

Glycoprotein 96 Perpetuates the Persistent Inflammation of Rheumatoid Arthritis

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Objective. The mechanisms that contribute to the persistent activation of macrophages in rheumatoid arthritis (RA) are incompletely understood. The aim of this study was to determine the contribution of endogenous gp96 in Toll-like receptor (TLR)–mediated macrophage activation in RA.

Methods. RA synovial fluid was used to activate macrophages and HEK–TLR-2 and HEK–TLR-4 cells. Neutralizing antibodies to TLR-2, TLR-4, and gp96 were used to inhibit activation. RA synovial fluid macrophages were isolated by CD14 negative selection. Cell activation was measured by the expression of tumor necrosis factor α (TNF α) or interleukin-8 messenger RNA. Arthritis was induced in mice by K/BxN serum transfer. The expression of gp96 was determined by immunoblot analysis, enzyme-linked immunosorbent assay, and immunohistochemistry. Arthritis was treated with neutralizing anti-gp96 antiserum or control serum.

Results. RA synovial fluid induced the activation of macrophages and HEK–TLR-2 and HEK–TLR-4 cells. RA synovial fluid–induced macrophage and HEK–TLR-2 activation was suppressed by neutralizing anti-gp96 antibodies only in the presence of high

(>800 ng/ml) rather than low (<400 ng/ml) concentrations of gp96. Neutralization of RA synovial fluid macrophage cell surface gp96 inhibited the constitutive expression of TNF α . Supporting the role of gp96 in RA, joint tissue gp96 expression was induced in mice with the K/BxN serum–induced arthritis, and neutralizing antibodies to gp96 ameliorated joint inflammation, as determined by clinical and histologic examination.

Conclusion. These observations support the notion that gp96 plays a role as an endogenous TLR-2 ligand in RA and identify the TLR-2 pathway as a therapeutic target.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that, if not successfully treated, leads to cartilage and bone destruction (1–3). Recent observations suggest that RA is initiated in genetically predisposed individuals who possess HLA–DR β 1 alleles that contain the shared epitope, following environmental exposure such as cigarette smoke or periodontal disease (4–6). The environmental exposure results in protein citrullination, and these modified proteins are selectively presented by shared epitope–positive antigen-presenting cells, resulting in anti–citrullinated protein antibodies (ACPAs) that are characteristic of RA (3,5). Recent studies have demonstrated that immune complexes containing ACPAs are capable of inducing inflammation, by activating macrophages through cell surface Fc receptors (7,8).

Once inflammation is initiated, several regulatory and structural molecules are up-regulated locally within the joint (9). Accumulating data suggest that some of these molecules may contribute to the persistence and destruction observed in RA by serving as endogenous Toll-like receptor (TLR) ligands (9). However, a functional candidate from RA synovial fluid (SF) has not been directly identified. TLRs include cell surface receptors (e.g., TLR-2 and TLR-4) and endosomal recep-

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tors (e.g., TLR-3, TLR-7, and TLR-9), which were originally identified in mammals for their ability to bind microbial ligands. TLR ligation results in the activation of transcription factors such as NF- κ B, JNK, ERK, and p38, which promote the expression of proinflammatory chemokines, cytokines, and matrix metalloproteinases (10,11). Prior studies have demonstrated increased expression of TLR-2 and TLR-4 by RA synovial macrophages and an increased response to TLR-2 or TLR-4 microbial ligands (12).

However, the contribution of endogenous SF ligands to TLR-2 or TLR-4 activation has not been directly shown, although several potential endogenous TLR ligands have been identified in the joints of patients with RA, including Hsp60, Hsp70, high mobility group box chromosomal protein 1, tenascin C, and fibrinogen (13–18). However, none of these potential TLR ligands present in RA SF has been shown to bind and activate through the TLR signaling pathway. Although recombinant Hsp60 and Hsp70 activated TLR-4 (13,17), subsequent studies using ultrapure recombinant proteins failed to detect TLR-4 activation (19,20). This underscores the risk of microbial TLR ligand contamination when using recombinant proteins expressed in *Escherichia coli* as TLR agonists, further supporting the importance of using SF.

We recently demonstrated that the endoplasmic reticulum-associated stress response protein gp96 is highly expressed in the synovial tissue and SF of patients with RA (21). Both macrophage-expressed gp96 and the recombinant gp96 N-terminal domain were capable of binding to TLR-2 in pull-down experiments. Furthermore, highly purified gp96 N-terminal domain activated macrophages mediated through TLR-2 and induced the expression of TLR-2, tumor necrosis factor α (TNF α), and interleukin-8 (IL-8) by RA SF macrophages. However, no prior studies have demonstrated the ability of a specific potential endogenous TLR ligand present in RA SF to activate macrophages and HEK 293 cells through TLR-2 or TLR-4.

In the current study, we demonstrate that elevated gp96 levels present in RA SFs promote TLR-2-dependent macrophage activation. We further show that gp96 expression is also increased in an experimental mouse model of RA, and that neutralizing gp96 in vivo ameliorates the arthritis. These observations identify gp96 as a clinically relevant endogenous TLR-2 ligand in RA and suggest that the TLR signaling pathway is a viable target in RA.

PATIENTS AND METHODS

Patients and specimens. Synovial fluid samples were obtained from the inflamed joints of 12 patients with RA diagnosed according to the American College of Rheumatology classification criteria (22). The SF specimens were obtained during routine clinical care, as part of ongoing treatment for an arthritis flare. Ten of the 12 patients were women. At the time of SF collection, the mean age of the patients was 59 years (range 30–85 years), and the mean disease duration was 13 years (range 0.5–25 years). Seven patients were tested for rheumatoid factor, and 2 of these patients had positive results. Of the 2 patients tested for anti-cyclic citrullinated peptide antibodies, 1 had a positive result. Six patients were receiving prednisone at a dosage of ≤ 10 mg/day, 1 patient was receiving methotrexate alone, 1 patient was being treated with methotrexate plus plaquenil, 5 patients were receiving methotrexate plus a TNF inhibitor (plus sulfasalazine in 1 patient), 1 patient was being treated with leflunomide alone, and 2 patients were not receiving any medication at the time of SF aspiration. Two patients had stopped treatment with methotrexate or methotrexate plus a TNF inhibitor 2 months and 4 months, respectively, prior to joint aspiration. At the time when SF was obtained, the mean swollen joint count of the patients was 2.9.

The SF specimens were first centrifuged at 800g for 10 minutes to obtain the cells and cell-free SF. The SF cells were then fractionated on a Histopaque 1077 density gradient to collect mononuclear cells, followed by further purification to obtain CD14+ macrophages by negative selection (StemCell Technologies), as previously described (21). Peripheral blood mononuclear cells and monocytes from normal control subjects and patients with RA were isolated by the same procedures. Primary human macrophages (control macrophages) were obtained from normal peripheral blood monocytes, isolated by elutriation, followed by in vitro differentiation for 7 days, as previously described (12,23–28). All patients were recruited from the Northwestern Medical Faculty Foundation or the Rehabilitation Institute of Chicago, and all patients provided informed consent. These studies were reviewed and approved by the Northwestern University Institutional Review Board.

Cell activation and detection. Macrophages were incubated with 25% RA SF in RPMI 1640 for 4 hours. To identify the presence of TLR-2 or TLR-4 ligands in the SF, rat monoclonal anti-TLR-2 or anti-TLR-4 antibodies (InvivoGen) or control rat IgG (10 μ g/ml) was incubated with macrophages for 30 minutes prior to the addition of SF. To determine whether gp96 in the SF contributed to macrophage activation, the SF specimens were preincubated (1:50) with control rabbit serum or neutralizing rabbit anti-gp96 anti-serum (21) for 30 minutes prior to the addition to macrophages. Macrophage activation was determined by TNF α and IL-8 messenger RNA expression, using quantitative reverse transcription-polymerase chain reaction (RT-PCR) (21). Known microbial TLR-2 and TLR-4 ligands were used as positive controls in each experiment (data not shown).

HEK-TLR-2 or HEK-TLR-4 cells were incubated with 50% SF in Dulbecco's modified Eagle's medium for 20 hours. To identify gp96-mediated activation, the SF samples were preincubated (1:50) with control rabbit serum or rabbit

anti-gp96 antiserum for 30 minutes prior to incubation with HEK cells. Activation of HEK-TLR-2 or HEK-TLR-4 cells was determined by quantitative RT-PCR for IL-8. Microbial TLR ligands were used as positive controls in each experiment (data not shown).

Cell surface gp96 expression. Cell surface gp96 expression was examined by 2-color flow cytometry (12). Mononuclear cells were isolated from the peripheral blood of healthy control subjects and patients with RA and from RA SF. Monocytes and macrophages were identified by fluorescein isothiocyanate-labeled anti-CD14. Cell surface gp96 expression was detected with a rat anti-gp96 monoclonal antibody (LabVision) or an isoform-matched rat IgG control followed by phycoerythrin (PE)-labeled anti-rat IgG. Data were acquired on a BD LSR II flow cytometer with FACSDiva software (BD Biosciences) and were analyzed using FlowJo (Tree Star). The level of macrophage surface gp96 expression was determined as the mean fluorescence intensity (MFI) of PE on the CD14+ or CD14- cell population (12).

K/BxN serum-transfer arthritis model. K/BxN mice were generated, and anti-glucose-6-phosphate isomerase (anti-GPI) antibody-positive serum was collected at 8–9 weeks of age, as previously described (29,30). Arthritis was induced by intraperitoneal injection of 150 μ l anti-GPI antibody-positive serum on day 0 and day 2 or by intraperitoneal injection of 100 μ l on day 0 only. The development of arthritis was assessed by measuring the hind ankle thickness with a caliper and grading the clinical index of all 4 ankles/paws on a scale of 0–3 for each ankle/paw (maximum score = 12), as previously described (29,30). At the time of harvesting, the ankles were quickly dissected and the skin was removed, followed by storage either in 10% neutral buffered formalin for histologic analysis or at -80°C for enzyme-linked immunosorbent assay (ELISA) or immunoblot analysis.

Histopathologic analysis. After storage in 10% neutral buffered formalin, the ankles were incubated in EDTA-formalin decalcification buffer for 2 weeks, embedded in paraffin, and then 4- μ m sections were stained with hematoxylin and eosin. Ankle sections were evaluated in a blinded manner by a pathologist, who assigned a score from 0 to 5 for each of the following: joint and extraarticular inflammation, pannus formation, bone erosion, median synovial line thickness, and cartilage destruction (29,30). Images were obtained using an Olympus BX41 microscope and a DP2-BSW camera.

Detection of gp96 and IL-1 β . Ankles were homogenized in phosphate buffered saline supplemented with a protease inhibitor cocktail (Sigma). Supernatants were collected by centrifugation at 12,000g for 5 minutes at 4°C , and the protein concentration was determined using bicinchoninic acid protein assay reagents (Thermo Scientific). The concentrations of gp96 were quantified by ELISA, as previously described (21); the assay was modified to recognize mouse gp96 by replacing the capture antibody with 100 μ l of rat anti-gp96 monoclonal antibody (LabVision) at 2 μ g/ml. The IL-1 β in the same ankle homogenate was quantified by ELISA (DuoSets; R&D Systems). The concentrations of gp96 (ng/ml) and IL-1 β (pg/ml) were adjusted to milligrams of total ankle protein. The expression of gp96 in RA SF was detected by immunoprecipitation with protein G-Sepharose-immobilized rabbit anti-gp96 polyclonal antibody-positive antiserum or control rabbit serum, followed by immunoblot analysis with a goat anti-gp96

antibody, as previously described (21). The expression of gp96 in murine ankle joints was also detected by immunohistochemistry (21).

Statistical analysis. Comparisons between multiple groups were performed using one-way analysis of variance followed by a Tukey-Kramer test. Comparisons between 2 groups were performed using 2-sided *t*-tests. For samples that failed the normality test, Mann-Whitney rank testing was performed. Correlations were determined using Spearman's nonparametric correlation, because the data analyzed had a non-Gaussian distribution. *P* values less than 0.05 were considered significant.

RESULTS

Activation of macrophages by RA SF through TLR-2 and TLR-4. We previously identified gp96 as a potential endogenous TLR-2 ligand that is present in RA SF and tissue (21). Therefore, primary human macrophages were used to directly determine whether RA SF is capable of activating macrophages through TLR-2 or TLR-4, and whether the gp96 that is present in SF is responsible for TLR activation. Quantitative RT-PCR was used to screen RA SF samples containing >100 ng/ml of gp96 for their ability to activate macrophages (defined as a ≥ 2 -fold induction of TNF α and IL-8) (Figure 1A). Activation of macrophages by RA SF was suppressed following incubation with neutralizing antibodies to TLR-2 or TLR-4 (Figure 1B), demonstrating that triggering through these TLRs contributes to macrophage activation.

We next determined whether gp96 expression in RA SF contributed to macrophage activation. We previously demonstrated that recombinant gp96 N-terminal domain (1 μ g/ml) was capable of activating TLR-2, and that the mean level of gp96 in RA SF was 817 ng/ml (21). Therefore, we divided the SF samples into those with high (>800 ng/ml) or low (<400 ng/ml) levels of gp96. SF samples were preincubated with neutralizing anti-gp96 antibody-positive serum or control rabbit serum prior to incubation with human macrophages. Although there was no difference in macrophage activation by the SF with high or low concentrations of gp96, neutralizing anti-gp96 antibodies suppressed the induction of TNF α and IL-8 (Figure 1C) only by the RA SF samples that contained high levels of gp96 and not those with concentrations of <400 ng/ml. Immunoprecipitation was used to confirm the specific interaction between anti-gp96 antibodies and gp96 in SF (Figure 1D). These analyses demonstrated that RA SF activates macrophages through TLR-2 or TLR-4 and by gp96 at a concentration of >800 ng/ml; however, they did not

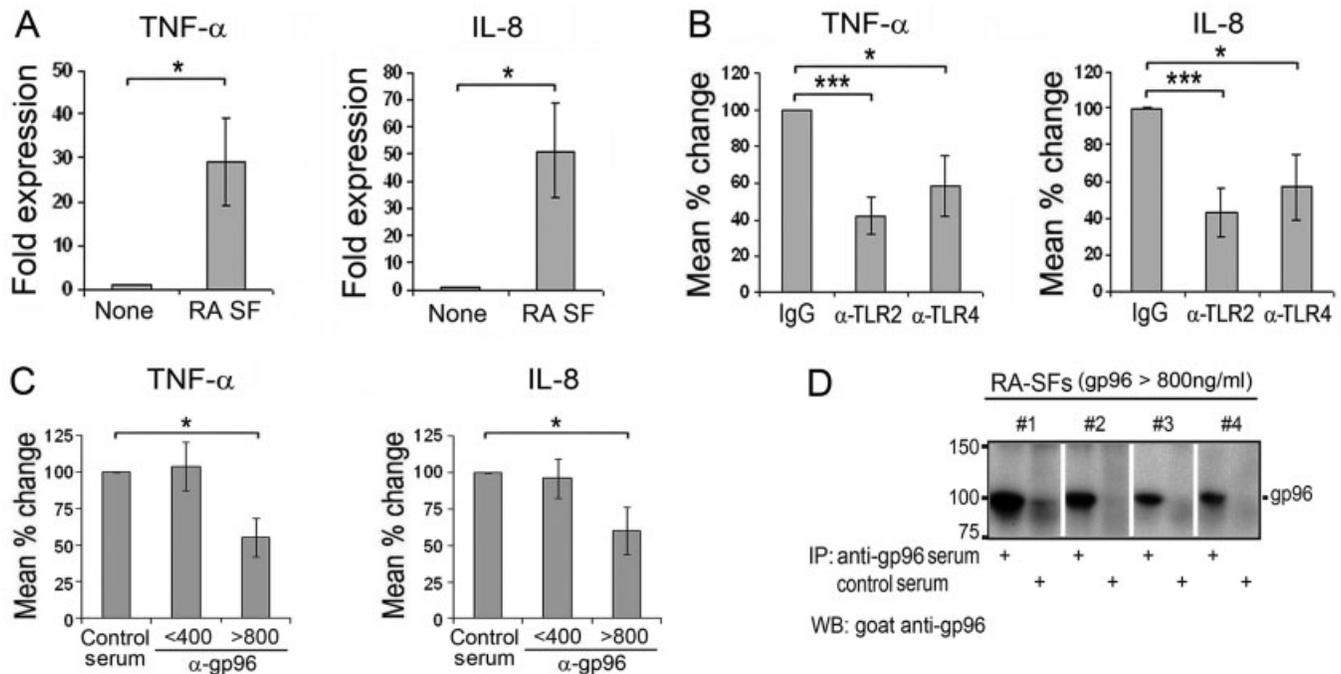


Figure 1. Macrophage activation by rheumatoid arthritis (RA) synovial fluid (SF) is suppressed by neutralizing antibodies to Toll-like receptor 2 (TLR-2), TLR-4, and gp96. **A**, Expression of tumor necrosis factor α (TNF α) and interleukin-8 (IL-8) mRNA in RA SF samples containing >100 ng/ml of gp96, as determined by quantitative reverse transcription–polymerase chain reaction. Results are presented as the mean \pm SEM fold expression compared with medium alone (None). **B**, Effect of neutralizing antibodies to TLR-2 or TLR-4 on activation of macrophages by RA SF. Results are presented as the mean \pm SEM percent change in TNF α and IL-8 expression in the presence of anti-TLR antibodies compared with IgG controls (100%). **C**, Macrophage activation by RA SF specimens with low concentrations (<400 ng/ml) and high concentrations (>800 ng/ml) of gp96. Results are presented as the mean \pm SEM percent suppression of TNF α and IL-8 in the presence of anti-gp96 compared with control rabbit serum (100%). **A–C**, All experiments were repeated at least twice. **D**, Expression of gp96 in 4 RA SF specimens with >800 ng/ml gp96, as detected by immunoprecipitation (IP) with anti-gp96 antibody–positive antiserum or control rabbit serum, followed by Western blot (WB) analysis with a second anti-gp96 antibody. * = $P < 0.05$; *** = $P < 0.001$.

discern whether the activation by gp96 is mediated through TLR-2 or TLR-4.

To directly determine whether the gp96 that is present in SF activates through TLR-2 or TLR-4, we incubated SF samples containing >800 ng/ml or <400 ng/ml of gp96 with HEK–TLR-2 and HEK–TLR-4 cell lines (Figure 2). There was no difference in the intensity of TLR-2 or TLR-4 activation by the SF samples that contained high concentrations of gp96 and those that contained low concentrations of gp96 (for HEK–TLR-2, mean \pm SEM 15.3 \pm 10.3-fold versus 13.3 \pm 4.1-fold; for HEK–TLR-4, 16.4 \pm 9.0-fold versus 17.8 \pm 7.2-fold). Nevertheless, the activation of HEK–TLR-2 cells induced by SF containing high, but not low, concentrations of gp96 was significantly suppressed by the neutralizing anti-gp96 antibody–positive antiserum compared with control rabbit serum (Figure 2A). In contrast, anti-gp96 did not suppress activation of HEK–

TLR-4 cells by RA SF (Figure 2B), indicating that the gp96 that is present in RA SF activates through TLR-2 but not TLR-4 or an alternative mechanism.

Promotion of macrophage activation by gp96 present on the cell surface of RA SF macrophages. Glycoprotein 96 is an endoplasmic reticulum–resident protein in homeostatic settings, and our prior studies demonstrated that macrophages are one of the sources of gp96 (21). Therefore, we used flow cytometry to examine macrophages from RA SF for cell surface–expressed gp96. Glycoprotein 96 was present on normal and RA peripheral blood CD14+ monocytes as well as RA SF macrophages (Figure 3A). Quantitative analysis of the MFI demonstrated that cell surface gp96 expression was significantly greater on RA SF macrophages compared with RA or control monocytes, while it was very low on CD14– mononuclear cells regardless of the source (Figure 3B).

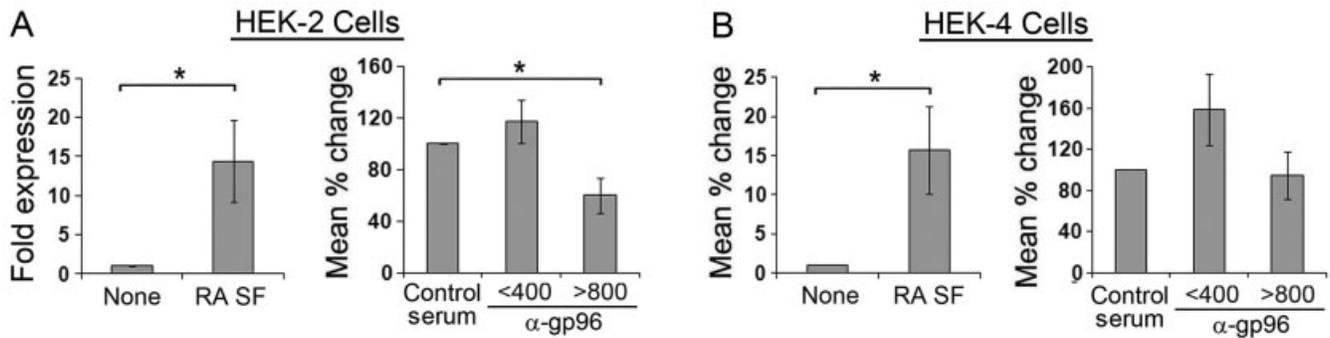


Figure 2. HEK-TLR-2 (HEK-2) cell activation by RA SF is suppressed by anti-gp96. **A and B, Left,** To evaluate gp96-mediated activation, HEK-TLR-2 or HEK-TLR-4 (HEK-4) cells were incubated with RA SF diluted 1:2 for 20 hours, and activation was determined by quantitative reverse transcription-polymerase chain reaction for IL-8. **Right,** To determine the ability of gp96 to activate HEK cells, RA SF specimens with low concentrations (<400 ng/ml) and high concentrations (>800 ng/ml) of gp96 were preincubated with control rabbit serum or rabbit anti-gp96 antibody-positive antiserum for 30 minutes prior to incubation with HEK-TLR-2 or HEK-TLR-4 cells. The percent inhibition by anti-gp96 was determined by comparison with control rabbit serum (100%). Values are the mean \pm SEM of 2–3 repeated experiments for each individual fluid. * = $P < 0.05$. See Figure 1 for other definitions.

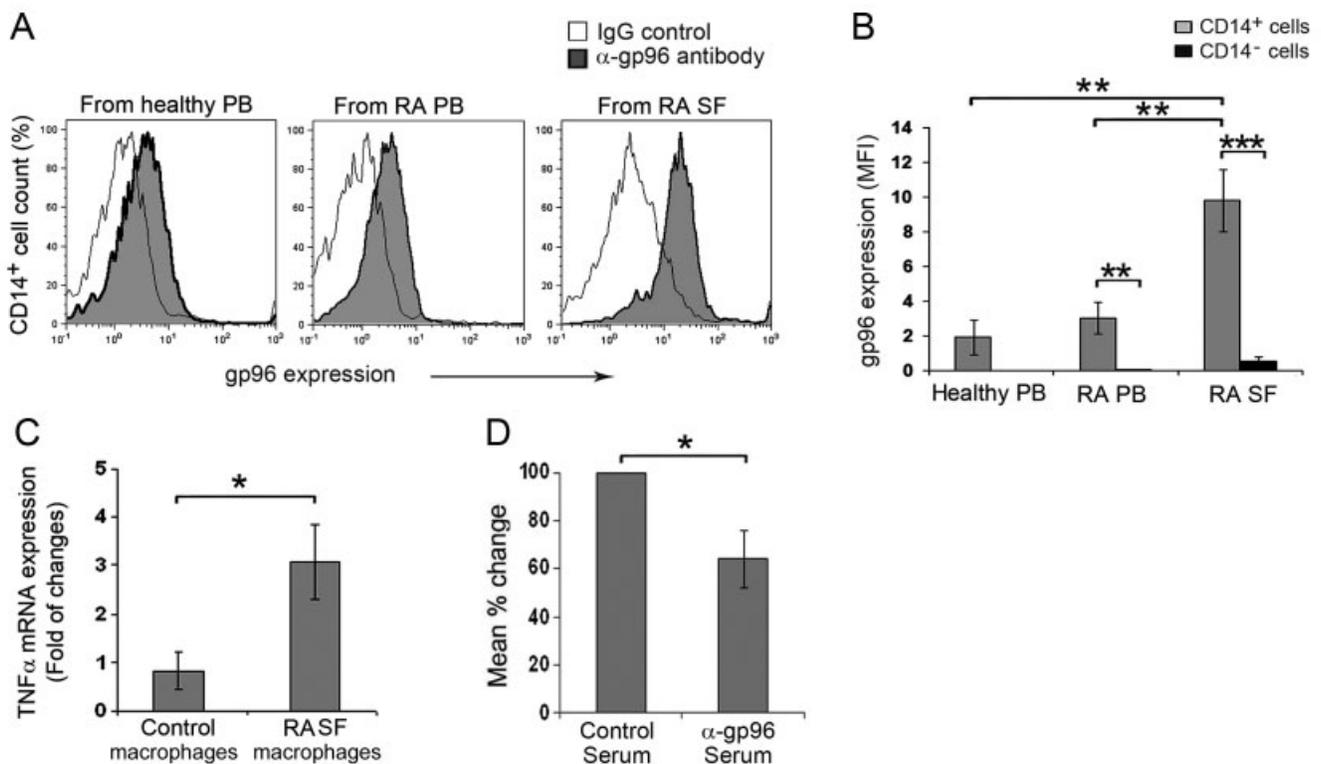


Figure 3. Blocking of cell surface gp96 expression on RA SF macrophages suppresses cell activation. **A,** Representative histograms showing gp96 expression on the cell surface of mononuclear cells isolated from the peripheral blood (PB) of healthy control subjects, the peripheral blood of patients with RA, and RA SF. **B,** Mean fluorescence intensity (MFI) of gp96 expression on the surface of CD14⁺ or CD14⁻ mononuclear cells from the peripheral blood of healthy control subjects ($n = 7$) and patients with RA ($n = 10$), and RA SF macrophages ($n = 9$). **C,** Constitutive expression of TNF α in RA SF macrophages ($n = 4$) compared with control in vitro-differentiated macrophages ($n = 5$), as determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR). **D,** Suppressed activation of CD14⁺ macrophages from RA SF by preincubation of cells with neutralizing anti-gp96 antiserum ($n = 4$) compared with cells incubated with control rabbit serum (100%), as determined by quantitative RT-PCR. Values in **B–D** are the mean \pm SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. See Figure 1 for other definitions.

We previously demonstrated by flow cytometry that TNF α was constitutively expressed by RA SF macrophages, but its expression was negligible in control in vitro–differentiated macrophages (12). Consistent with these observations, the constitutive expression of TNF α was 3-fold higher in RA SF macrophages compared with control in vitro–differentiated macrophages, as determined by quantitative RT-PCR (Figure 3C). To determine whether cell surface–expressed gp96 contributed to the constitutive expression of TNF α , we preincubated RA SF macrophages with control or neutralizing anti-gp96 antibody–positive serum prior to incubation. Compared with control serum, anti-gp96 antibody–positive serum suppressed the constitutive expression of TNF α by RA SF macrophages (Figure 3D) (12). These observations suggested that cell surface gp96 on RA SF macrophages is capable of promoting macrophage activation within the RA joint.

Synergistic RA SF macrophage activation. Our data suggested that endogenous TLR-2 and TLR-4 ligands are present in RA SF. We previously demonstrated that RA SF macrophages exhibit an enhanced response to microbial TLR-2 or TLR-4 ligands compared with control macrophages or those from the joints of patients with psoriatic arthritis or ankylosing spondylitis (12). We therefore investigated the potential synergistic activation of control and RA SF macrophages, using suboptimal concentrations of the TLR-2 ligand peptidoglycan (PG) and the TLR-4 ligand lipopolysaccharide (LPS). The induction of IL-6 was slightly greater than additive and not significantly different for TNF α using control in vitro–differentiated macrophages (Figure 4). In contrast, the combination of suboptimal concentrations of PG and LPS synergistically activated RA SF macrophages, as demonstrated by the induction of TNF α and IL-6 expression that was significantly greater than that observed for control macrophages (Figure 4). These observations suggested that relatively low levels of endogenous TLR-2 and TLR-4 ligands present in the RA joint, including gp96, may act synergistically to promote local macrophage activation.

Correlation of gp96 expression with disease activity in anti-GPI antibody–induced arthritis. To further elucidate the role of gp96 in disease pathogenesis, we examined the expression of gp96 in the joints of mice following the induction of anti-GPI antibody–positive K/BxN serum–transfer arthritis. Arthritis developed in all of the mice injected with anti-GPI antibody–positive serum. Although gp96 was not detected by ELISA prior

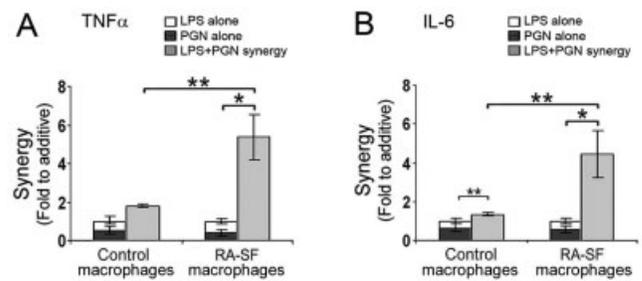


Figure 4. The combination of suboptimal concentrations of the TLR-2 ligand peptidoglycan (PGN) and the TLR-4 ligand lipopolysaccharide (LPS) synergistically activates RA SF macrophages. **A** and **B**, Expression of TNF α (**A**) and IL-6 (**B**), as determined by enzyme-linked immunosorbent assay. In vitro–differentiated control macrophages or CD14+ macrophages isolated from RA SF were incubated with a suboptimal concentration of LPS (0.1 ng/ml) or PGN (0.2 μ g/ml), individually or in combination, for 4 hours. Results are presented as the mean \pm SEM fold increase with the combination of LPS plus PGN compared with the sum of the response to the individual ligands ($n = 5$ control macrophages and $n = 4$ RA SF macrophages). * = $P < 0.05$; ** = $P < 0.01$. See Figure 1 for other definitions.

to the induction of arthritis, it was minimally detected on days 5 and 7, as the severity of arthritis was increasing (as determined by ankle thickness); it was highly expressed between days 9 and 14 (the peak of the arthritis), with expression decreasing by day 19 (Figure 5A). The expression of gp96 in the joints was confirmed by immunoblot analysis and immunohistochemistry (Figure 5B) and was highly correlated ($r = 0.71$, $P < 0.001$) with clinical arthritis (Figure 5C). Furthermore, anti-GPI antibody–positive serum–transfer arthritis is dependent on the expression of IL-1 β , which was increased in inflamed joints and correlated ($r = 0.63$, $P < 0.002$) with the expression of gp96 (Figure 5D). These results demonstrated that the local expression of gp96 is highly correlated with inflammation, as documented by clinical examination, and with the presence of IL-1 β .

Ameliorated progression of anti-GPI antibody–induced arthritis by neutralizing anti-gp96 antibody. To directly determine the potential role of gp96 in arthritis progression, we investigated whether neutralizing anti-gp96 can ameliorate the disease. We treated mice with neutralizing anti-gp96 antibody–positive serum or control serum on days 4, 6, and 8. Arthritis developed in all of the mice. Both the clinical scores and ankle thickness were significantly reduced ($P < 0.01$) in mice treated with anti-gp96 antibodies compared with mice treated with control serum. Improvement was noticeable within 4 days of gp96 neutralization and lasted throughout the

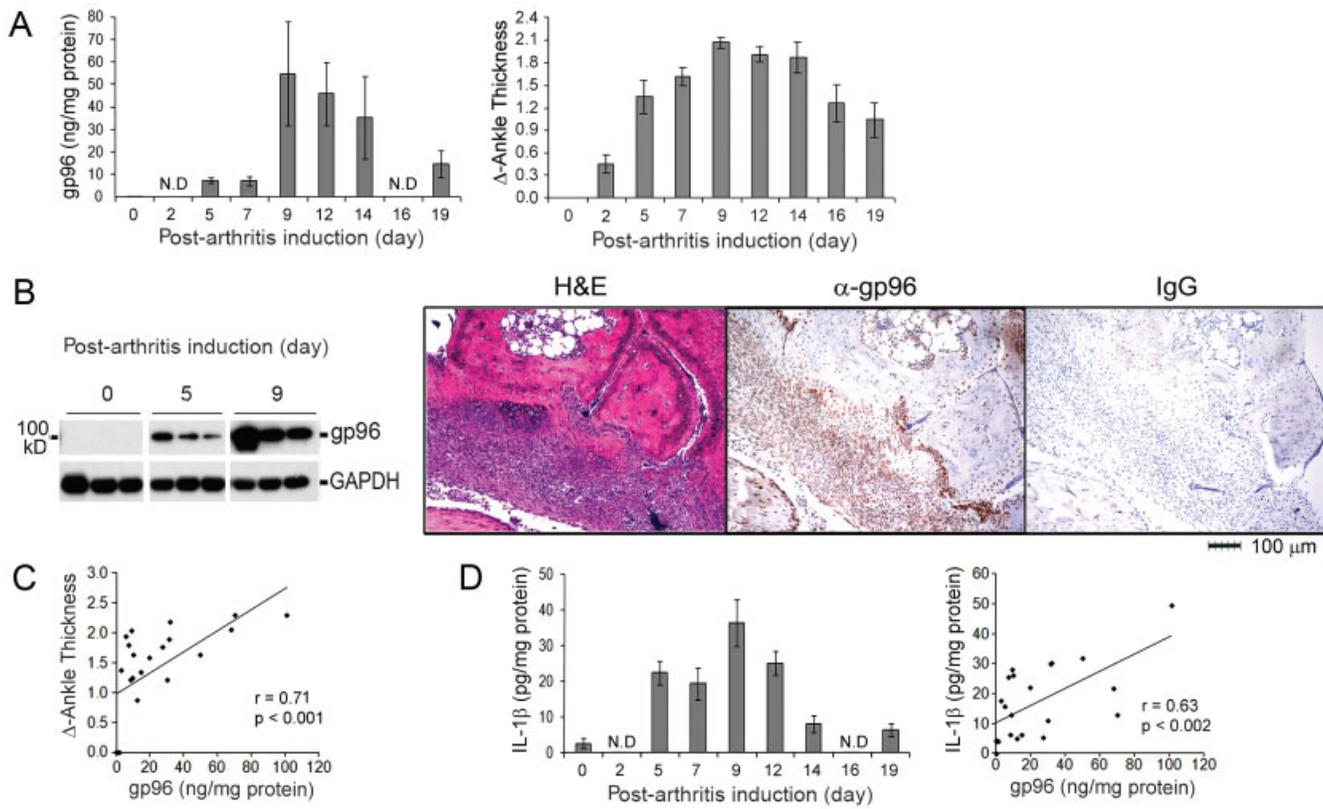


Figure 5. Glycoprotein 96 expression in the inflamed ankle of anti-glucose-6-phosphate isomerase (anti-GPI) antibody-induced arthritis correlates with disease activity. Serum-transfer arthritis was induced in K/BxN mice ($n = 19$) by intraperitoneal injection of anti-GPI antibody-positive serum ($150 \mu\text{l}$) on days 0 and 2, and joint swelling (ankle thickness) was measured over time. Three mice were killed at each time point except on day 19, when 4 mice were killed. One ankle from each mouse was collected at the indicated time points and homogenized, and the concentration of gp96 was determined by enzyme-linked immunosorbent assay (ELISA). The other ankle was used for immunohistochemical analysis. **A**, Increasing severity of arthritis, as demonstrated by increasing gp96 expression and ankle thickness. **B**, Expression of gp96, as confirmed by immunoblotting and immunohistochemical analysis. **C**, Correlation between the concentration of gp96 and ankle thickness. **D**, Left, Expression of IL-1 β before and after induction of arthritis, as determined by ELISA. Right, Correlation between the concentration of gp96 and IL-1 β expression. Bars in **A** and **D** are the mean \pm SEM. ND = not determined; H&E = hematoxylin and eosin (see Figure 1 for other definitions).

course of the disease (Figure 6A). Histologic examination of joints obtained on day 12 demonstrated a significant ($P < 0.05$) reduction in joint inflammation and extraarticular inflammation in mice treated with anti-gp96 antibodies (Figures 6B and C). However, as expected, neither pannus formation nor bone erosion was reduced on day 12, because joint damage cannot be repaired during this short time period. These observations provide evidence of a role of gp96 in the progression and persistence of the arthritis observed in the anti-GPI antibody-positive serum-transfer model of RA.

DISCUSSION

This study is the first to document that gp96, when present in RA SF at concentrations of >800 ng/ml,

is an endogenous TLR-2 ligand capable of activating macrophages. The activation of macrophages by RA SF was inhibited by neutralizing antibodies to TLR-2 and TLR-4 as well as by neutralizing antibodies to gp96. Additionally, neutralizing antibodies to gp96 suppressed RA SF-induced activation of HEK-TLR-2 cells but not HEK-TLR-4 cells, demonstrating specificity for TLR-2. Further supporting the relevance of these observations, neutralization of cell surface gp96 on RA SF macrophages suppressed the constitutive expression of TNF α . Importantly, the gp96 that was present in RA SF was free of the potential endotoxin contamination that might affect the results when recombinant proteins are used, directly demonstrating the pathogenic potential of this endogenous TLR ligand in RA.

We previously demonstrated that gp96 was de-

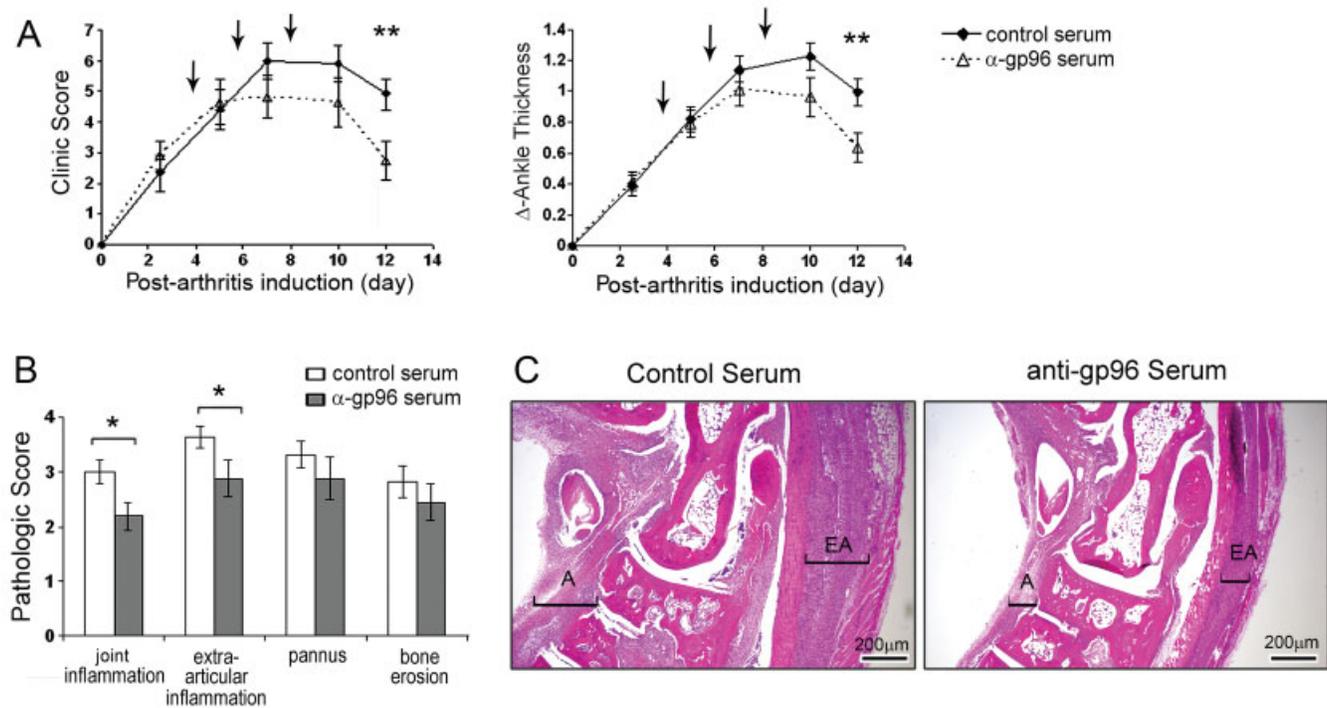


Figure 6. Neutralizing anti-gp96 antiserum ameliorates the progression of anti-glucose-6-phosphate isomerase (anti-GPI) antibody-induced arthritis. **A**, Course of arthritis as determined by the clinical activity score (maximum 12) and swelling of the 2 hind ankles (ankle thickness). **Arrows** indicate the time of intraperitoneal treatment with either control or the anti-gp96 antiserum. Results are the mean \pm SEM of 2 independent experiments ($n = 12$ mice per group). **B**, Pathology scores for inflammation, pannus formation, and bone erosion of ankles collected on day 12 after induction of arthritis. Values are the mean \pm SEM ($n = 8$ mice). **C**, Representative hematoxylin and eosin-stained sections of joints collected on day 12 from mice that received control rabbit serum or anti-gp96 antiserum. **A** = articular inflammation; **EA** = extraarticular inflammation. * = $P < 0.05$; ** = $P < 0.01$.

tected in RA SF, with a mean \pm SD concentration of 817 ± 362 ng/ml (21). SF from patients with other forms of inflammatory arthritis, including psoriatic arthritis and ankylosing spondylitis, demonstrated significantly less gp96 (mean \pm SD 206 ± 46 ng/ml), while the expression of gp96 was even lower in osteoarthritis SF (mean \pm SD 71 ± 20). Therefore, although gp96 is present in the SF of patients with a variety of forms of arthritis, only RA SF possessed concentrations capable of activating TLR-2. However, it is possible that within synovial tissue, where gp96 is being released to the extracellular space, the concentrations are sufficient to activate TLR-2 in diseases other than RA. Our studies demonstrate that RA SF macrophages are significantly more responsive to microbial TLR-2 and TLR-4 ligands compared with macrophages isolated from the joints of patients with other forms of inflammatory arthritis, control peripheral blood monocytes, or in vitro-differentiated macrophages (12). Expanding on these observations, the ability of microbial TLR-2 and

TLR-4 ligands to synergistically activate RA SF macrophages was significantly greater than the ability to activate control macrophages. This increased sensitivity may be attributable to decreased production of IL-10 or to increased expression of interferon- γ by RA SF macrophages (31,32). Taken together, these observations suggest that RA macrophages that are present at the site of joint inflammation may respond to concentrations of endogenous TLR ligands that are even lower than those documented in this study.

The activation of macrophages by RA SF was suppressed by neutralizing TLR-2 and TLR-4 antibodies, and both HEK-TLR-2 and HEK-TLR-4 cells were activated by RA SF, suggesting that both endogenous TLR-2 and TLR-4 ligands are present in RA SF. Although other factors such as cytokines might contribute to HEK-TLR cell activation, the suppression of RA SF-mediated activation of HEK-TLR-2 cells but not HEK-TLR-4 cells by neutralizing anti-gp96 antibodies demonstrated that gp96 in RA SF was activating through

TLR-2. The observation that functional TLR-2 and TLR-4 ligands are present in the RA joint is supported by earlier studies. Other investigators have shown that RA SF is capable of activating HEK-TLR-4 cells, and that neutralizing antibodies to TLR-2 or TLR-4 suppressed the constitutive expression of proinflammatory TNF α by RA synovial tissue culture explants (33,34). Further supporting the role of gp96 in RA, neutralizing anti-gp96 antibodies suppressed the constitutive expression of TNF α by isolated RA SF macrophages. We are aware of no data identifying gp96 as binding to other endogenous TLR ligands or cell-expressed molecules, such as myeloid differentiation protein 2, in the process of activating through TLR-2. Taken together, these studies document the ability of endogenous TLR ligands, in particular gp96, to promote inflammation in RA.

The mechanism by which gp96 is released from the endoplasmic reticulum in RA is not clear. The expression of gp96 on the surface of a variety of tumor cells has been described (35). Furthermore, photodynamic therapy induced the expression of cell surface gp96, which was capable of inducing the expression of TNF α by macrophages (36). Under homeostatic conditions, gp96 binds to KDEL receptors in the Golgi apparatus and is returned to the endoplasmic reticulum. Aminoacyl-transfer RNA synthetase-interacting multifunctional protein 1 (AIMP-1) promotes the retention of gp96 in the endoplasmic reticulum, and AIMP-1-deficient cells demonstrate increased expression of cell surface gp96 (37). Recently, TLR-4-mediated activation of a macrophage cell line resulted in increased cell surface gp96 expression that was mediated by JNK-induced phosphorylation of AIMP-1, which resulted in disruption of the interaction between gp96 and AIMP-1 (38). However, gp96 has been shown to bind to monocytes (39), and it is possible that the cell surface gp96 present on RA SF macrophages may be secondary to released gp96 bound to scavenger receptor class A (40,41) or to TLR-2 itself. Although gp96 is released from necrotic cells (42), necrosis is not a common feature in RA. Therefore, the mechanism responsible for the release of gp96 to the cell surface and into the extracellular space and the SF remains to be determined.

Supporting the notion that it has a role in RA, gp96 expression was very low prior to the induction of experimental arthritis, as determined by ELISA or immunoblot analysis. Glycoprotein 96 was weakly expressed early in the clinical course of disease and was highly expressed by the time of maximal inflammation, and its expression strongly correlated with joint swelling

on clinical examination and the expression of IL-1 β . Consistent with the relevance of gp96 in the pathogenesis of joint inflammation, neutralizing antibodies to gp96 resulted in amelioration of arthritis and decreased joint and periarticular inflammation. Although anti-gp96 was injected beginning on day 4, a significant clinical difference was not observed until day 12. This is most likely attributable to the fact that a marked increase in gp96 expression was not observed until day 9. Furthermore, no significant reduction in bone erosion was noted, most likely due to the rapid clinical course associated with this model of arthritis, in which erosions are already observed early in the disease (43). These observations support the notion that gp96 has a role in promoting chronic inflammation.

Our data suggest that TLR-2 or gp96 may be a therapeutic target in RA. However, a concern associated with neutralizing TLR-2 is the increased joint inflammation observed in *Il1rn*^{-/-} *Tlr2*^{-/-} mice compared with *Il1rn*^{-/-} *Tlr2*^{+/-} mice (33). In contrast, a neutralizing anti-TLR-2 antibody suppressed zymosan-induced arthritis (44) and suppressed myocardial ischemia/reperfusion injury and inflammation in an experimental stroke model (45,46). Additionally, neutralizing antibodies to TLR-2 suppressed the activation of macrophages by RA SF and the constitutive expression of TNF α by RA synovial tissue explants (34). Further supporting the role of endogenous TLR ligands in joint destruction, TLR-2 is strongly expressed in the RA pannus as it erodes into bone (47). Consistent with the role of TLR-2 in disease progression, our unpublished data demonstrate increased inflammation and joint destruction when low doses of microbial TLR-2 ligands are injected into the ankles of mice with anti-GPI-induced arthritis. Taken together, these observations suggest that inflammation promotes a positive feedback loop in RA by inducing endogenous TLR ligands that are released or expressed on the cell surface, promoting progressive, ongoing TLR-mediated inflammation that results in further joint destruction and damage, identifying the TLR signaling pathway as a potential target in RA.

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. Pope 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. Huang, Perlman, Stehlik, Nicchitta, Pope.

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