ARTHRITIS & RHEUMATISM
Vol. 65, No. 10, October 2013, pp 2583–2593
DOI 10.1002/art.38087

Toll-like Receptor 2 Controls Acute Immune Complex–Driven Arthritis in Mice by Regulating the Inhibitory Fcγ Receptor IIB

Shahla Abdollahi-Roodsaz, Marije I. Koenders, Birgitte Walgreen, Judith Bolscher, Monique M. Helsen, Liduine A. van den Bersselaar, Peter L. van Lent, Fons A. J. van de Loo, and Wim B. van den Berg

Objective. **Previous studies have demonstrated a protective role of Toll-like receptor 2 (TLR-2) and a proinflammatory function of TLR-4 during chronic T cell–driven arthritis. The involvement of TLRs in T cell–independent arthritic processes, however, remains unclear. This study was undertaken to determine the functional significance of TLR-2 and TLR-4 in T cell–independent immune complex–driven arthritis.**

Methods. **Serum-transfer arthritis was induced in wild-type and TLR-deficient mice by intraperitoneal injections of arthritogenic K/BxN mouse serum. Arthritis was assessed macroscopically and by histologic analysis. The influence of TLR-2 on macrophage cytokine profile, Fcγ receptor (FcγR) expression, and response to immune complexes was determined.**

Results. **While TLR-4, unexpectedly, did not play any significant role, TLR-2 deficiency accelerated the onset and markedly increased the severity of acute immune complex–driven arthritis in mice. TLR-2 deficiency resulted in a substantial increase in joint inflammation, bone erosion, and cartilage pathology, indicating a protective function of TLR-2 in passive FcγR-driven disease. Ex vivo study of the macrophage inflammatory phenotype revealed increased production of tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) despite similar levels of IL-10, along with a significant increase in FcγR-specific response, in TLR-2$^{-/-}$ mouse macrophages early in the disease. Although distinct FcγR messenger RNA expression was not affected, cell surface protein expression of the inhibitory FcγRIIB in TLR-2$^{-/-}$ naive primary macrophages was specifically diminished, resulting in a higher proinflammatory response. Accordingly, TLR-2 stimulation specifically up-regulated FcγRIIB, but not the activating FcγR, on macrophages.**

Conclusion. **TLR-2 regulates acute immune complex–driven arthritis by controlling macrophage FcγR response. Our findings indicate that the protective role of TLR-2 is extended beyond its previously described role in promoting Treg cells during T cell–mediated arthritis.**

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation and progressive cartilage and bone destruction in multiple joints. The pathogenesis of RA is grounded in a complex network of the components of innate and adaptive immune systems. Among the innate immune receptors, Toll-like receptors (TLRs) have recently gained attention due to a growing body of evidence indicating their involvement in RA.

TLRs are a family of pattern-recognition receptors evolved to recognize conserved microbe-associated molecular patterns. Increased expression of TLR-2, TLR-3, TLR-4, and TLR-7 in RA synovial tissue supports the idea that these receptors are relevant in the disease (1–3). Some TLRs can be activated by endoge-

Supported by The Netherlands Organization for Scientific Research (Veni grant 916.12.039) and the European Innovative Medicines Initiative (BTCure project grant 115142-2) within the framework of TI Pharma project D1-101.

Shahla Abdollahi-Roodsaz, PhD, Marije I. Koenders, PhD, Birgitte Walgreen, BSc, Judith Bolscher, MSc, Monique M. Helsen, BSc, Liduine A. van den Bersselaar, BSc, Peter L. van Lent, PhD, Fons A. J. van de Loo, PhD, Wim B. van den Berg, PhD: Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

Address correspondence to Shahla Abdollahi-Roodsaz, PhD, Rheumatology Research and Advanced Therapeutics, Department of Rheumatology, 272, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500HB Nijmegen, The Netherlands. E-mail: s.abdollahi-roodsaz@reuma.umcn.nl.

Submitted for publication December 23, 2012; accepted in revised form June 25, 2013.

nous "danger" molecules associated with inflammation and tissue destruction. Many of these endogenous TLR agonists have been found in the joints and serum of patients with RA and show a positive correlation with disease activity scores (1,4–8). TLR activation generally leads to a rapid and robust production of multiple inflammatory cytokines and matrix metalloproteinases (MMPs). In this regard, TLR-2 and TLR-4 have been shown to drive spontaneous production of tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) by intact RA synovial tissue explants cultured ex vivo (9,10).

In recent years, significant progress has been made in understanding the role of TLRs during arthritis. In vivo research has unraveled the role of TLR-4 in driving synovial inflammation and joint destruction in experimental models where T and B cell responses are actively involved (11,12). TLR-2, in contrast, was shown to inhibit T cell–mediated arthritis by promoting the suppressive function of Treg cells, thereby hampering the production of interferon-γ and IL-17 by T cells (9). Despite evidence of the involvement of TLR-2 and TLR-4 in regulating T cell–driven arthritis, the potential role and relevance of these receptors in T cell–independent arthritic processes remains poorly understood to date.

In the present study, we aimed to identify the functional significance of TLR-2 and TLR-4 in passive (T cell–independent) experimental arthritis induced by arthritogenic serum from K/BxN mice (13). The K/BxN serum contains autoantibodies directed against the ubiquitously expressed glucose-6-phosphate isomerase, forming IgG-containing immune complexes that activate another critical receptor family, Fcγ receptors (FcγR), on innate immune cells including monocytes and macrophages (13). Activation of FcγR by immune complexes has previously been shown to drive joint inflammation and severe cartilage destruction in experimental arthritis (14,15). Since RA is characterized by high levels of circulating IgG autoantibodies, activation of FcγR may play a major role and, therefore, the influence of TLRs on FcγR response to immune complexes will be of high relevance to understanding the pathogenesis of the disease.

In mice, FcγR comprise 4 classes: the activating receptors I, III, and IV, which among other effects induce cytokine production and oxidative burst upon triggering, and the inhibitory receptor IIB, which blocks the activating receptors through its intracellular immunoreceptor tyrosine–based inhibition motif (16). The balance between these receptors is a critical determinant of the net response to immune complexes. In addition, specific FcγR have been shown to play distinct roles in antibody-induced K/BxN serum-transfer arthritis. Moreover, FcγRII$^{-/-}$ mice manifest severe accelerated arthritis, whereas FcγRIII$^{-/-}$ mice develop an attenuated disease with delayed kinetics (17,18). One previous study demonstrated a crucial role of FcγR on bone marrow–derived rather than joint resident cells, and another study showed the involvement of macrophages in recognition of immune complexes and joint pathology in this model (17,19).

In the present study, we used the serum-transfer model to investigate the roles of TLR-2 and TLR-4 in the effector phase of arthritis devoid of T cell involvement and instead mediated via FcγR, and to study the regulation of FcγR expression and response in macrophages by TLR-2.

MATERIALS AND METHODS

Induction of K/BxN serum–transfer arthritis. C57BL/6 wild-type mice were purchased from Janvier. TLR-2$^{-/-}$ and TLR-4$^{-/-}$ mice on a C57BL/6 background were kindly provided by Professor S. Akira (Immunology Frontier Research Center, Osaka University, Osaka, Japan). Wild-type TLR-4$^{+/+}$ littermates were generated in house by crossing TLR-4$^{-/-}$ mice with wild-type C57BL/6 mice. Mice were housed in filter-top cages, and water and food were provided ad libitum. KRN T cell receptor–transgenic mice were a kind gift from Drs. C. Benoist and D. Mathis (Harvard Medical School, Boston, MA), and were maintained on a C57BL/6 background (K/B). Arthritic K/BxN mice were obtained by crossing K/B mice with NOD/Lt animals in the Nijmegen animal facility. Arthritis was induced by 2 intraperitoneal injections, 1 on day 0 and 1 on day 2, of 200 μl arthritogenic serum derived from arthritic K/BxN mice. The presence of endogenous TLR-2 and TLR-4 ligands in K/BxN serum was excluded using wild-type and TLR-deficient macrophages. Arthritis was macroscopically scored on a scale of 0–2 in each paw 3 times per week by 2 observers in a blinded manner. Animal studies were approved by the Animal Ethics Review Board of Radboud University Nijmegen.

Isolation and culture of peritoneal and bone marrow–derived macrophages (BMMs). Primary macrophages were isolated on days 2 and 4 after the first serum injection or from naive mice by lavage of peritoneal cavity using 10 ml of ice-cold phosphate buffered saline containing 10% fetal calf serum (FCS). Adherent cells were harvested and used for stimulations as described below. BMMs were generated by culturing bone marrow cells derived from femur in the presence of 15 ng/ml of macrophage colony-stimulating factor (R&D Systems) for 6 days. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FCS, 1 m*M* sodium pyruvate, and 50 mg/liter gentamicin.

Stimulation of macrophages and measurement of cytokine levels. To assess the macrophage cytokine profile during arthritis, mouse peritoneal macrophages were stimulated with phorbol myristate acetate (PMA; 50 ng/ml) (Sigma) and

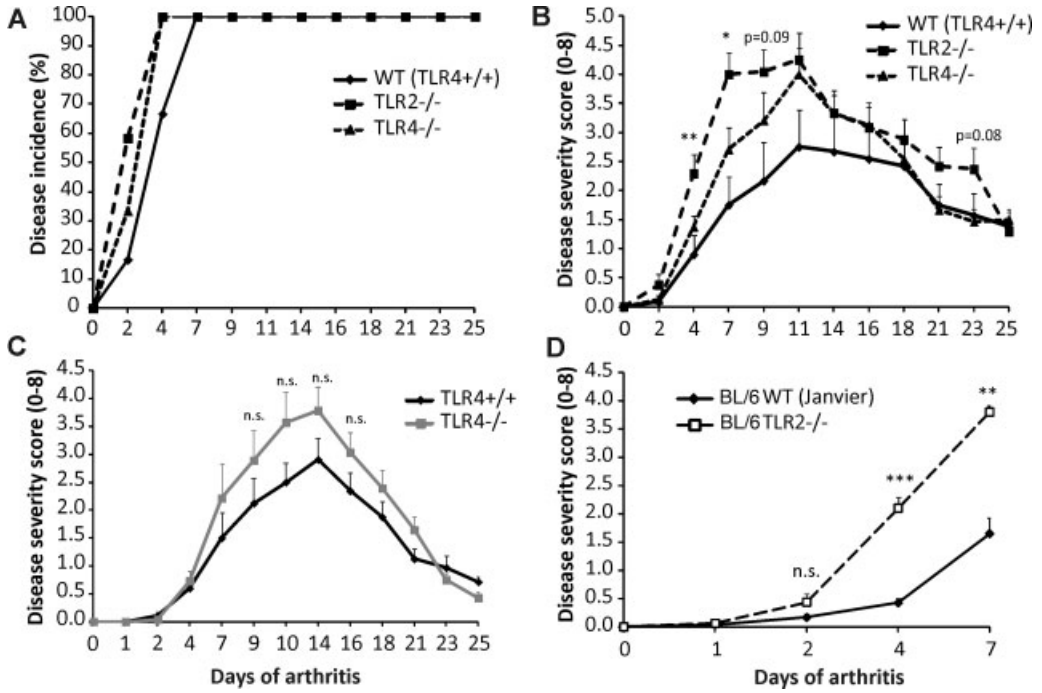

Figure 1. Toll-like receptor 2 (TLR-2) negatively regulates the severity of acute serum-transfer arthritis. Passive serum-transfer arthritis was induced in mice by 2 intraperitoneal injections, 1 on day 0 and 1 on day 2, of 200 μl arthritogenic serum from K/BxN mice. **A,** Arthritis incidence in the wild-type (WT), TLR-2$^{-/-}$, and TLR-4$^{-/-}$ mouse groups (n = 12 mice per group). **B–D,** Arthritis severity, scored macroscopically on a scale of 0–2 for each paw 3 times per week, in the indicated mouse strains. Values are the mean ± SEM (n = 12 mice per group in **B**; n = 7 or more mice per group in **C**; n = 15 mice per group on day 0 [5 mice per group were killed on days 2 and 4 for macrophage isolation] in **D**). $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ versus wild-type mice, by Kruskal-Wallis test in **B** and by Mann-Whitney U test in **C** and **D**. NS = not significant.

ionomycin (1 μg/ml; Sigma) for 24 hours. To activate FcγR, bovine serum albumin (BSA)–anti-BSA immune complexes were used. (BSA was obtained from Sigma.) Immune complexes were formed by incubating 500 μg/ml BSA with 100 μg/ml anti-BSA antibodies (Serotec) for 50 minutes at 37°C, then mixed with polymyxin B (10 μg/ml; Sigma) for 10 minutes before adding to the culture in order to block the effects of any potential endotoxin contamination. IL-1β (10 ng/ml; R&D Systems) and poly(I-C) (25 mg/ml; InvivoGen) stimulations were used as control. TLR-2 stimulation was achieved using Pam_3Cys (1 and 10 ng/ml) and fibroblast-stimulating lipopeptide 1 (FSL-1; 10 ng/ml) (both from EMC Microcollections). Cytokine concentrations in culture supernatants as well as mouse sera were determined using Milliplex cytokine assays according to the recommendations of the manufacturer (Millipore).

RNA isolation and quantitative polymerase chain reaction (qPCR). Synovial biopsy samples were obtained from the mouse knee joints using a 3-mm punch (Stiefel) on day 4 after the first serum injection or from naive mice. Total RNA isolation and real-time qPCR using SYBR Green Master Mix in an ABI Prism 7000 sequence detection system (Applied Biosystems) were performed as previously described (20). Primer sequences (forward and reverse, respectively) were as follows: for GAPDH (housekeeping gene), 5′-GGC-AAA-TTC-AAC-GGC-ACA-3′ and 5′-GTT-AGT-GGG-GTC-TCG-CTC-TG-3′; for IL-1β, 5′-GGA-CAG-AAT-ATC-AAC-CAA-CAA-GTG-ATA-3′ and 5′-GTG-TGC-CGT-CTT-TCA-TTA-CAC-AG-3′; for IL-6, 5′-CAA-GTC-GGA-GGC-TTA-ATT-ACA-CAT-G-3′ and 5′-ATT-GCC-ATT-GCA-CAA-CTC-TTT-TCT-3′; for TNFα, 5′-CAG-ACC-CTC-ACA-CTC-AGA-TCA-TCT-3′ and 5′-CCT-CCA-CTT-GGT-GGT-TTG-CTA-3′; for MMP-3, 5′-TGG-AGC-TGA-TGC-ATA-AGC-CC-3′ and 5′-TGA-AGC-CAC-CAA-CAT-CAG-GA-3′; for MMP-9, 5′-GGA-ACT-CAC-ACG-ACA-TCT-TCC-A-3′ and 5′-GAA-ACT-CAC-ACG-CCA-GAA-GAA-TTT-3′; for MMP-13, 5′-AGA-CCT-TGT-GTT-TGC-AGA-GCA-CTA-C-3′ and 5′-CTT-CAG-GAT-TCC-CGC-AAG-AG-3′; for FcγRI, 5′-ACA-CAA-TGG-TTT-ATC-AAC-GGA-ACA-3′ and 5′-TGG-CCT-CTG-GGA-TGC-TAT-AAC-T-3′; for FcγRIIB, 5′-GAC-AGC-CGT-GCT-AAA-TCT-TGC-T-3′ and 5′-GTG-TCA-CCG-TGT-CTT-CCT-TGA-G-3′; for FcγRIII, 5′-GAC-AGG-CAG-AGT-GCA-GCT-CTT-3′ and 5′-TGT-CTT-CCT-TGA-GCA-CCT-GGA-T-3′; and for FcγRIV, 5′-CCA-GAG-TTA-AGG-ACA-GTG-GAA-TGT-AC-3′ and 5′-GCA-ATA-GCC-AGC-CCA-TAT-GG-3′. The threshold cycle (C_t) value of the gene of interest was corrected for that of GAPDH to obtain the ΔC_t. Relative messenger RNA (mRNA) expression was calculated as $2^{-\Delta C_t}$.

Histologic analysis. Total ankle joints from mice were fixed in 4% formaldehyde for 4 days, then decalcified in 5% formic acid and embedded in paraffin. Tissue sections of 7 μm

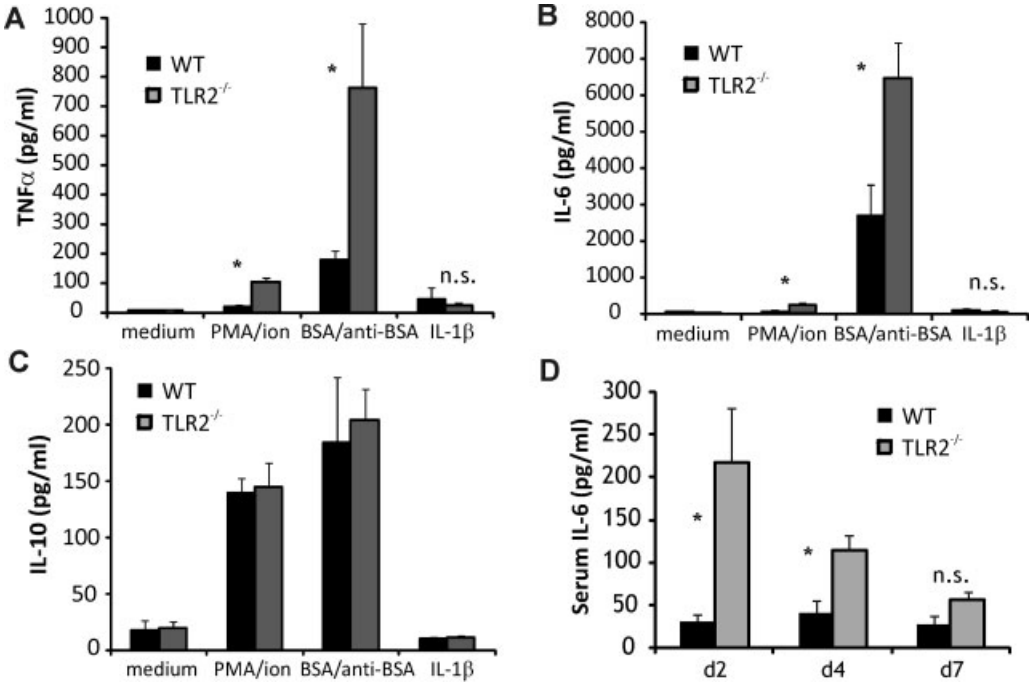

Figure 2. Toll-like receptor 2 (TLR-2) deficiency potentiates the inflammatory phenotype and Fcγ receptor (FcγR)–specific response of macrophages during serum-transfer arthritis. **A–C**, Levels of tumor necrosis factor α (TNFα) (**A**), interleukin-6 (IL-6) (**B**), and IL-10 (**C**) in peritoneal macrophages harvested from wild-type (WT) and TLR-$2^{-/-}$ mice on day 2 of serum-transfer arthritis. Cells were stimulated with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (ion; 1 μg/ml), bovine serum albumin (BSA)–anti-BSA immune complexes (500 μg/ml BSA + 100 μg/ml anti-BSA) mixed with polymyxin B (10 μg/ml), or IL-1β (10 ng/ml) for 24 hours. Cytokine concentrations in culture supernatants were determined using the Milliplex cytokine array. IL-1β was produced at low levels only in a few samples. Bars show the mean ± SEM. $* = P < 0.05$, wild-type versus TLR-$2^{-/-}$ mice, by Mann-Whitney U test. **D**, IL-6 concentrations in mouse sera at various time points during serum-transfer arthritis. Bars show the mean ± SEM (n = 5 mice per group per time point). $* = P < 0.05$, wild-type versus TLR-$2^{-/-}$ mice, by Mann-Whitney U test. NS = not significant.

were stained using hematoxylin and eosin to study inflammatory cell influx and chondrocyte death or using Safranin O to determine proteoglycan depletion, cartilage destruction, and bone erosion. Each parameter was scored on a scale of 0–3 by 2 observers in a blinded manner.

Fluorescence-activated cell sorting (FACS) analysis. Cell surface expression of distinct FcγR on macrophages was determined using allophycocyanin-conjugated anti-F4/80 (Serotec), Alexa Fluor 647–conjugated anti-FcγRI (X54-5/7.1; BD PharMingen), Alexa Fluor 488–conjugated anti-FcγRIIB (clone K9.361 anti-Ly17.2) (kindly provided by Dr. S. Verbeek, Leiden University Medical Centre, Leiden, The Netherlands), fluorescein isothiocyanate–conjugated anti-FcγRIII (clone 275003; R&D Systems), and Alexa Fluor 647–conjugated anti-FcγRIV (clone 9E9) (a kind gift from Professor F. Nimmerjahn, University of Erlangen-Nuremberg, Erlangen, Germany). Cells were incubated with the antibodies described above or the appropriate isotype-matched control antibodies for 30 minutes at 4°C and analyzed on a FACSCalibur using CellQuest software (BD Biosciences PharMingen).

Statistical analysis. Group measures are expressed as the mean ± SEM. Statistical significance was assessed using the Mann-Whitney 2-tailed U test to compare 2 experimental groups and the Kruskal-Wallis test to compare more than 2 groups. Analysis was performed using GraphPad Prism 4.0 software. *P* values less than 0.05 were considered significant.

RESULTS

Significantly enhanced severity of immune complex–mediated arthritis in TLR-$2^{-/-}$, but not TLR-$4^{-/-}$, mice. To assess the involvement of TLR-2 and TLR-4 in T cell–independent immune complex–driven arthritis, passive K/BxN arthritis was induced in wild-type and TLR-deficient mice. TLR-$4^{-/-}$, and especially TLR-$2^{-/-}$, mice showed accelerated onset of the disease, reaching 100% incidence as early as 4 days after the first serum injection (Figure 1A). The mean time point of arthritis onset was 4.7, 2.8, and 3.3 days for the wild-type, TLR-$2^{-/-}$, and TLR-$4^{-/-}$ groups, respectively. Interestingly, the disease severity score was markedly increased in TLR-$2^{-/-}$ mice during the acute phase (Figure 1B), indicating a negative regulatory function for TLR-2 during disease induction. The arthritis sever-

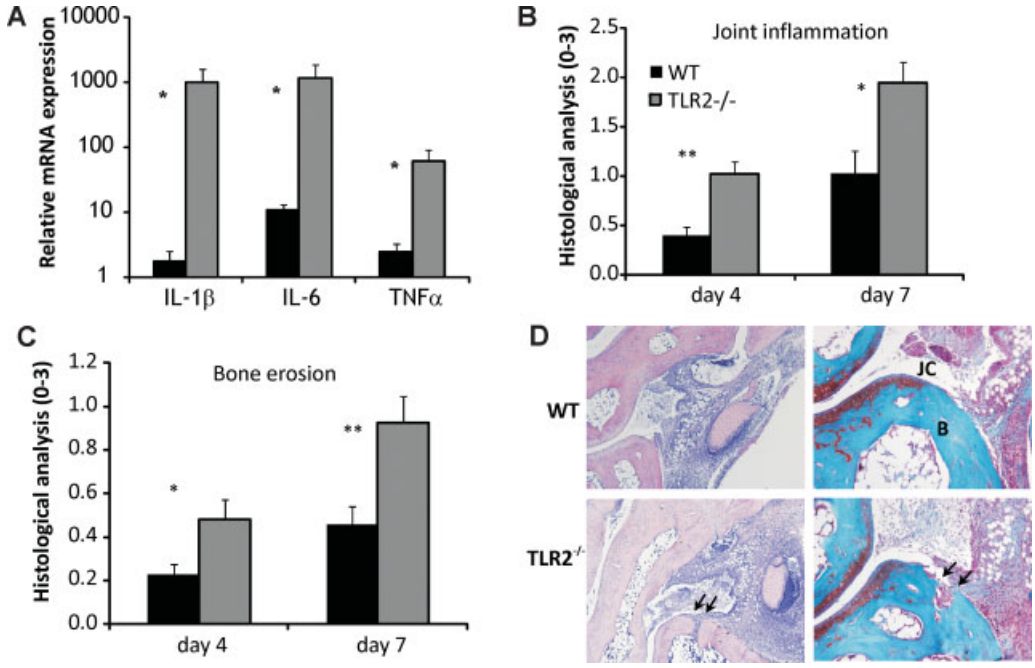

Figure 3. TLR-2 regulates joint inflammation and arthritic bone erosion in mice during passive immune complex–mediated arthritis. **A**, Synovial expression of proinflammatory genes on day 4 of serum-transfer arthritis in wild-type and TLR-$2^{-/-}$ mice, as determined by quantitative polymerase chain reaction. The ΔC_t values after correction for the housekeeping gene GAPDH were used to calculate relative mRNA expression ($2^{-\Delta C_t}$). **B** and **C**, Histologic assessment of joint inflammation (**B**) and bone erosion (**C**), scored on a scale of 0–3. Sections of mouse ankle joints isolated on days 4 and 7 were stained with hematoxylin and eosin (H&E) to score joint inflammation and with Safranin O to score bone erosion. In **A–C**, bars show the mean ± SEM (n = 5 mice per group). $* = P < 0.05$; $** = P < 0.01$, wild-type versus TLR-$2^{-/-}$ mice, by Mann-Whitney U test. **D,** Representative images of H&E-stained sections (left) and Safranin O–stained sections (right) obtained from mice with arthritis on day 7, illustrating increased inflammation (**arrows** in left panels) and arthritic bone erosion (**arrows** in right panels) in TLR-$2^{-/-}$ mice. **JC** = joint cavity; **B** = bone. Original magnification × 100. See Figure 2 for other definitions.

ity score was also significantly higher in TLR-$2^{-/-}$ mice when only the mice with disease were considered, ruling out the influence of increased incidence.

In contrast to TLR-2, arthritis severity was, unexpectedly, not altered significantly by TLR-4 deficiency (Figure 1B). Although previous findings in other experimental models provide support for a proinflammatory role of TLR-4, repeating the study using TLR-$4^{+/+}$ littermates confirmed that passive arthritis was not diminished, but rather tended to increase in TLR-$4^{-/-}$ mice (Figure 1C). The considerable protective role of TLR-2 during acute immune complex–mediated arthritis was also reproduced in subsequent experiments regardless of the source of wild-type control mice (Figure 1D). In all experiments, disease scores during the resolution phase appeared not to be influenced by TLR-2 or TLR-4 deficiency (Figures 1B and C).

Potentiated inflammatory profile and FcγR-specific response of macrophages during passive arthritis upon TLR-2 deficiency. The processes underlying the increased arthritis severity observed in TLR-$2^{-/-}$ mice were further investigated during the acute phase. To this end, the cytokine profile of macrophages as important mediators of K/BxN passive arthritis was determined ex vivo by nonspecific activation with PMA and ionomycin. TLR-$2^{-/-}$ peritoneal macrophages isolated as early as day 2 of disease induction, i.e., prior to any significant change in arthritis scores, produced much higher levels of TNFα and IL-6 than did wild-type mouse macrophages (Figures 2A and B). Notably, production of the antiinflammatory cytokine IL-10 was not affected in TLR-$2^{-/-}$ mouse macrophages (Figure 2C). Of great interest, the FcγR-specific proinflammatory response upon triggering with BSA–anti-BSA immune complexes was markedly increased in TLR-$2^{-/-}$ mouse macrophages at this early time point (Figures 2A and B). The production of cytokines by macrophages stimulated with other agents, such as IL-1β and poly(I-C), was similar in wild-type and TLR-$2^{-/-}$ cells (Figures 2A–C and data not shown), suggesting that the regulatory role of TLR-2 in macrophage proinflammatory response is specific to FcγR stimulation.

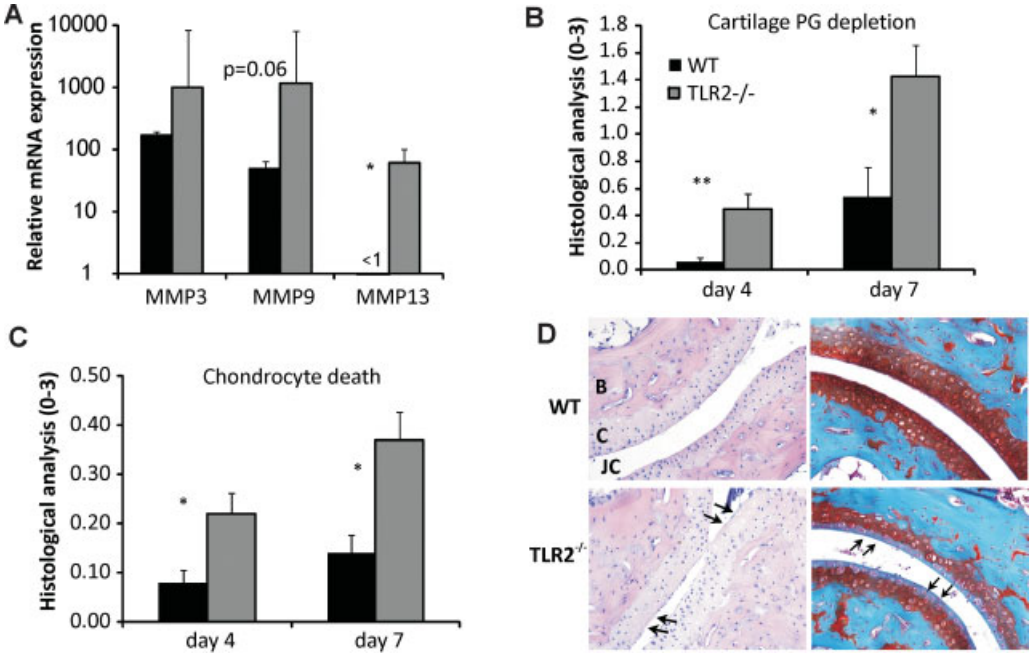

Figure 4. Toll-like receptor 2 (TLR-2) controls cartilage pathology during passive immune complex–mediated arthritis. **A**, Synovial expression of matrix metalloproteinases (MMPs) on day 4 of serum-transfer arthritis in wild-type (WT) and TLR-$2^{-/-}$ mice, as determined by quantitative polymerase chain reaction. The ΔC_t values after correction for the housekeeping gene GAPDH were used to calculate relative mRNA expression ($2^{-\Delta C_t}$). **B** and **C**, Histologic assessment of cartilage proteoglycan (PG) depletion (**B**) and chondrocyte cell death (**C**), scored on a scale of 0–3. Sections of mouse ankle joints isolated on days 4 and 7 were stained with Safranin O to score PG depletion and hematoxylin and eosin (H&E) to score chondrocyte death. In **A–C**, bars show the mean ± SEM (n = 5 mice per group). $* = P < 0.05$; $** = P < 0.01$, wild-type versus TLR-$2^{-/-}$ mice, by Mann-Whitney U test. **D**, Representative images of H&E-stained sections (left) and Safranin O–stained sections (right) obtained from mice with arthritis on day 7, illustrating increased chondrocyte death (**arrows** in left panels) and PG depletion (**arrows** in right panels) in TLR-$2^{-/-}$ mice. **B** = bone; **C** = cartilage; **JC** = joint cavity. Original magnification × 200.

The potentiated inflammatory profile and FcγR response of TLR-$2^{-/-}$ primary macrophages was clearly visible on day 4 of arthritis as well. The mean ± SEM stimulation index compared to medium control, e.g., for TNFα production, was 3.6 ± 0.3 in wild-type mice versus 13.8 ± 1.7 in TLR-$2^{-/-}$ mice upon PMA/ionomycin stimulation and 2.9 ± 0.4 in wild-type mice versus 56.3 ± 11.9 in TLR-$2^{-/-}$ mice upon FcγR stimulation ($P < 0.05$ for both stimulations). Production of IL-6 was also significantly increased upon stimulation with PMA/ionomycin or FcγR; however, the effect of IL-1 stimulation on IL-6, as well as TNFα, production remained unaltered by TLR-2 deficiency (data not shown). In addition, increased production of IL-6 along with the keratinocyte-derived chemokine in TLR-$2^{-/-}$ mice with passive arthritis was evident in serum at early stages (Figure 2D and data not shown). TNFα was undetectable in serum.

Regulation of joint inflammation and arthritic bone erosion by TLR-2 during passive immune complex–mediated arthritis in mice. The involvement of TLR-2 in joint pathology induced by T cell–independent immune complex–mediated arthritis was investigated locally. Synovial tissue from TLR-$2^{-/-}$ mice expressed higher transcript levels of mRNA for IL-1β, TNFα, and IL-6 than synovial tissue from wild-type mice (Figure 3A). K/BxN serum-transfer arthritis was accompanied by mild synovial inflammation and bone erosion in wild-type mice on day 4, which increased in time toward day 7 (Figures 3B and C). Importantly, TLR-2 deficiency resulted in a substantial increase in joint inflammation as well as bone pathology (Figures 3B and C). Figure 3D shows representative images of joint histopathology on day 7 in wild-type mice as compared to TLR-$2^{-/-}$ mice.

TLR-2 protects against cartilage pathology during immune complex–mediated arthritis in mice. Synovial expression of mRNA for the cartilage-degrading enzymes MMP-3 and MMP-9 was induced during serum-transfer arthritis and tended to be higher in TLR-$2^{-/-}$ mice (Figure 4A). MMP-13 expression was low in wild-type mice but increased significantly in TLR-$2^{-/-}$ mice (Figure 4A). Next, histologic analysis of several features of cartilage pathology, including matrix proteoglycan depletion, chondrocyte cell death, and

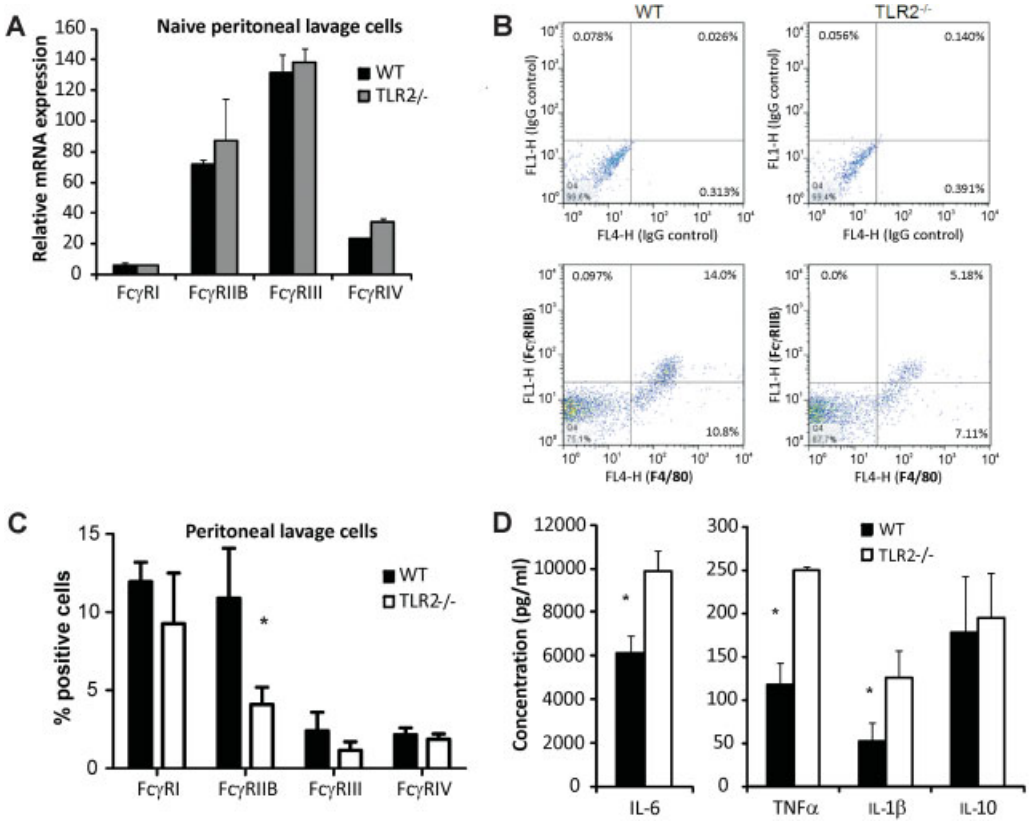

Figure 5. TLR-2 negatively regulates FcγRIIB cell surface expression and response in naive mouse peritoneal lavage cells. **A**, Relative expression of mRNA ($2^{-\Delta C_t}$) for Fcγ receptors in peritoneal lavage cells from naive wild-type and TLR-$2^{-/-}$ mice. **B**, Representative flow cytometry plots showing FcγRIIB cell surface expression on wild-type and TLR-$2^{-/-}$ mouse macrophages. **C**, Expression of distinct FcγR proteins on the surface of mouse peritoneal lavage cells, as measured by flow cytometry. **D**, Levels of cytokines in peritoneal cells from wild-type and TLR-$2^{-/-}$ mice. Cells were stimulated with BSA–anti-BSA immune complexes (500 μg/ml BSA + 100 μg/ml anti-BSA) mixed with polymyxin B (10 μg/ml) for 24 hours. Cytokine concentrations in culture supernatants were determined using the Milliplex cytokine array. Bars in **A**, **C**, and **D** show the mean ± SEM (n = 5 mice per group from a representative experiment). $* = P < 0.05$, wild-type versus TLR-$2^{-/-}$ mice, by Mann-Whitney U test. See Figure 2 for definitions.

cartilage surface erosions, was performed. TLR-$2^{-/-}$ cartilage showed more severe proteoglycan depletion as evidenced by loss of red Safranin O staining, and higher degrees of chondrocyte death as represented by empty lacunae (Figures 4B and C). Only 2 of 5 TLR-$2^{-/-}$ mice showed signs of very mild cartilage surface erosions. Increased immune complex–driven cartilage pathology upon TLR-2 deficiency is illustrated in Figure 4D.

Increase in FcγRIIB expression and negative regulation of FcγR response by TLR-2 in naive mouse primary macrophages. Considering the notable protective role of TLR-2 in passive immune complex–mediated arthritis, we examined the involvement of TLR-2 in regulating the expression of activating and inhibitory FcγR. Quantitative PCR analysis showed that basal expression of mRNA transcripts for FcγR under naive conditions was not affected by TLR-2 deficiency in mouse peritoneal lavage cells or synovial tissue (Figure 5A and data not shown). However, measurement of cell surface expression of the 4 distinct FcγR classes revealed significant and specific reduction of FcγRIIB expression on TLR-$2^{-/-}$ cells from peritoneal lavage, while the activating receptors were unaltered (Figures 5B and C). Stimulation of peritoneal cells from naive nonarthritic mice with immune complexes resulted in higher concentrations of IL-6, TNFα, and IL-1β, but comparable IL-10 production, in TLR-$2^{-/-}$ mice versus wild-type mice (Figure 5D).

Since cells isolated from the mouse peritoneal cavity might potentially have been exposed to low concentrations of endogenous or gut-derived TLR agonists, we investigated FcγR expression and response in naive unstimulated mouse BMMs. A similar specific reduction in FcγRIIB, but not in FcγRI, III, or IV, was observed in

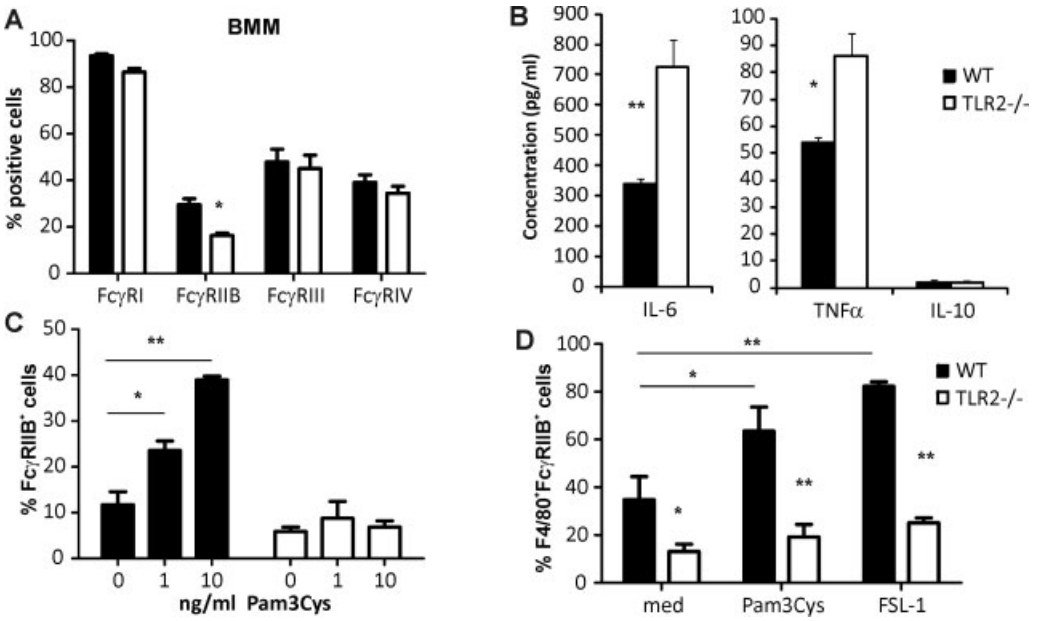

Figure 6. TLR-2 increases FcγRIIB expression and negatively regulates FcγR response in bone marrow–derived macrophages (BMMs) from naive mice. **A**, Expression of 4 distinct FcγR proteins on BMMs, as measured by fluorescence-activated cell sorting (FACS), in wild-type and TLR-$2^{-/-}$ mice. **B**, Levels of cytokines in the culture supernatants of BMMs from wild-type and TLR-$2^{-/-}$ mice. BMMs were stimulated with BSA–anti-BSA immune complexes (500 μg/ml BSA + 100 μg/ml anti-BSA) mixed with polymyxin B (10 μg/ml) for 24 hours. **C**, Percentages of mouse BMMs expressing FcγRIIB, as determined by FACS analysis, after 24 hours of stimulation with the TLR-1/2 agonist Pam_3Cys at the indicated concentrations. **D**, Percentages of mouse F4/80+ BMMs expressing FcγRIIB, as determined by FACS analysis, after 24 hours of stimulation with the TLR-1/2 agonist Pam_3Cys or the TLR-2/6 agonist fibroblast-stimulating lipopeptide 1 (FSL-1) (both 10 ng/ml). Bars show the mean ± SEM (n = 5 mice per group from a representative experiment in **A** and **B**; n = 3 mice per group in **C**; n = 4 or more mice per group in **D**). $* = P < 0.05$; $** = P < 0.01$, wild-type versus TLR-$2^{-/-}$ mice except where indicated otherwise in **C** and **D**, by Mann-Whitney U test. See Figure 2 for other definitions.

TLR-$2^{-/-}$ mouse BMMs as compared to wild-type mouse cells (Figure 6A). The production of cytokines in response to immune complexes was less potent in naive BMMs than in peritoneal cells, but nevertheless clearly confirmed the negative regulatory function of TLR-2 in macrophage FcγR response (Figure 6B).

Consistent with these observations, stimulation of TLR-2 on naive mouse BMMs induced a specific up-regulation of FcγRIIB in a dose-dependent manner, revealing a mean ± SEM of 39.0 ± 0.8% FcγRIIB+ cells at the highest ligand concentration used (10 ng/ml) as compared to 11.7 ± 2.8% in medium control (Figure 6C). Expression of the activating FcγR was, however, either not affected or very marginally affected (for FcγRI, 78.0 ± 4.2% versus 80.7 ± 1.1%; for FcγRIII, 7.2 ± 0.8% versus 8.3 ± 1.0%; and for FcγRIV, 7.9 ± 2.1% versus 11.5 ± 3.5% in medium control and after stimulation with 10 ng/ml TLR-2 agonist, respectively). Furthermore, induction of FcγRIIB expression was achieved by both Pam_3Cys engaging a TLR-1/TLR-2 heterodimer and FSL-1 activating a TLR-2/TLR-6 functional heterodimer (Figure 6D). Taken together, these data indicate a negative regulatory function of TLR-2, in conjunction with either TLR-1 or TLR-6 as partner, in FcγR response through specific induction of the inhibitory FcγRIIB.

DISCUSSION

Considerable evidence has accumulated over the last decade to support the involvement of TLR-2 and TLR-4 in the pathogenesis of RA. Identification of specific RA-related processes to which these receptors contribute would help clarify the disease mechanisms involved and allow therapeutic interventions to be targeted to specific phases of the disease or specific subgroups of patients.

Importantly, TLR-2 activation has recently been shown to promote angiogenesis in RA synovial explants and to induce endothelial cell adhesion and invasion (21). Despite a tremendous amount of data indicating a proinflammatory and tissue-destructive role of TLR-4 in

various, mainly T cell–driven, experimental models, in vivo studies of the role of TLR-2 during arthritis are very limited. TLR-2 is responsible for acute arthritis directly induced by intraarticular injection of microbial components such as streptococcal cell wall fragments and zymosan, which are specifically recognized by this receptor (20,22,23). However, in experimental models devoid of direct microbial TLR triggering but with additional adaptive immune activation, the function of TLR-2 is likely to be different. Since TLR-2 is important for the optimal differentiation and function of Treg cells (24,25), TLR-$2^{-/-}$ mice develop a severe destructive disease when T cell immunity is involved (9).

This study was undertaken to unravel the as-yet largely unknown role of TLR-2 and TLR-4 in T cell–independent arthritic processes. Activation of FcγR during the effector phase of the disease is a relevant aspect in this context. In contrast to the active T cell–dependent K/BxN arthritis resulting from the KRN T cell receptor transgene, the FcγR-dependent serum-transfer model can be optimally induced in T cell– and B cell–deficient ($RAG^{0/0}$) mice (13) and was therefore used in this study. Although serum-transfer arthritis can be augmented by external addition of KRN T cells, these autoreactive T cells are not present in non-KRN–transgenic mice (26). The spleens of BL/6 mice with serum-transfer arthritis lack significant levels of Th17 cells, and the disease is independent of IL-17 in the absence of transferred KRN T cells (26). These characteristics enabled us to investigate the T cell/Th17–independent role of TLR-2 and TLR-4 in immune complex–mediated arthritis.

There is some controversy regarding the role of TLR-4 in passive immune complex–induced arthritis. TLR-4 mutant (C3H/HeJ) mice were shown to exhibit less severe arthritis during the chronic (resolution) phase of the K/BxN serum-transfer model than wild-type mice, while having a similar initiation phase (27). Two other studies using TLR-4 gene–deficient mice showed, however, either clinical scores and ankle inflammation similar to those seen in wild-type mice throughout the entire disease course (28), or a protected phenotype from the early induction phase (29). Consistent with the findings of Christianson et al (28), our study revealed that TLR-4 does not promote immune complex–mediated arthritis. In fact, TLR-$4^{-/-}$ mice tended to develop slightly more severe disease. The nonsignificant role of TLR-4 in serum-transfer arthritis is distinct from its evident role in other arthritis models, including collagen-induced arthritis, methylated BSA antigen–induced arthritis, and chronic streptococcal cell wall–induced arthritis, as well as the spontaneous arthritis in IL-1 receptor antagonist–deficient (IL-$1Ra^{-/-}$) mice, all of which strongly rely on T cell activation and development of pathogenic Th17 cells (9,11,12,20,30–32). Hence, the data regarding the redundancy of TLR-4 in serum-transfer arthritis clarify its divergent role in distinct disease-related processes.

The interaction between TLRs and FcγR has been the subject of several studies. Immune complexes containing citrullinated fibrinogen have been shown to induce TNFα production in human and murine macrophages via concomitant engagement of TLR-4 and FcγR (33). A recent study showed that cotriggering of FcγRIIA amplified the cytokine response of human dendritic cells to TLR-2, TLR-4, and TLR-5 in order to promote Th17 differentiation (34). Considering the potent and robust inflammatory effects of both TLRs and FcγR, tight control of their response is desired. In fact, counterregulation of TLR response by FcγRIIB has been suggested before. In this context, FcγR ligation can inhibit the responses of TLR-2 and TLR-4 to their respective agonists via activation of the phosphatidylinositol 3-kinase/Akt pathway (35–37). However, it has so far been unclear whether TLRs also pursue a counterregulatory mechanism to control FcγR response. To our knowledge, this is the first study to show that TLR-2 indeed limits FcγR activation by immune complexes, a process with notable consequences for antibody-driven joint pathology.

TLR-2 deficiency exaggerated the inflammatory phenotype and FcγR response in primary macrophages at a very early stage of the disease. This resulted in increased expression of TNFα and IL-1β, for which relevant roles in this serum-induced model have been demonstrated (27,38). TLR-2 stimulation was found to specifically up-regulate the inhibitory FcγRIIB, which dampens the response of other FcγR. Accordingly, TLR-$2^{-/-}$ mice have reduced FcγRIIB expression on macrophages and exhibit a phenotype very similar to that previously observed in FcγRII$^{-/-}$ mice (17). Since TLR-2 has been reported to induce alternative macrophage activation via IL-4Rα and STAT-6 (39), expression of a variety of markers for alternatively activated macrophages including arginase 1, CD163, YM1, and IL-1Ra and IL-1RII was analyzed along with markers for classically activated macrophages such as inducible nitric oxide synthase and CD86 (40). However, no evidence of reduced alternatively activated macrophages and skewing toward the classically activated macrophage phenotype was found in TLR-$2^{-/-}$ mice (data not shown).

It remains to be determined exactly how TLR-2 controls cell surface expression of FcγRIIB. Various endogenous TLR-2 agonists have been described in the context of inflammatory diseases such as RA; however, it is unclear whether these agonists are at play in our model. The observation that FcγRIIB expression is already altered in naive unstimulated BMMs differentiated from BM cells ex vivo tends to exclude an indirect effect of ligand-mediated TLR-2–induced cytokines modulating FcγR expression. Extensive flow cytometric analyses showed similar proportions of FcγR-bearing myeloid blasts, granulocytes, and monocytes in the bone marrow of wild-type and TLR-$2^{-/-}$ mice with unaltered FcγR expression (data not shown). However, the influence of TLR-2 deficiency on other mature FcγR-expressing cells, such as mast cells and neutrophils, that contribute to disease is not excluded. A previous study showed that triggering with IgG immune complexes causes direct physical association of TLR-4 with FcγRIII, an effect required for optimal response to immune complexes in macrophages and polymorphonuclear cells (41). Decreased FcγRIIB expression on naive unstimulated TLR-$2^{-/-}$ BMMs suggests the possibility of such a physical interaction between these 2 receptors. However, this has not been examined so far and remains an intriguing subject for future studies.

Taken together, our findings reveal a clear protective role of TLR-2 in immune complex–mediated arthritis beyond its previously described Treg cell–dependent protective function in T cell–driven disease. A regulatory role of TLR-2 and redundancy of TLR-4 in FcγR-mediated joint inflammation and destruction is highly relevant when considering therapeutic interventions targeting these receptors in the future.

ACKNOWLEDGMENTS

We are grateful to Professor S. Akira (Osaka University, Osaka, Japan) for providing TLR-$2^{-/-}$ and TLR-$4^{-/-}$ mice, and to Professor F. Nimmerjahn (University of Erlangen-Nuremberg, Erlangen, Germany) and Dr. S. Verbeek (Leiden University Medical Centre, Leiden, The Netherlands) for providing antibodies for FcγR detection.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Abdollahi-Roodsaz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Abdollahi-Roodsaz, Koenders, van de Loo, van den Berg.

Acquisition of data. Abdollahi-Roodsaz, Walgreen, Bolscher, Helsen, van den Bersselaar.

Analysis and interpretation of data. Abdollahi-Roodsaz, Koenders, van Lent, van de Loo, van den Berg.

REFERENCES

1. Brentano F, Kyburz D, Gay S. Toll-like receptors and rheumatoid arthritis. Methods Mol Biol 2009;517:329–43.
2. Radstake TR, Roelofs MF, Jenniskens YM, Oppers-Walgreen B, van Riel PL, Barrera P, et al. Expression of Toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-γ. Arthritis Rheum 2004;50:3856–65.
3. Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, van Lieshout AW, Sprong T, van den Hoogen FH, et al. The expression of Toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of Toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. Arthritis Rheum 2005;52:2313–22.
4. Brentano F, Schorr O, Gay RE, Gay S, Kyburz D. RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. Arthritis Rheum 2005;52:2656–65.
5. Foell D, Wittkowski H, Roth J. Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. Nat Clin Pract Rheumatol 2007;3:382–90.
6. Huang QQ, Sobkoviak R, Jockheck-Clark AR, Shi B, Mandelin AM, Tak PP, et al. Heat shock protein 96 is elevated in rheumatoid arthritis and activates macrophages primarily via TLR2 signaling. J Immunol 2009;182:4965–73.
7. Shiozawa K, Hino K, Shiozawa S. Alternatively spliced EDA-containing fibronectin in synovial fluid as a predictor of rheumatoid joint destruction. Rheumatology (Oxford) 2001;40:739–42.
8. Youssef P, Roth J, Frosch M, Costello P, Fitzgerald O, Sorg C, et al. Expression of myeloid related proteins (MRP) 8 and 14 and the MRP8/14 heterodimer in rheumatoid arthritis synovial membrane. J Rheumatol 1999;26:2523–8.
9. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, Devesa I, Roelofs MF, Radstake TR, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. J Clin Invest 2008;118:205–16.
10. Ultaigh SN, Saber TP, McCormick J, Connolly M, Dellacasagrande J, Keogh B, et al. Blockade of Toll-like receptor 2 prevents spontaneous cytokine release from rheumatoid arthritis ex vivo synovial explant cultures. Arthritis Res Ther 2011;13:R33.
11. Abdollahi-Roodsaz S, Joosten LA, Roelofs MF, Radstake TR, Matera G, Popa C, et al. Inhibition of Toll-like receptor 4 breaks the inflammatory loop in autoimmune destructive arthritis. Arthritis Rheum 2007;56:2957–67.
12. Midwood K, Sacre S, Piccinini AM, Inglis J, Trebaul A, Chan E, et al. Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. Nat Med 2009;15:774–80.
13. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, et al. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity 1999;10:451–61.
14. Nabbe KC, Blom AB, Holthuysen AE, Boross P, Roth J, Verbeek S, et al. Coordinate expression of activating Fcγ receptors I and III and inhibiting Fcγ receptor type II in the determination of joint inflammation and cartilage destruction during immune complex–mediated arthritis. Arthritis Rheum 2003;48:255–65.
15. Van Lent PL, Grevers L, Lubberts E, de Vries TJ, Nabbe KC, Verbeek S, et al. Fcγ receptors directly mediate cartilage, but not bone, destruction in murine antigen-induced arthritis: uncoupling

of cartilage damage from bone erosion and joint inflammation. Arthritis Rheum 2006;54:3868–77.
16. Nimmerjahn F, Ravetch JV. Fcγ receptors as regulators of immune responses. Nat Rev Immunol 2008;8:34–47.
17. Corr M, Crain B. The role of FcγR signaling in the K/B × N serum transfer model of arthritis. J Immunol 2002;169:6604–9.
18. Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA, et al. Arthritis critically dependent on innate immune system players. Immunity 2002;16:157–68.
19. Solomon S, Rajasekaran N, Jeisy-Walder E, Snapper SB, Illges H. A crucial role for macrophages in the pathology of K/B × N serum-induced arthritis. Eur J Immunol 2005;35:3064–73.
20. Abdollahi-Roodsaz S, Joosten LA, Helsen MM, Walgreen B, van Lent PL, van den Bersselaar LA, et al. Shift from Toll-like receptor 2 (TLR-2) toward TLR-4 dependency in the erosive stage of chronic streptococcal cell wall arthritis coincident with TLR-4–mediated interleukin-17 production. Arthritis Rheum 2008;58:3753–64.
21. Saber T, Veale DJ, Balogh E, McCormick J, NicAnUltaigh S, Connolly M, et al. Toll-like receptor 2 induced angiogenesis and invasion is mediated through the Tie2 signalling pathway in rheumatoid arthritis. PLoS One 2011;6:e23540.
22. Frasnelli ME, Tarussio D, Chobaz-Peclat V, Busso N, So A. TLR2 modulates inflammation in zymosan-induced arthritis in mice. Arthritis Res Ther 2005;7:R370–9.
23. Joosten LA, Koenders MI, Smeets RL, Heuvelmans-Jacobs M, Helsen MM, Takeda K, et al. Toll-like receptor 2 pathway drives streptococcal cell wall-induced joint inflammation: critical role of myeloid differentiation factor 88. J Immunol 2003;171:6145–53.
24. Liu H, Komai-Koma M, Xu D, Liew FY. Toll-like receptor 2 signaling modulates the functions of $CD4^+CD25^+$ regulatory T cells. Proc Natl Acad Sci U S A 2006;103:7048–53.
25. Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 2006;116:485–94.
26. Jacobs JP, Wu HJ, Benoist C, Mathis D. IL-17-producing T cells can augment autoantibody-induced arthritis. Proc Natl Acad Sci U S A 2009;106:21789–94.
27. Choe JY, Crain B, Wu SR, Corr M. Interleukin 1 receptor dependence of serum transferred arthritis can be circumvented by Toll-like receptor 4 signaling. J Exp Med 2003;197:537–42.
28. Christianson CA, Dumlao DS, Stokes JA, Dennis EA, Svensson CI, Corr M, et al. Spinal TLR4 mediates the transition to a persistent mechanical hypersensitivity after the resolution of inflammation in serum-transferred arthritis. Pain 2011;152:2881–91.
29. Kim HS, Chung DH. TLR4-mediated IL-12 production enhances IFN-gamma and IL-1beta production, which inhibits TGF-β production and promotes antibody-induced joint inflammation. Arthritis Res Ther 2012;14:R210.
30. Joosten LA, Abdollahi-Roodsaz S, Heuvelmans-Jacobs M, Helsen MM, van den Bersselaar LA, Oppers-Walgreen B, et al. T cell dependence of chronic destructive murine arthritis induced by repeated local activation of Toll-like receptor–driven pathways: crucial role of both interleukin-1β and interleukin-17. Arthritis Rheum 2008;58:98–108.
31. Koenders MI, Devesa I, Marijnissen RJ, Abdollahi-Roodsaz S, Boots AM, Walgreen B, et al. Interleukin-1 drives pathogenic Th17 cells during spontaneous arthritis in interleukin-1 receptor antagonist–deficient mice. Arthritis Rheum 2008;58:3461–70.
32. Pierer M, Wagner U, Rossol M, Ibrahim S. Toll-like receptor 4 is involved in inflammatory and joint destructive pathways in collagen-induced arthritis in DBA1J mice. PLoS One 2011;6:e23539.
33. Sokolove J, Zhao X, Chandra PE, Robinson WH. Immune complexes containing citrullinated fibrinogen costimulate macrophages via Toll-like receptor 4 and Fcγ receptor. Arthritis Rheum 2011;63:53–62.
34. Den Dunnen J, Vogelpoel LT, Wypych T, Muller FJ, de Boer L, Kuijpers TW, et al. IgG opsonization of bacteria promotes Th17 responses via synergy between TLRs and FcγRIIa in human dendritic cells. Blood 2012;120:112–21.
35. Polumuri SK, Toshchakov VY, Vogel SN. Role of phosphatidylinositol-3 kinase in transcriptional regulation of TLR-induced IL-12 and IL-10 by Fcγ receptor ligation in murine macrophages. J Immunol 2007;179:236–46.
36. Wenink MH, Santegoets KC, Roelofs MF, Huijbens R, Koenen HJ, van Beek R, et al. The inhibitory FcγIIb receptor dampens TLR4-mediated immune responses and is selectively up-regulated on dendritic cells from rheumatoid arthritis patients with quiescent disease. J Immunol 2009;183:4509–20.
37. Zhang Y, Liu S, Liu J, Zhang T, Shen Q, Yu Y, et al. Immune complex/Ig negatively regulate TLR4-triggered inflammatory response in macrophages through FcγRIIb-dependent PGE_2 production. J Immunol 2009;182:554–62.
38. Ji H, Pettit A, Ohmura K, Ortiz-Lopez A, Duchatelle V, Degott C, et al. Critical roles for interleukin 1 and tumor necrosis factor α in antibody-induced arthritis. J Exp Med 2002;196:77–85.
39. Shirey KA, Cole LE, Keegan AD, Vogel SN. Francisella tularensis live vaccine strain induces macrophage alternative activation as a survival mechanism. J Immunol 2008;181:4159–67.
40. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity 2010;32:593–604.
41. Rittirsch D, Flierl MA, Day DE, Nadeau BA, Zetoune FS, Sarma JV, et al. Cross-talk between TLR4 and FcγReceptorIII (CD16) pathways. PLoS Pathog 2009;5:e1000464.