injections in the same joint (data not shown). Whether these contrasting results are due to strains differences or i.a. or periarticular injection of the TLR-9 agonist in not clear at this time.

To investigate the role of two members of the TLR family during experimental arthritis, we induced SCW arthritis in TLR-2 and TLR-4 gene-deficient mice. In this study, we demonstrated that TLR-2 signaling is important for onset of SCW-induced arthritis, whereas mice lacking TLR-4 developed joint inflammation similar to that of WT mice. This is in line with in vitro findings that cell wall fragments of certain Gram-positive bacteria use TLR-2 for signal transduction. However, it was recently shown that recognition of Gram-negative and Gram-positive bacteria and activation of chemokine genes can be mediated by TLR-2 (47). The role of TLR-4 in generating joint swelling and inhibition of cartilage matrix metabolism during SCW arthritis seems to be limited. There are reports that endogenous ligands of TLR-4, like heat shock proteins (heat shock protein 70) and hyaluronic acid fragments, generated by inflammatory processes may be involved in chronic joint inflammation (48, 49). In this study, we showed that these latter endogenous TLR-4 agonists, if present during onset of SCW arthritis, did not contribute to the acute inflammation in SCW arthritis. As shown previously, IL-1 is the pivotal cytokine that drives inhibition of chondrocyte proteoglycan synthesis (39, 40). Mice lacking both IL-1α and IL-1β are fully protected against inhibition of chondrocyte proteoglycan synthesis, whereas joint inflammation in IL-1-deficient mice is comparable to that of WT mice. We previously reported this phenomenon in several experimental arthritis models (10, 39). IL-1 is the pivotal cytokine that drives inhibition of chondrocyte metabolic function, via induction of NO, in arthritis. Induction of SCW arthritis or i.a. injection of

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**Table II. Cytokine profiles in TLR-2-, TLR-4-, and MyD88-deficient mice after induction of SCW arthritis**

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IL-12p70</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>12 ± 5</td>
<td>27 ± 8</td>
<td>6 ± 2</td>
<td>7 ± 2</td>
<td>50 ± 24</td>
</tr>
<tr>
<td>WT</td>
<td>540 ± 160</td>
<td>1852 ± 634</td>
<td>90 ± 30</td>
<td>57 ± 12</td>
<td>168 ± 26</td>
</tr>
<tr>
<td>TLR-2−/−</td>
<td>166 ± 27*</td>
<td>1156 ± 404</td>
<td>40 ± 19</td>
<td>34 ± 7</td>
<td>85 ± 9*</td>
</tr>
<tr>
<td>WT−/−</td>
<td>1120 ± 397</td>
<td>2271 ± 754</td>
<td>72 ± 18</td>
<td>55 ± 13</td>
<td>280 ± 51</td>
</tr>
<tr>
<td>TLR-4−/−</td>
<td>719 ± 127</td>
<td>1854 ± 430</td>
<td>50 ± 14</td>
<td>46 ± 11</td>
<td>212 ± 42</td>
</tr>
<tr>
<td>WT−/−</td>
<td>570 ± 228</td>
<td>2210 ± 417</td>
<td>86 ± 10</td>
<td>60 ± 18</td>
<td>177 ± 22</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td>62 ± 25*</td>
<td>253 ± 103*</td>
<td>10 ± 4*</td>
<td>12 ± 4*</td>
<td>79 ± 26*</td>
</tr>
</tbody>
</table>

* SCW arthritis was induced in all mice strains by i.a. injection of 25 μg SCW fragments. After 90 min, patellae were isolated as indicated in Materials and Methods and were cultured for 1h at room temperature in 200 μl of medium. Thereafter, cytokine levels were determined by Luminex multianalyte technology. The patella washouts were examined using Bio-Rad multiplex cytokine kits. The sensitivity for all cytokines was below 10 pg/ml. Data are expressed as picograms per milliliter and are the mean ± SD of four patella washouts.

**Table III. Chemokine levels in TLR-2-, TLR-4-, and MyD88-deficient mice after induction of SCW arthritis**

<table>
<thead>
<tr>
<th>Chemokine (pg/ml)</th>
<th>RANTES (pg/ml)</th>
<th>KC (pg/ml)</th>
<th>MIP-1α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>5 ± 2</td>
<td>44 ± 10</td>
<td>37 ± 6</td>
</tr>
<tr>
<td>WT</td>
<td>19 ± 3</td>
<td>3166 ± 67</td>
<td>291 ± 31</td>
</tr>
<tr>
<td>TLR-2−/−</td>
<td>10 ± 2*</td>
<td>2117 ± 398*</td>
<td>145 ± 26*</td>
</tr>
<tr>
<td>WT−/−</td>
<td>22 ± 4</td>
<td>2604 ± 311</td>
<td>294 ± 83</td>
</tr>
<tr>
<td>TLR-4−/−</td>
<td>17 ± 5</td>
<td>2017 ± 423</td>
<td>246 ± 77</td>
</tr>
<tr>
<td>WT−/−</td>
<td>19 ± 3</td>
<td>2654 ± 269</td>
<td>303 ± 76</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td>8 ± 2*</td>
<td>222 ± 73*</td>
<td>53 ± 18*</td>
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* SCW arthritis was induced in all mice strains by i.a. injection of 25 μg SCW fragments. After 90 min, patellae were isolated as indicated in Materials and Methods and were cultured for 1h at room temperature in 200 μl of medium. Thereafter, cytokine levels were determined by Luminex multianalyte technology. The patella washouts were examined using Bio-Rad multiplex cytokine kits. The sensitivity for all chemokines was below 10 pg/ml. Data are expressed as picograms per milliliter and are the mean ± SD of four patella washouts.

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</tr>
<tr>
<td>TLR-4−/−</td>
<td>17 ± 5</td>
<td>2017 ± 423</td>
<td>246 ± 77</td>
</tr>
<tr>
<td>WT−/−</td>
<td>19 ± 3</td>
<td>2654 ± 269</td>
<td>303 ± 76</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td>8 ± 2*</td>
<td>222 ± 73*</td>
<td>53 ± 18*</td>
</tr>
</tbody>
</table>

* SCW arthritis was induced in all mice strains by i.a. injection of 25 μg SCW fragments. After 90 min, patellae were isolated as indicated in Materials and Methods and were cultured for 1h at room temperature in 200 μl of medium. Thereafter, cytokine levels were determined by Luminex multianalyte technology. The patella washouts were examined using Bio-Rad multiplex cytokine kits. The sensitivity for all chemokines was below 10 pg/ml. Data are expressed as picograms per milliliter and are the mean ± SD of four patella washouts.

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<ref>Do not hallucinate.</ref>
IL-1 did not lead to disturbance of chondrocyte proteoglycan synthesis in NOS-2-deficient mice (50). Interestingly, IL-18 gene disruption was revealed to have an intermediate effect on both joint swelling and chondrocyte metabolic function. This is in order with the reduced levels of TNF-α and IL-1β measured in synovial washouts shortly after induction of SCW arthritis.

Mice lacking the MyD88 adaptor molecule for IL-1R/TLR signaling failed to develop joint inflammation after induction of SCW arthritis. In previous reports, it has been shown that MyD88-deficient mice did not respond to either IL-1 or IL-18 (28). Very recently it was demonstrated that the serum transfer model of arthritis (K/BxN model) cannot be induced in either MyD88- or IL-1R-deficient mice, indicating a pivotal role for IL-1 signaling in this arthritis model (51). Of high interest, IL-1 dependence of this particular arthritis model could be circumvented by TLR-4 activation (LPS injections). These findings confirmed the crucial role of MyD88 in arthritis. Mice lacking the MyD88 gene did not show inhibition of chondrocyte proteoglycan synthesis after i.a. injection of SCW fragments. Moreover, cartilage explants from Myd88-deficient mice were revealed to have enhanced proteoglycan synthesis (140% of control) after exposure to SCW fragments. This indicates that when suppression of chondrocyte proteoglycan metabolism mediated via the IL-1R/TLR pathway is blocked, it allows anabolic factors, like TGF-β, to stimulate cartilage matrix production (52).

Analysis of local cytokine and chemokine production after induction of SCW arthritis in TLR-2−/−, TLR-4−/−, and MyD88−/− mice revealed that both TLR-2−/− and MyD88−/− mice showed significantly reduced levels of TNF-α and IL-1β. This corresponds with reduction of joint swelling and less inhibition of chondrocyte anabolic function. Furthermore, MyD88 gene-deficient mice had also strongly reduced IL-6 and IL-12p70 levels, determined shortly after induction of SCW arthritis. In previous reports, it has been shown that MyD88-deficient mice did not respond to either IL-1 or IL-18 (28). This is in order with the reduced levels of TNF-α and IL-1β measured in synovial washouts shortly after induction of SCW arthritis.

Histopathology in IL-1−/−, IL-18−/−, TLR-2−/−, TLR-4−/−, and MyD88−/− deficient mice after induction of SCW arthritis

<table>
<thead>
<tr>
<th>Influx of Inflammatory Cells</th>
<th>Proteoglycan Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joint cavity</td>
<td>Synovial tissue</td>
</tr>
<tr>
<td>WT+</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>IL-1−/−</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>IL-18−/−</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>WT+</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>TLR-2−/−</td>
<td>0.8 ± 0.4*</td>
</tr>
<tr>
<td>WT−/−</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>TLR-4−/−</td>
<td>1.0 ± 0.4*</td>
</tr>
<tr>
<td>WT−/−</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td>0.4 ± 0.3*#</td>
</tr>
</tbody>
</table>

*SCW arthritis was induced in all mice strains by i.a. injection of 25 μg SCW fragments. Histology was performed at day 2 after induction of arthritis. Cell influx of both joint cavity and synovial tissue was scored on a scale ranging from 0 to 3. Loss of cartilage matrix proteoglycans was determined on safranin O-stained sections and scored on a scale of 0–3.

a WT, C57BL/6 mice.
b WT, C57BL/6 mice.
c WT, C57BL/10 mice.
d WT, MyD88−/− mice.
e p < 0.05, Mann-Whitney U test compared to WT mice.
f p < 0.001, Mann-Whitney U test compared to WT mice.
FIGURE 7. MyD88 \textsuperscript{-/-} mice are protected against matrix proteoglycan depletion. Analysis of matrix proteoglycan content by safranin O staining at day 2 after injection of SCW fragments. A, Knee joint of WT C57BL/6 mouse. B, TLR-2 \textsuperscript{-/-} mouse. C, TLR-4 \textsuperscript{-/-} mouse. D, MyD88 \textsuperscript{-/-} mouse. Of high interest, no loss of matrix proteoglycans was seen in MyD88-deficient mice at day 2 of SCW arthritis. Safranin O staining; original magnification, ×400.

P, Patella; C, cartilage. Arrows indicates loss of matrix proteoglycans.

after onset of arthritis. This chemokine, the murine equivalent of IL-8, is one of the key players in the attraction of PMNs to sites of inflammation (53). RANTES and MIP-1\textalpha, chemokines involved in the influx of monocytes and T cells were also significantly reduced in both TLR-2 and MyD88 gene knockout mice. It has been shown previously that C-C chemokine production, such as RANTES, is dependent on TLR-2 signaling (54). Interestingly, loss of matrix proteoglycan from cartilage layers can be seen after induction of SCW arthritis, starting from day 2. At later time points (days 4 and 7), more severe proteoglycan loss is noted. IL-1 is the pivotal inflammatory cytokine. The in vivo role of IL-1 in SCW arthritis, starting from day 2. At later time points (days 4 and 7), more severe proteoglycan loss is noted. IL-1 is the pivotal inflammatory cytokine. The in vivo role of IL-1 in SCW arthritis. The role of IL-1 in the development of joint inflammation. Targeting of the IL-1R/TLR pathway by interference with the MyD88 adaptor molecule may be a novel therapy in inflammatory joint diseases such as RA.

References


