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Heat Shock Protein 96 Is Elevated in Rheumatoid Arthritis and Activates Macrophages Primarily via TLR2 Signaling

Qi-Quan Huang,* Rudina Sobkoviak,* Angela R. Jockheck-Clark,§ Bo Shi,* Arthur M. Mandelin II,* Paul Peter Tak,† G. Kenneth Haines III,‡ Christopher V. Nicchitta,§ and Richard M. Pope2*

Macrophages are important mediators of chronic inflammation and are prominent in the synovial lining and sublining of patients with rheumatoid arthritis (RA). Recently, we demonstrated increased TLR2 and TLR4 expression and increased response to microbial TLR2 and TLR4 ligands in macrophages from the joints of RA. The current study characterized the expression of the 96-kDa heat shock glycoprotein (gp96) in the joints of RA and its role as an endogenous TLR ligand to promote innate immunity in RA. gp96 was increased in RA compared with osteoarthritis and arthritis-free control synovial tissues. The expression of gp96 strongly correlated with inflammation and synovial lining thickness. gp96 was increased in synovial fluid from the joints of RA compared with disease controls. Recombinant gp96 was a potent activator of macrophages and the activation was mediated primarily through TLR2 signaling. The cellular response to gp96 was significantly stronger with RA synovial macrophages compared with peripheral blood monocytes from RA or healthy controls. The transcription of TLR2, TNF-α, and IL-8, but not TLR4, was significantly induced by gp96, and the induction was significantly greater in purified RA synovial macrophages. The expression of TLR2, but not TLR4, on synovial fluid macrophages strongly correlated with the level of gp96 in the synovial fluid. The present study documents the potential role of gp96 as an endogenous TLR2 ligand in RA and provides insight into the mechanism by which gp96 promotes the chronic inflammation of RA, identifying gp96 as a potential new therapeutic target.


Macrophages, that are prominent in the lining and sublining of joints with rheumatoid arthritis (RA)1 are important mediators of chronic inflammation. Synovial macrophages express high levels of cytokines and chemokines such as IL-1β, TNF-α, GM-CSF, IL-6, and IL-8, which mediate inflammation and cartilage and bone destruction (1, 2). Although the mechanisms contributing to the persistent activation of macrophages in RA are not clear, a number of factors have been described, such as T cell-released cytokines IL-17 and IFN-γ, autoantibodies and resulting immune complexes, and cell-cell contact between macrophages and T cells or macrophages and fibroblasts (3–6). The potential role of TLR has been suggested since TLR2 and TLR4 are expressed in RA synovial tissue (7–10). We recently demonstrated that macrophages from the joints of patients with RA expressed more TLR2 and TLR4 and that their activation by microbial TLR2 and TLR4 ligands was increased compared with control macrophages (11). Furthermore, bacterial cell wall fragments and peptidoglycan have been identified in RA synovial tissue and in other forms of arthritis, in macrophages and other APCs (12, 13).

In addition to the potential role of microbial TLR ligands, a number of potential endogenous stress response proteins such as heat shock protein (HSP) 60 (14, 15), HSP70 (16, 17), and the extracellular matrix component biglycan (18) have been implicated as potential endogenous TLR ligands in RA. However, their role in the perpetuation of the chronic inflammation observed in the RA joint is not clear. Recently, the 96-kDa heat shock glycoprotein (gp96), an endoplasmic reticulum localized stress response protein also known as GRP 94, has been implicated in Ag presentation, tumor immunity, and activation of innate immunity (19–21). gp96 participates in the folding and assembly of many secretory and membrane proteins and is essential for the cell surface expression of TLR, including TLR2 and TLR4 (22). gp96 has been reported to activate professional APCs, such as dendritic cells (23–25) and macrophages (21, 26–29), as well as neutrophils and monocytes (30), promoting the induction of IL-1β and TNF-α (28). Although gp96 is generally localized to the endoplasmic reticulum, it may gain extracellular access after cell activation (31), virus infection (32), or necrotic cell death (28). However, the potential role of gp96 in human disease, such as RA, has not been previously identified.

In this study, we identified by immunohistochemistry, immuno- blot, and ELISA that gp96 was highly expressed in the RA joint, while other HSPs that might serve as endogenous TLR ligands were less readily detected in RA synovial fluid (SF). gp96 induced the expression of TLR2 and cytokines in control and RA SF macrophages. The response by RA SF macrophages was significantly greater than observed with control macrophages and with peripheral blood monocytes from RA or
Materials and Methods

Patients and cell isolation

SFs were obtained from the inflamed joints of patients with RA, with other forms of chronic inflammatory arthritis including psoriatic arthritis, ankylosing spondylitis, arthritis associated with ulcerative colitis and Crohn’s disease, and patients with osteoarthritis (OA) and acute gout. Peripheral blood was collected from patients with RA and healthy donors. Synovial tissues were obtained from patients with RA and OA at the time of arthroplasty or by arthroscopy of actively inflamed joints. Synovial tissues from the arthritis-free controls were obtained at the time of autopsy or surgery for injury from the National Disease Research Interchange. RA patients were diagnosed according to the criteria of the American College of Rheumatology (33). The patients were recruited from the Northwestern Medical Faculty Foundation, the Rehabilitation Institute of Chicago, Northwestern Memorial Hospital, and the Academic Medical Center (Amsterdam, The Netherlands). These studies have been reviewed and approved by the institutional review boards.

The cell-free SF or plasma was isolated by centrifugation to separate the cells and stored at −80°C. The cells were fractionated by Histopaque-1077 density gradient to isolate mononuclear cells, as described previously (11). Peripheral blood monocytes or SF macrophages were purified by negative selection using a monocyte enrichment system (StemCell Technologies). Abs to CD14, CD3, CD20, CD56, CD66b, CD123, and glycopphin attached to magnetic beads were used to deplete cells positive for these markers, leaving CD14+ macrophages from SF or CD14+ monocytes from peripheral blood. Primary human macrophages (control macrophages) were obtained from normal peripheral blood monocytes, isolated by elution followed by in vitro differentiation for 7 days, as previously described (11). Peripheral blood monocytes or SF macrophages were purified by negative selection using a monocyte enrichment system (StemCell Technologies). Abs to CD14, CD3, CD20, CD56, CD66b, CD123, and glycopphin attached to magnetic beads were used to deplete cells positive for these markers, leaving CD14+ macrophages from SF or CD14+ monocytes from peripheral blood. Primary human macrophages (control macrophages) were obtained from normal peripheral blood monocytes, isolated by elution followed by in vitro differentiation for 7 days, as previously described (11).

Recombinant gp96 N-terminal domain

Recombinant canine gp96 N-terminal domain (aa 22–337, gp96-NTD, or gp96) was expressed in Escherichia coli strain BL21-21 as previously described (21, 27, 40). This fragment is 98.7% identical to the amino acid sequence of human gp96, with the sequence difference comprising three conserved substitutions, and mirrors the capacity of the full-length molecule to activate murine dendritic cells (41). Purification of recombinant gp96 was performed as previously described, with minor modifications, as described (27). Purification of recombinant gp96 was performed as previously described, with minor modifications, as described (27).

Immunoblotting

Cells were incubated with gp96 (10 or 50 µg/ml) for 4 h or overnight, as identified in the text. The microbial TLR2 ligands peptidoglycan from Staphylococcus aureus (PGN-SA, 20 µg/ml), Pam3CSK4 (Pam3, 50 ng/ ml), and lipoteichoic acid from S. aureus (LTA-SA, 5 µg/ml) (all from InvivoGen), and TLR4 ligands LPS (1 ng/ml; Sigma-Aldrich) or ultrapure LPS from E. coli K12 (LPS-UP, 5 ng/ml; InvivoGen) were used as positive controls. For intracellular staining, brefeldin A (10 µg/ml; Sigma-Aldrich) was applied along with the ligands. For Ab neutralization, either the monoclional rat anti-TLR2 or TLR4 Abs (InvivoGen) or control rat IgG (10 µg/ml) was added into the macrophage culture 30 min before the addition of gp96 for macrophage activation. All reagents used in this study were examined for endotoxin contamination by the Limulus Amebocyte Lysate QCL-1000 Kit (BioWhittaker) according to the manufacturer’s instructions. All reagents used for the experiments with gp96 possessed endotoxin levels below the detectable level (< 0.1 EU/ml). In some experiments, TLR2, TLR4, TNF-α, and IL-8 mRNA was determined by quantitative real-time RT-PCR (11).

ELISA for detecting gp96 in SF

A double Ab sandwich ELISA was developed to detect full-length gp96 in SF (gp96-ELISA). Microtiter plates (Corning) were coated with 100 µl of goat polyclonal Ab against gp96 C terminus (capture Ab; Santa Cruz Biotechnology) at 0.5 µg/ml in PBS and incubated at room temperature overnight. The blocking and diluent buffer was PBS containing 0.1% BSA and 0.1% Tween 20. All samples were 1/40 diluted in diluent buffer and loaded at 100 µl/well in duplicate for 2 h at room temperature. The wells were then incubated with polyclonal rabbit anti-gp96 antisera, which recognized the N terminus of gp96 (27), for 2 h at room temperature. After washing, HRP-conjugated anti-rabbit IgG (GE Healthcare) at 1/5000 dilution was incubated for 2 h. The color reaction was developed using 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich) for 10–15 min and stopped by adding 50 µl of 2 N H2SO4. The concentration of gp96 was determined using a standard curve generated with recombinant canine gp96 (Stressgen). The correlation coefficient of the standard curve is >0.99 for all experiments. The concentration of gp96 in the tested fluids is interpolated from a standard curve included on each testing plate. Control gp96 did not show any reactivity if either capturing or detecting Ab was absent from the reaction.

Immunoblotting

The cell-free SFs were incubated with hyaluronidase (250 U/ml, type VI from bovine testes; Sigma-Aldrich) for 10 min at 37°C. The fluids were added to SDS-PAGE sample buffer and heated at 90°C for 5 min. The samples were loaded at the equivalent of 4 µl of original fluid. Controls were cell lysates from control macrophages (15 µg), synovial fibroblasts (15 µg), and recombinant proteins (20–50 ng). The protein extracts were resolved by 10% SDS-PAGE. Following transfer to polyvinylidene difluoride membranes (Immobilon-P; Millipore), the blots were probed with goat Abs to TLR2 or TLR4 plus a vector expressing alkaline phosphatase reporter gene under the control of an inducible NF-κB promoter. The TLR2 and TLR4 cell lines were incubated with gp96-NTD (2.5–20 µg/ml), PGN-SA (0.625–5 µg/ml), and LPS-UP (0.125–2 ng/ml) at 37°C for 24 h. The activation of the TLR2 and TLR4 cascade was determined by measuring alkaline phosphatase (OD655 nm) or IL-8 (by ELISA) in the culture supernatants.

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Recombinant TLR-Fc fusion proteins and protein-interactive precipitation assay

The cDNAs encoding the N-terminal extracellular domain of human TLR2 and TLR4 were generated by PCR and cloned into the pFUSE-hIgG1-Fc1 vector (InvivoGen) in frame to the Fc portion of human IgG1. The control vector contains only signal peptide from TLR2 cloned in frame to the
IgG1-Fc. The physical interaction between gp96 and the extracellular domains of TLR2 and TLR4 were determined by the protein-protein interaction precipitation (IP) assay. Recombinant TLR2-Fc, TLR4-Fc, and Fc control proteins were isolated from culture supernatants of stably transfected Chinese hamstery ovary cell lines by affinity binding to protein G-agarose (Roche Diagnostics), followed by washing and incubation with macrophage lysates (precleaned with protein G-agarose) or the recombinant gp96. The arrows identify positive cells in the OA synovial tissue. A, Representative staining for tissues from patients with RA, OA, and arthritis-free controls. The expression of gp96 is presented as the product of the frequency and intensity in the lining and the sublining. C, Disease activity is presented as the median lining thickness and the inflammatory score (0–4). *p < 0.05; **p < 0.01, and ***p < 0.001. D, gp96 expression correlates with lining thickness and inflammation. The relationship between gp96 expression in the lining to the lining thickness and sublining to inflammation is demonstrated for all tissues examined.

Immunohistochemistry

Local expression of gp96 in paraffin- and OCT-embedded synovial tissue was characterized by immunohistochemistry as described previously (40–42), with modifications. Paraffin-embedded synovial tissues were deparaffinized and rehydrated, and Ag retrieval was accomplished by boiling in 10 mM citrated buffer (pH 6.0) with 0.05% Tween 20 for 20 min. The endogenous peroxidase activity was quenched by 3% H2O2 for 5 min, followed by incubation with a monoclonal rabbit anti-gp96 Ab or IgG control. A, Representative staining for tissues from patients with RA, OA, and arthritis-free controls. The arrows identify positive cells in the OA synovial tissue. B, The expression of gp96 is presented as the product of the frequency and intensity in the lining and the sublining. C, Disease activity is presented as the median lining thickness and the inflammatory score (0–4). *p < 0.05; **p < 0.01, and ***p < 0.001. D, gp96 expression correlates with lining thickness and inflammation. The relationship between gp96 expression in the lining to the lining thickness and sublining to inflammation is demonstrated for all tissues examined.

Statistical analysis

ANOVA followed by the Tukey test was used for comparison between groups. Pearson’s product-moment correlation was used with R software (www.r-project.org) to determine the relationship between variables. The two-sided paired t test was used to analyze differences before and after stimulation. Significance levels were set at 0.05.

Results

gp96 is highly expressed in RA synovial tissue

Synovial tissues were obtained from patients with RA and controls to characterize the expression of gp96 by immunohistochemistry. The expression of gp96 was significantly increased in the synovial lining and the sublining of patients with RA compared with those with OA (p < 0.05 and 0.01) or the arthritis-free controls (p < 0.001; Fig. 1, A and B). Only scattered cells within the lymphoid aggregates were positive for gp96. The majority of cells expressing gp96 in the sublining had the morphological appearance of macrophages. The expression of gp96 in the synovial lining was highly correlated (r = 0.71, p < 0.05) with lining thickness in tissues from patients with RA examined alone and when tissues from the three groups were
gp96 was readily detected in the RA and OA SFs (Fig. 3B). In contrast to HSP60, HSP70, and biglycan, full-length gp96 was readily detected in lysates from the RA synovial fibroblasts (Fig. 2A) using Coomassie blue staining to assess equality of loading. Controls are macrophage (M0) and RA synovial fibroblast (FB) lysates (15 μg) and recombinant proteins for each Ab (20–50 ng). Each SF was loaded equivalent to 4 μl of original fluid. B, Coomassie blue R250 staining is presented as a loading control. C, Results of densitometry analysis of gp96 expression in the RA and OA SFs, normalized to 4 μl of SF. D, SFs were examined by ELISA for gp96. Samples are from patients with RA (total n = 32), with other forms of inflammatory arthritis (OIA) (n = 17) and with OA (n = 8). The mean ± 1 SE of gp96 concentration in each group is presented. **, p < 0.01; ***, p < 0.005 between the indicated groups.

FIGURE 2. gp96 is increased in RA SFs by immunoblot analysis and gp-96 ELISA. A, Immunoblot with monospecific Abs to gp96, HSP60, and HSP70 and biglycan. Randomly selected SFs from patients with RA (samples 1–5) and OA (samples 6–10) were resolved by 10% SDS-PAGE and detected for the presence of candidate endogenous TLR ligands by immunoblot analysis. Controls are macrophage (M0) and RA synovial fibroblast (FB) lysates (15 μg) and recombinant proteins for each Ab (20–50 ng). Each SF was loaded equivalent to 4 μl of original fluid. B, Coomassie blue staining is presented as a loading control. C, Results of densitometry analysis of gp96 expression in the RA and OA SFs, normalized to 4 μl of SF. D, SFs were examined by ELISA for gp96. Samples are from patients with RA (total n = 32), with other forms of inflammatory arthritis (OIA) (n = 17) and with OA (n = 8). The mean ± 1 SE of gp96 concentration in each group is presented. **, p < 0.01; ***, p < 0.005 between the indicated groups.

gp96 is increased in RA SF

For endogenous TLR ligands to activate through TLR2 or TLR4, they must reach the extracellular space. Therefore, we examined randomly selected cell-free SFs from patients with RA and OA for the presence of gp96, as well as for HSP60, HSP70, and biglycan, which have been implicated as potential TLR ligands that may be important in RA. All of the HSPs, including gp96, were readily detected in the control macrophage lysates, while biglycan was readily detected in lysates from the RA synovial fibroblasts (Fig. 2A) using Coomassie blue staining to assess equality of loading (Fig. 2B). In contrast to HSP60, HSP70, and biglycan, full-length gp96 was readily detected in the RA and OA SFs (Fig. 3A) using goat polyclonal Ab to the C terminus of gp96. Quantification of the gp96 bands by densitometry suggested that gp96 was increased (p < 0.05) in the RA SFs, compared with those from patients with OA (Fig. 2C).

To more precisely quantify the gp96 in SFs, an ELISA was developed which detected full-length gp96 (Fig. 2D). When multiple fluids were available from individual patients, only the first fluid available was included in this analysis. The concentration of gp96 detected in the SFs of patients with RA (817 ± 362 ng/ml, n = 32) was significantly greater compared with patients with other forms of inflammatory arthritis (206 ± 46 ng/ml, n = 17, p < 0.004) and patients with OA (71 ± 20 ng/ml, n = 8, p < 0.005). The level of gp96 in the fluids from patients with other forms of inflammatory arthritis was higher compared with those with OA, but this difference did not reach significance. Some patients had SFs obtained at multiple time points which were available for analysis. When all available fluids were examined, the results were similar to those presented in Fig. 2D: gp96 was 687 ± 193 ng/ml in RA (n = 62), 255 ± 53 in other forms of inflammatory arthritis (n = 25, p < 0.002 vs RA), and 71 ± 16 in OA (n = 11, p < 0.001 vs RA). Of interest, gp96 was low in the acute inflammatory fluids from three patients with gout (131 ± 73 ng/ml). Together, these observations suggest that gp96 is released in the environment of chronically inflamed joints and that gp96 is increased in the fluids of patients with RA compared with those with other forms of inflammatory arthritis and OA.

gp96 activates macrophages

Previous studies have provided variable results concerning the ability of gp96 to activate macrophages through cell surface TLRs (21, 27, 44). Using very low endotoxin (<0.5 EU/mg) gp96 (10 μg/ml), macrophages differentiated in vitro from normal monocytes (control macrophages) were readily activated, defined by the secretion of IL-6 and TNF-α (Fig. 3A). Preincubation of gp96 with monospecific Abs to gp96 suppressed the induction of TNF-α and IL-6 by gp96 (Fig. 3B), but not by LPS,
FIGURE 3. gp96 activates macrophages. A. In vitro-differentiated control macrophages (n = 11) were incubated with medium alone or with gp96 (10 μg/ml) for 4 h. B, The gp96 or LPS was preincubated with rabbit anti-gp96 antisera at 1/100 or control rabbit IgG before addition to macrophages for 4 h (n = 3–4). C, gp96 or LPS-UP (10 ng/ml) was incubated with macrophages in the presence of polymyxin B (1 μg/ml), or treated by heating (55°C for 1 h), or with proteinase K (50 μg/ml, 55°C for 45 min) before addition to macrophages for 4 h (n = 2). Results for B and C are presented as the percentage of IgG or medium alone control (none). The release of IL-6 and TNF-α in the culture supernatants was quantified by ELISA. **, p < 0.01 and ***, p < 0.001 compared between the indicated groups.

supporting the specificity of gp96 in macrophage activation. Even though the level of endotoxin in the gp96 was >20-fold less than that needed to activate macrophages (data not shown), additional studies were performed to evaluate the potential contribution of endotoxin. Treatment with polymyxin B had no effect on macrophage activation by gp96 while markedly suppressing activation by LPS (Fig. 3C). Furthermore, heating and proteinase K greatly reduced macrophage activation by gp96 but not by LPS (Fig. 3C). These observations demonstrate that macrophage activation by gp96 is not mediated by endotoxin.

**gp96 activates macrophage primarily through TLR2**

Experiments were performed to determine whether macrophage activation was mediated through TLR2 or TLR4, using neutralizing mAbs. The specificity of anti-TLR2 was documented by the inhibition of microbial TLR2 ligands PGN, Pam3CSK4, and LTA-SA, but not TLR4-mediated activation by LPS-UP, while the anti-TLR4 Ab suppressed activation by LPS-UP but not the TLR2 ligands (Fig. 4, A and B). The production of IL-6 and TNF-α was significantly (p < 0.001, respectively) reduced when the macrophages were preincubated with neutralizing anti-TLR2 Ab before the addition of gp96, compared with preincubation with control IgG (Fig. 4, A and B). There was a modest but significant (p < 0.05) reduction of gp96-induced IL-6 (Fig. 4A), but not TNF-α (Fig. 4B) by preincubation with anti-TLR4.

**gp96 activates and binds TLR2 and TLR4**

To further document which TLR signaling pathway was responsible for activation by gp96, HEK-TLR2 and HEK-TLR4 cell lines were used. The cell lines were activated as expected by the respective microbial TLR ligands (Fig. 5, A and B, insets). Concentrations of gp96 as low as 5 μg/ml resulted in significant activation of the HEK-TLR2 cell line determined by NF-κB activation (Fig. 5A) or the secretion of IL-8 (Fig. 5B). The addition of gp96 to the HEK-TLR4 cells also resulted in significant activation at concentrations of 10 μg/ml and greater (Fig. 5, A and B). The activation of the TLR2-expressing cell line by gp96 was significantly greater than observed with the HEK-TLR4 cells. Together, these results indicate that the activation induced by gp96 is mediated primarily through TLR2 signaling.

Previous studies (22) demonstrated that cellular gp96 binds to full-length TLR2 and TLR4 and is responsible for chaperoning these receptors to the cell surface (29). Recombinant TLR2-Fc and TLR4-Fc fusion proteins, expressing only the extracellular domains of TLR2 and TLR4, were used to determine their ability to bind to gp96. Both the wild-type gp96 from human macrophages (Fig. 5C) and the recombinant gp96 (Fig. 5D) were precipitated by binding to TLR2-Fc and TLR4-Fc, but not the control Fc. These observations demonstrate that gp96 is capable of interacting with the extracellular domains of both TLR2 and TLR4.

FIGURE 4. gp96 activates macrophages primarily through TLR2. Macrophages were incubated with neutralizing Abs to TLR2 and TLR4 (10 μg/ml) or isotype control IgG for 30 min before activation by gp96 (10 μg/ml, n = 7), PGN-SA (20 μg/ml, n = 4), Pam3CSK4 (Pam3, 50 ng/ml, n = 5), LTA-SA (5 μg/ml, n = 3), and LPS-UP (10 ng/ml, n = 6) for 4 h. The release of IL-6 (A) and TNF-α (B) in the culture supernatants was quantified by ELISA. Results are presented as the percentage of control IgG. The 100% values for IL-6 (pg/ml) are: gp96: 269 ± 75, PGN-SA: 207 ± 91, Pam3: 453 ± 101, LTA-SA: 285 ± 88, LPS-UP: 901 ± 158; for TNF-α (pg/ml), the 100% values are: gp96: 531 ± 131, PGN-SA: 528 ± 214, Pam3: 3310 ± 464, LTA-SA: 1392 ± 407, LPS-UP: 2542 ± 473. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 compared with IgG controls.
FIGURE 5. gp96 activates HEK-TLR cell lines and interacts with the extracellular domain of TLR2 and TLR4. A and B, HEK-TLR2 (HEK-2) and HEK-TLR4 (HEK-4) cell lines were incubated with medium alone (0) or with increasing concentrations of gp96 (2.5–20 μg/ml) at 37°C for 24 h. Activation of the cell lines was identified by activation of NF-κB alkaline phosphatase promoter-reporter, which is quantified by the spectrophotometer at OD 655 nm (A), or determined by the production of IL-8, quantified by ELISA (B). The results of PGN-SA and LPS-UP controls are presented in the insets. Results are the mean of two to three experiments performed in duplicate or triplicate, presented as fold increase compared with medium alone. *, p < 0.05; **, p < 0.01; and ###, p < 0.001 compared with medium alone. #, p < 0.05 and ##, p < 0.01 comparing reactivity between HEK-TLR2 and HEK-TLR4 cell lines. C and D, Protein G-agarose-bound recombinant fusion proteins TLR2-Fc, TLR4-Fc, and the Fc control were used for IP with macrophage (Mφ) lysate (C, upper panel) or with recombinant gp96 (D). The IP between gp96 and the extracellular domain of TLR2 and TLR4 was determined by probing the blots with Abs to gp96. The presence of TLR2-Fc (87 kDa), TLR4-Fc (93 kDa), and Fc (30 kDa) in the macrophage IPs was identified by probing the same blot using an anti-human Fc Ab (C, lower panel).

Activation of RA SF macrophages by gp96
To define the potential relevance of gp96 to RA, studies were performed to compare the response of RA SF macrophages to gp96 with normal and RA peripheral blood monocytes. Mononuclear cells were incubated with gp96 and the activation of monocytes and macrophages was determined by the expression of TNF-α in CD14+ cells examined by flow cytometry (Fig. 6A). Following the addition of gp96, there was a significant increase of TNF-α/CD14+ cells with RA peripheral blood and SF, and the frequency (Fig. 6B) and intensity (data not shown) of TNF-α in CD14+ cells was significantly greater (p < 0.02) in RA SF compared with normal, but not RA peripheral blood.

To examine the response of isolated macrophages to gp96, RA SF macrophages were isolated by negative selection and compared with in vitro-differentiated control macrophages. Incubation with gp96 induced the expression of both TNF-α and IL-8 in control and RA synovial macrophages determined by quantitative real-time RT-PCR (Fig. 7, A and B). The activation of RA synovial macrophages was significantly (p < 0.05 and 0.02) greater with observed control macrophages. Since activation of macrophages by gp96 was mediated through TLR2, the effect of gp96 on the expression of TLR2 and TLR4 was also examined. Incubation of both control and RA synovial macrophages induced the expression of TLR2 in a dose-dependent fashion and the induction of TLR2 was greater (p < 0.02) in RA synovial, compared with control macrophages (Fig. 7C). There was no induction of TLR4 with either control or RA synovial macrophages (Fig. 7D). These observations support the potential role of gp96 in the pathogenesis of RA mediated by inducing proinflammatory cytokines and TLR2.

The concentration of gp96 in SF correlates with the intensity of the expression of TLR2 on macrophages
Since gp96 is a chaperone for the expression of TLRs in macrophages (29) and since gp96 induced TLR2 and cytokine genes through TLR2, the in vivo relationship between macrophage cell surface TLR2 and SF gp96 was examined. TLR2 expression on macrophages from RA SFs (Fig. 8A) and those from patients with other forms of inflammatory arthritis (Fig. 8B) were analyzed by flow cytometry. A, Representative histograms of mononuclear cells from RA SF with or without gp96 stimulation stained with PE-labeled anti-TNF-α or control IgG, which was analyzed by flow cytometry. B, Summary of the induction of TNF-α in CD14+ monocytes from normal peripheral blood (n = 4), RA peripheral blood (n = 4), and CD14+ macrophages (Mφs) from RA-SF (n = 7). Data are expressed as the percentage (percent) of double-positive cells. **, p < 0.01 between the indicated groups.

FIGURE 6. gp96 induces TNF-α in RA peripheral blood monocytes and synovial macrophages. Mononuclear cells isolated from peripheral blood (PB) of healthy donors (NL) and RA patients and from RA SF were stimulated with medium only (none) or gp96 (50 μg) in the presence of brefeldin A (10 μg/ml) overnight. Two-color intracellular Ab staining was performed with FITC-labeled anti-CD14 and PE-labeled anti-TNF-α or control IgG, which was analyzed by flow cytometry. A, Representative histograms of mononuclear cells from RA SF with or without gp96 stimulation stained with PE-labeled anti-TNF-α Abs. B, Summary of the induction of TNF-α in CD14+ monocytes from normal peripheral blood (n = 4), RA peripheral blood (n = 4), and CD14+ macrophages (Mφs) from RA-SF (n = 7). Data are expressed as the percentage (percent) of double-positive cells. **, p < 0.01 between the indicated groups.
demonstrated significant (\(p < 0.01\) and \(0.05\), respectively) linear correlations (\(r = 0.62\) and 0.65, respectively) with the concentration of gp96 in the same fluids. However, there is no correlation between macrophage TLR4 and gp96 level in RA (Fig. 8A) or other inflammatory arthritis fluids (Fig. 8B). These observations support the potential role of gp96 in the chronic activation of macrophages in the RA joint, which contributes to the pathogenesis of the self-perpetuating inflammation.

Discussion
The mechanisms contributing to the persistent activation of macrophages in the joints of patients with chronic inflammatory arthritis have not been fully elucidated. This study demonstrates that gp96 is highly expressed in RA synovial tissue and that the level of gp96 correlates with lining thickness and inflammation. Furthermore, gp96 is significantly increased in RA SF, demonstrating that this endoplasmic reticulum-localized chaperone is released during chronic inflammation. Very low endotoxin (\(<0.5\) EU/mg) gp96 activated macrophages primarily through TLR2 and the activation of RA SF macrophages was significantly increased compared with the response by control macrophages or normal peripheral blood monocytes. In addition to signaling through TLR2, gp96 induced the expression of TLR2, which was greater with RA SF compared with control macrophages. Furthermore, supporting a potential role of gp96 in vivo, the concentration of gp96 in RA SF correlates with the level of TLR2 expressed on synovial macrophages. These observations suggest that in RA gp96 is released and functions as an endogenous TLR2 ligand capable of promoting the self-perpetuating activation of synovial macrophages.

Other HSPs have been implicated as potential TLR ligands which may contribute to the pathogenesis of RA. To activate through TLR2 or TLR4, endogenous TLR ligands should be either expressed on the cell surface or released. In this study, we examined cell-free SF to identify endogenous TLR ligands released from cells, even though the concentration of any potential ligand might be much greater in the extracellular matrix of the synovial tissue. Earlier studies identified HSP70 in RA synovial tissue and fluid (16, 45). However, a more recent study, using highly specific mAbs, failed to identify increased HSP70 in RA synovial tissue (46). HSP60 has been implicated in RA, because it may be capable of activating monocytes of RA patients and controls through TLR2 or TLR4 (7, 15). However, in screening SFs from patients with RA and OA, neither HSP60 nor HSP70 were detected by immunoblot analysis, while each was readily detected in macrophage lysates. Additionally, biglycan, a small leucine-rich proteoglycan, recently identified as signaling through TLR2 and TLR4, although present in RA synovial fibroblast lysates, was not detected by immunoblot analysis of the SFs. In contrast, gp96 was readily detected by immunoblot analysis and was significantly increased in RA SFs, compared with those from patients with OA. The release of gp96 into the SF was not specific for RA since gp96 was detected by...
ELISA in fluids from patients with other forms of inflammatory arthritis and OA. However, the increased concentration of gp96 in the SFs appears to be related to chronic inflammation, since gp96 in the fluids of patients with acute gout was similar to those with OA. Other HSPs might contribute to the pathogenesis of RA. Recently, low molecular mass HSP22 was identified in RA synovial tissue and was capable of dendritic cell activation mediated through TLR4 (47). Supporting its potential role in RA, HSP22 was also readily detected in RA SF (data not shown). It is possible that endogenous TLR2 and TLR4 ligands might cooperate to promote the persistent activation of macrophages observed in RA.

The mechanisms promoting the increased expression and release of gp96 in the chronically inflamed joints are not fully understood. Inflammatory cytokines such as IFN-γ and IL-2, which may be expressed in early RA, are capable of inducing the expression of gp96 (48, 49). Photodynamic therapy induced the cell surface localization of gp96 on macrophages, suggesting that cellular stress may lead to externalization of gp96 (31). Another mechanism regulating cell surface expression of gp96 is aminoacyl-tRNA synthetase-interaction multifunctional protein 1, which promotes targeting of gp96 to the endoplasmic reticulum, and its deficiency promotes cell surface localization (50). Necrosis, but not apoptosis, was shown to release gp96 (28). However, neither apoptosis nor necrosis is characteristic of RA (1). In fact, the induction of HSPs by cellular stress may protect against apoptosis mediated by both the extrinsic or intrinsic pathways (51). Therefore, although inflammatory mediators may promote the expression of gp96, the mechanism(s) responsible for its release remain to be determined.

Our results document the ability of gp96 to activate primary human macrophages, differentiated in vitro from peripheral blood mononuclear cells (control macrophages). Earlier studies demonstrated that gp96 was capable of activating human peripheral blood monocytes (30) and human mononuclear cell lines (52). However, contaminating endotoxin may have confounded the interpretation of these studies. Subsequent studies demonstrated that low endotoxin gp96 induced ERK phosphorylation, but did not activate NF-κB using the RAW264.7 murine macrophage cell line (27). The low endotoxin recombinant gp96 used in this study activated murine bone marrow-derived dendritic cells, but only at 50 μg/ml (21). In the present study, using primary human macrophages, activation was strongly induced at10 μg/ml. Macrophage activation was mediated primarily through TLR2 since Abs to TLR2 suppressed the induction of both IL-6 and TNF-α and gp96 strongly activated HEK-TLR2 cells. Nonetheless, some activation through TLR4 was observed, since Abs to TLR4 modestly suppressed the gp96-induced expression of IL-6, although no reduction of TNF-α was observed. Supporting a potential minor role for TLR4, gp96 also weakly activated HEK-TLR4 cells. Our data clearly demonstrate that any activation that may occur through TLR4 was not mediated through contaminating endotoxin. Consistent with its ability to activate both pathways, recombinant and wild-type gp96 each interacted with the extracellular domains of both TLR2 and TLR4.

Although our data suggest a potential minor role for TLR4 signaling in gp96-mediated macrophage activation, a recent study demonstrated that neither adeno viral expressed cell surface gp96 nor low endotoxin-soluble gp96 were capable of activating HEK293 cells expressing TLR4 (44). Furthermore, the mechanism responsible for the activation of human macrophages is distinct from the systemic autoimmune disease observed in mice transgenic for cell surface gp96 (53), which is mediated by the increased expression of TLR4 and activated by commensal flora and not through the cell surface expression of gp96 (44). Together, these observations suggest that differences in the methods of isola-


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