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Heat Shock Protein 96 is Elevated in Rheumatoid Arthritis and Activates Macrophages primarily via TLR2 Signaling

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Abstract

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 toll like receptor (TLR) 2 and 4 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 to peripheral blood monocytes from RA or healthy controls. The transcription of TLR2, TNFα 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.

Keywords

gp96; grp94; macrophages; TLR2; TLR4; Rheumatoid Arthritis

Introduction

Macrophages, which are prominent in the lining and sublining of joints from patients with rheumatoid arthritis (RA), are important mediators of chronic inflammation. Synovial macrophages express high levels of cytokines and chemokines such as IL-1β, TNFα, GM-CSF, IL-6 and IL-8, which mediate inflammation and cartilage and bone destruction (1,2). Although

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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 IFNγ, autoantibodies and resulting immune complexes, and cell-cell contact between macrophages and T cells or macrophages and fibroblasts (3-6). The potential role of toll-like receptors (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 to control macrophages (11). Further, bacterial cell wall fragments and peptidoglycan have been identified in RA synovial tissue, and in other forms of arthritis, in macrophages and other antigen presenting cells (12,13).

In addition to the potential role of microbial TLR ligands, a number of potential endogenous stress response proteins such heat shock (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 antigen presentation, tumor immunity and activation of innate immunity (19-21). Gp96 participates in the folding and assembly of many secretary and membrane proteins, and is essential for the cell-surface expression of TLRs including TLR2 and TLR4 (22). Gp96 has been reported to activate professional antigen-presenting cells, 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 TNFα (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 identify by immunohistochemistry, immunoblot and ELISA that gp96 is highly expressed in the RA joint, while other HSPs that might serve as endogenous TLR ligands were less readily detected in RA synovial fluid. Gp96 induced the expression of TLR2 and cytokines in control and RA synovial fluid macrophages. The response by RA synovial fluid macrophages was significantly greater than observed with control macrophages and with peripheral blood monocyte from RA or healthy controls. We also demonstrate that macrophage activation by gp96 is primarily mediated through TLR2 signaling. Further, cell surface TLR2 expression on macrophages from synovial fluid correlates with the level of gp96 present in the synovial fluid, suggesting a relationship between gp96 and the expression of TLR2. These novel findings provide insights into the mechanism by which gp96 promotes chronic inflammation in RA, and identify gp96 as a potential new therapeutic target for RA.

Materials and Methods

Patients and cell isolation

Synovial fluids were obtained from the inflamed joints of patients with RA, with other forms of chronic inflammatory arthritis including psoriatic arthritis, ankylosing spondylosis, arthritis associated with ulcerative colitis and Crohn's disease, and patients with osteoarthritis and acute gout. Peripheral blood was collected from patients with RA and healthy donors. Synovial tissue was obtained from patients with RA and osteoarthritis at the time of arthroplasty, or by arthroscopy of actively inflamed joints. Synovial tissue from the arthritis-free controls was 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 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

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Academic Medical Center, Amsterdam, Netherlands. These studies have been reviewed and approved by the Institutional Review Boards.

The cell-free synovial fluid 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 synovial fluid macrophages were purified by negative selection employing a monocyte enrichment system (StemCell Technologies, Vancouver, Canada). Antibodies to CD2, CD3, CD20, CD56, CD66b, CD123 and glycophorin attached to magnetic beads were used to deplete cells positive for these markers, leaving CD14+ macrophages from synovial fluid or CD14+ monocytes from peripheral blood. 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 (11,34-39). Unless otherwise indicated, all cells were maintained in RPMI-1640 culture medium supplemented with 20% FBS, 100U penicillin, 100 μg/ml streptomycin at 37°C in 5% CO_2.

Recombinant gp96 N-terminal domain

Recombinant canine gp96 N-terminal domain (amino acids 22-337, gp96.NTD or gp96) was expressed in *E.* coli strain BL-21 as previously described (21,27,40). This fragment is 98.7% identical to the amino acid sequence of human gp96, containing 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 follows (21,27). Bacterial pellets were resuspended in 50 mM dextrose, 50 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole and homogenates prepared in a French press. The homogenate was incubated with an equal volume of 10 mM Tris, pH 8.0, 50 mM KCl, 0.5% (v/v) Tween 20, 0.5% (v/v) Triton X-100, 300 mM NaCl, 10 mM imidazole for 30 minutes on ice and insoluble debris subsequently removed by centrifugation at 40,000 × g for 30 minutes at 4°C. The supernatant fraction was filtered (0.45 μm) and the recombinant protein bound to nickel-Sepharose (GE Healthcare). Columns were washed with 30 column volumes of 0.2% (v/v) Tween 20, 0.2% (v/v) Triton X-100, PBS, 40 mM imidazole, pH 8.0 and subsequently in depyrogenation solution (1% (v/v) Triton X-114 in sterile PBS, pH 7.4). Columns were then rinsed with sterile PBS until the absorbance at 280 nm returned to baseline. The recombinant protein was then eluted with sterile imidazole in PBS. Peak fractions were pooled and concentrated in a YM-30 spin column (Amicon). The level of endotoxin in the preparations employed in this study was < 0.5 EU/mg (21).

Macrophage/ monocyte activation and detection

Cells were incubated with gp96 (10 or 50 μg/ml) for 4 hours or over night, as identified in the text. The microbial TLR2 ligands Peptidoglycan from *S. aureus* (PGN-SA,(20 μg/ml), Pam_3CSK4 (Pam_3, 50ng/ml) and Lipoteichoic acid from *S. aureus* (LTA-SA, 5μg/ml) (all from InvivoGen, CA), and TLR4 ligands lipopolysaccharides (LPS, 1 ng/ml, Sigma St. Louis, MO) or ultrapure LPS from *E.coli* K12 (LPS-UP, 5 ng/ml, InvivoGen) were employed as positive controls. For intracellular staining, brefeldin A (10 μg/ml, Sigma) was applied together with the ligands. For antibody neutralization, either the monoclonal rat anti-TLR2 or TLR4 antibodies (InvivoGen) or control rat IgG (10 μg/ml) was added into macrophage culture 30 minutes prior to 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, Walkersville, MD) according to manufacturer's instructions. All reagents employed for the experiments with gp96 possessed endotoxin levels below the detectable level (< 0.1EU/ml). In some experiments the gp96 was pre-incubated with polymyxin B (1μg/ml), pre-heated at 55°C for 1 hour or incubated with proteinase K (50 μg/ml) at 55°C for 45 min prior to incubation with macrophages for 4 hours (n=2). LTA-SA was the only non-TLR4

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ligand with low endotoxin contamination, therefore, polymyxin B was incubated with the LTA-SA prior to addition to the cells.

Following activation of mononuclear cells, the expression of TNFα in CD14+ macrophages or monocytes was quantified by intracellular anti-TNFα antibody staining together with anti-CD14 immuno-phenotyping employing two color flow cytometry, as recently described (11). When employing isolated homogeneous populations of macrophages, the secretion of IL-6 and TNFα in culture supernatants was determined by ELISA (DuoSets, R&D, Minneapolis, MN), while the expression of TLR2, TLR4, TNFα and IL-8 mRNA was determined by quantitative real time RT-PCR (qRT-PCR)(11).

HEK-TLR2 and HEK-TLR4 cell activation and detection

HEK-TLR2 and HEK-TLR4 cell lines (InvivoGen) are engineered HEK293 cells stably transfected with vectors expressing human TLR2 or TLR4, plus a vector expressing alkaline phosphatase (AP) reporter gene under the control of an inducible NF-κB promoter. HEK-TLR2 and HEK-TLR4 cell lines were incubated with gp96.NTD (2.5 to 20 μg/ml), PGN-SA (0.625 to 5 μg/ml) and ultra pure LPS (0.125 to 2 ng/ml) at 37°C for 24 hours. The activation of TLR2 and TLR4 cascade was determined by measuring AP or IL-8 (by ELISA) in the culture supernatants.

ELISA for detecting gp96 in synovial fluid

A double antibody-sandwich ELISA was developed to detect full length gp96 in synovial fluid (gp96-ELISA). Microtiter plates (Corning Inc. NY) were coated with 100 μl of goat polyclonal antibody against gp96 C-terminus (capture antibody, Santa Cruz Biotechnology, Santa Cruz, CA) 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/per well in duplicate for 2 hours at room temperature. The wells were then incubated with polyclonal rabbit anti-gp96 antiserum, which recognized the N-terminus of gp96 (27), for 2 hours at room temperature. Afterward, HRP-conjugated anti-rabbit IgG (GE Healthcare, UK) at 1:5000 dilution was incubated for 2 hours. The reaction was developed employing 3,3′, 5,5′-Tetramethylbenzidine (TMB) substrate (Sigma) for 10-15 min and stopped by adding 50 μl of 2N H_2SO_4. The concentration of gp96 was determined utilizing a standard curve generated with recombinant canine gp96 (Stressgen, Victoria, Canada). Correlation coefficient of the standard curve is greater than 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 reaction if either capturing or detecting antibody was absent from the reaction.

Immunoblotting

The cell-free synovial fluids were incubated with hyaluronidase (250 U/ml, Type VI from bovine testes, Sigma) for 10 minutes at 37°C. The fluids were added to SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and heated at 90°C for 5 minutes. 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 (20-50ng). The protein extracts were resolved by 10% SDS-PAGE. Following transfer to PVDF membrane (immobilon-P, Millipore Co. Bedford, MA), the blots were probed with goat antibodies to the C-terminus of gp96 (Santa Cruz Biotechnology), monoclonal anti-HSP70 and anti-HSP60 (Stressgen, Victoria, B.C.), and monoclonal anti-biglycan (R&D Minneapolis, MN) antibodies. Densitometry analysis of immunoblots was performed by ImageJ software. The total protein contents of the fluids were visualized by Coomassie Blue R250 staining.

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Recombinant TLR-Fc fusion proteins and protein interactive precipitation assay

The cDNAs encoding N-terminal extracellular domain of human TLR2 and TLR4 were generated by PCR and cloned into pFUSE-hIgG1-Fc1 vector (InvivoGen) in frame to the Fc portion of human IgG1. The control vector contains only signal peptide from TLR4 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 interactive precipitation (IP) assay. TLR2-Fc, TLR4-Fc and the Fc control were isolated from CHO culture supernatant by affinity binding to protein-G agarose (Roche Diagnostics GmbH, Mannheim, Germany), followed by washing and incubation with macrophage lysates (pre-cleaned with protein-G agarose) or the recombinant gp96.NTD at 4°C overnight. The resulting agarose complexes were washed and the precipitates resolved by 10% SDS-PAGE, which were probed with a polyclonal rabbit anti-gp96 and antibodies to human Fc to identify the TLR2-Fc (87 kD), TLR4-Fc (93 kD) and the control Fc (30 kD).

Immunohistochemistry

Local expression of gp96 in paraffin and OCT embedded synovial tissue was characterized by immunochemistry, as described (40-42), with modifications. Paraffin embedded synovial tissues were de-paraffinized and rehydrated, and antigen retrieval was accomplished by boiling in 10mM citrated buffer (pH 6.0) with 0.05% Tween-20 for 20 min. The endogenous peroxidase activity was quenched by 3% H_2O_2 for 5 minutes followed by incubation with a monoclonal rat anti-gp96 (Lab Vision, Fremont, CA) or isotype matched rat IgG_{2a} control (eBioscience, San Diego, CA) at 2 μg/ml, diluted in PBS containing 1% of goat serum overnight at 4°C. OCT embedded sections were washed and then blocked by 0.3% H_2O_2, followed by incubation rat anti-gp96 or control antibody at 1 μg/ml overnight at 4°C. The secondary antibody employed was HRP-conjugated goat anti-rat IgG. Color was developed by DAB substrate, followed by counter staining with hematoxylin. The slides were read by a blinded observer for gp96 expression defined as the product of the frequency (%) and intensity (0-3) of staining in the lining or sublining. Additionally, median synovial lining thickness and inflammation (0-4) were scored as previously described (34,43).

Statistical Analysis

Analysis of variance between groups (ANOVA) followed by the Tukey test was employed for comparison between groups. Pearson's product-moment correlation was employed using R software to determine the relationship between variables. The two-sided paired t-test was employed to analyze differences before and after stimulation. Significance levels were set at 0.05.

Results

Gp96 is highly expressed in RA synovial tissue

Synovial tissue was obtained from patients with RA and controls to characterize the expression of gp96 by immunohistochemistry. Gp96 was strongly expressed in the synovial tissue of patients with RA compared with those with osteoarthritis or arthritis free controls (Figure 1). The expression of gp96 was significantly increased in the synovial lining and the sublining of patients with RA compared with those with osteoarthritis ($p< 0.05$ and 0.01) or the arthritis free controls ($p < 0.001$) (Figure 1A,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 3 groups were combined ($r = 0.80$, $p < 0.001$, Figure 1D). Further, the inflammatory score correlated with gp96 in the sublining of patients with RA

(r = 0.83, $p < 0.01$) and when all 3 groups of tissues were combined (r = 0.76, $p < 0.001$, Figure 1D).

Since these RA synovial tissues were obtained from patients at the time of joint replacement surgery, a second group of OCT embedded RA synovial tissues (n = 15), obtained from patients with clinical signs and symptoms of active disease before treatment, were analyzed by immunohistochemistry. Gp96 was highly expressed in the lining (gp96 expression score 144 ± 14) and the sublining (gp96 expression score 152 ± 22) of these tissues. The expression of gp96 in the sublining correlated (r = 0.69, $p < 0.01$) with inflammation, while gp96 in the lining correlated with median lining thickness (r = 0.57, $p < 0.05$). When the data for both groups of RA synovial tissues were combined these relationships remained significant (r = 0.56 and r = 0.62, $p < 0.01$). These observations demonstrate that gp96 is highly expressed in the synovial tissues of patients with RA and that the expression of gp96 is related to the severity of the disease assessed by inflammation and lining thickness.

Gp96 is increased in RA synovial fluid

For endogenous TLR ligands to activate through TLR2 or TLR4, they must reach the extracellular space. Therefore, we examined randomly selected cell-free synovial fluids from patients with RA and osteoarthritis for the expression 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 the HSPs, including gp96 were readily detected in the control macrophage lysates, while biglycan was readily detected in lysates from the RA synovial fibroblasts (Figure 2A), employing coomassie blue staining to assess equality of loading (Figure 2B). In contrast to HSP60, HSP70 and biglycan, full length gp96 was readily detected in the RA and osteoarthritis synovial fluids (Figure 3A) employing goat polyclonal antibody to the C-terminus of gp96. Quantification of the gp96 bands by densitometry, suggested that gp96 was increased ($p < 0.05$) in the RA synovial fluids, compared with those from patients with osteoarthritis (Figure 2C).

In order to more precisely quantify the gp96 in synovial fluids, an ELISA was developed which detected full length gp96 (Figure 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 synovial fluids 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 osteoarthritis (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 to those with osteoarthritis, but this difference did not reach significance. Some patients had synovial fluids obtained at multiple time points which were available for analysis. When all available fluids were examined the results were similar to those presented in Figure2D: 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 osteoarthritis (n = 11, $p < 0.001$ vs. RA). Of interest, gp96 was low in the acute inflammatory fluids from 3 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 osteoarthritis.

Gp96 activates macrophages

Previous studies have provided variable results concerning the ability of gp96 to activate macrophages through cell surface TLRs (21,27,44). Employing 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 TNFα (Figure 3A). Pre-incubation of gp96 with monospecific antibodies to gp96 suppressed the induction of

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TNFα and IL-6 by gp96 (Figure 3B), but not by LPS, 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 (Figure 3C). Further, heating and proteinase K greatly reduced macrophage activation by gp96 but not by LPS (Figure 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 if macrophage activation was mediated through TLR2 or TLR4, employing neutralizing monoclonal antibodies. The specificity of anti-TLR2 was documented by the inhibition of microbial TLR2 ligands PGN, Pam_3CSK4 and LTA-SA, but not TLR4-mediated activation by LPS-UP, while the anti-TLR4 antibody suppressed activation by LPS-UP but not the TLR2 ligands (Figure 4A,B). The production of IL-6 and TNFα was significantly ($p < 0.001$ respectively) reduced when the macrophages were pre-incubated with neutralizing anti-TLR2 antibody prior to the addition of gp96, compared with preincubation with control IgG (Figure 4A,B). There was a modest but significant ($p < 0.05$) reduction of gp96-induced IL-6 (Figure 4A), but not TNFα (Figure 4B) by pre-incubation 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 employed. The cell lines were activated as expected by the respective microbial TLR ligands (Figure 5A,B, insets). Concentrations of gp96 as low as 5μg/ml resulted in significant activation of HEK-TLR2 cell line determined by NF-κB activation (Figure 5A) or the secretion of IL-8 (Figure 5B). The addition of gp96 to the HEK-TLR4 cells also resulted in significant activation, at concentrations of 10 μg/ml and greater (Figure 5A,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 employed to determine their ability to bind to gp96. Both the wild type gp96 from human macrophages (Figure 5C) and the recombinant gp96 (Figure 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.

Activation of RA synovial fluid macrophages by gp96

To define the potential relevance of gp96 to RA, studies were performed to compare the response of RA synovial fluid macrophages to gp96 with normal and RA peripheral blood monocytes. Mononuclear cells were incubated with gp96 and the activation of monocytes and macrophages determined by the expression of TNFα in CD14+ cells, examined by flow cytometry (Figure 6A). The frequency of TNFα/CD14 double positive cells was significantly higher in RA synovial fluid compared with normal or RA peripheral blood (Figure 6B). Following the addition of gp96, there was a significant increase of TNFα/CD14+ cells with RA peripheral blood and synovial fluid, and the frequency (Figure 6B) and intensity (data not shown) of TNFα in CD14+ cells was significantly greater ($p < 0.02$) in RA synovial fluid compared to normal, but not RA, peripheral blood.

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In order to examine the response of isolated macrophages to gp96, RA synovial fluid macrophages were isolated by negative selection and compared with *in vitro* differentiated control macrophages. Incubation with gp96 induced the expression of both TNFα and IL-8 in control and RA synovial macrophages (Figure 7A, B). The activation of RA synovial macrophages was significantly ($p < 0.05$ and 0.02) greater than observed with 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 to control macrophages (Figure 7C). There was no induction of TLR4 with either control or RA synovial macrophages (Figure 7D). These observations support the potential role of gp96 in the pathogenesis of RA, mediated by inducing pro-inflammatory cytokines and TLR2.

The concentration of gp96 in synovial fluid 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 synovial fluid gp96 was examined. TLR2 expression on macrophages from RA synovial fluids (Figure 8A), and those from patients with other forms of inflammatory arthritis (Figure 8B), demonstrated significant ($p < 0.01$ and 0.05 respectively) linear correlations ($r = 0.62$ and 0.65) with the concentration of gp96 in the same fluids. However, there is no correlation between macrophage TLR4 and gp96 level in RA (Figure 8A) or other inflammatory arthritis fluids (Figure 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. Further, gp96 is significantly increased in RA synovial fluid, 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 synovial fluid macrophages was significantly increased compared to 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 synovial fluid compared with control macrophages. Further, supporting a potential role of gp96 *in vivo*, the concentration of gp96 in RA synovial fluid correlates with the level of TLR2 expressed in 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. In order 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 synovial fluid 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, employing highly specific monoclonal antibodies, failed to identity 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

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or TLR4 (7,15). However, in screening synovial fluids from patients with RA and osteoarthritis, neither HSP60 nor HSP70 were detected by immunoblot analysis, while each was readily detected in macrophage lysates. Additionally, biglycan, a small leucin-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 synovial fluids. In contrast gp96 was readily detected by immunoblot analysis and was significantly increased in RA synovial fluids, compared with those from patients with osteoarthritis. The release of gp96 into the synovial fluid was not specific for RA since gp96 was detected by ELISA in fluids from patients with other forms of inflammatory arthritis and osteoarthritis. However, the increased concentration of gp96 in the synovial fluids appears to be related to chronic inflammation, since gp96 in the fluids of patients with acute gout was similar to those with osteoarthritis. Other HSPs might contribute to the pathogenesis of RA. Recently low molecular weight 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 synovial fluid (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 IFNγ 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 of 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 monocytes (control macrophages). Earlier studies demonstrated that gp96 was capable of activating human peripheral blood monocytes (30) and human monocytic 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 employing the RAW264.7 murine macrophage cell line (27). The low endotoxin (< 0.5EU/mg) recombinant gp96 employed in this study activated murine bone marrow derived dendritic cells, but only at 50 μg/ml (21). With primary human macrophages activation was strongly induced at10 μg/ml. Macrophage activation was mediated primarily through TLR2 since antibodies to TLR2 suppressed the induction of both IL-6 and TNFα and gp96 strongly activated HEK-TLR2 cells. Nonetheless, some activation through TLR4 was observed, since antibodies to TLR4 modestly suppressed the gp96-induced expression of IL-6, although no reduction of TNFα 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 both interacted with the extracellular domains of both TLR2 and TLR4.

While our data suggests a potential minor role for TLR4 signaling in gp96-mediated macrophage activation, a recent study demonstrated that neither adenoviral expressed cell surface gp96 nor low endotoxin soluble gp96 were capable of activating HEK293 cells expressing TLR4 (44). Further, the mechanism responsible for the activation of human

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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 isolation or expression may determine whether or not gp96 is capable of activating through TLR4.

Our data strongly support a potential role for gp96 in the pathogenesis of RA. Not only was gp96 highly expressed in the RA synovial tissue, and extracellularly in the synovial fluid, but also the activation of RA synovial fluid macrophages was significantly increased compared with the control macrophages and normal peripheral blood monocytes. Our earlier studies also demonstrated increased expression of TLR2 on RA synovial macrophages, compared to control macrophages (11). Additionally, activation of RA synovial macrophages by a microbial TLR2 ligand, was increased compared to control macrophages and those from the synovial fluid of patients with other forms of inflammatory arthritis, suggesting the importance of TLR2 signaling in RA and that cell surface TLR2 was increased on RA synovial macrophages (11). In the present study, gp96 *in vitro* further induced the expression of TLR2 in RA synovial macrophages. Gp96 in the synovial fluid was highly correlated with the expression of TLR2, but not TLR4, on macrophages from the synovial fluid, suggesting that *in vivo* gp96 may contribute to the increased expression of TLR2. These observations were not unique to RA, since gp96 was present in the synovial fluids of patient with other forms of chronic inflammatory arthritis and TLR2 was also increased on the synovial fluid macrophages from these patients (11). The addition of a suboptimal concentration of gp96 to RA synovial fluid, resulted in greater than additive macrophage activation (data not shown), suggesting that other factors in the fluids are capable of collaborating with gp96 to activate macrophages.

In summary, increased expression and release of gp96 may promote a self-perpetuating chronic inflammatory response driven primarily through TLR2 activation. These observations suggest that gp96 and TLR2 signaling are potential therapeutic targets in RA, although the arthritis that develops in IL-1 receptor antagonist deficient mice is worse in mice lacking TLR2 (54). Nonetheless, supporting this approach, recent studies demonstrated that inhibition of TLR signaling ameliorated disease activity in collagen induced arthritis, an experimental model of RA (55)and in patients with RA (56).

Acknowledgments

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Abbreviations

gp96	96-kDa heat shock glycoprotein

RA	rheumatoid arthritis
OIA	Other forms of chronic Inflammatory Arthritis
OA	osteoarthritis
TLR	Toll-like receptor
PGN	peptidoglycan
LPS	lipopolysaccharide
SF	synovial fluid

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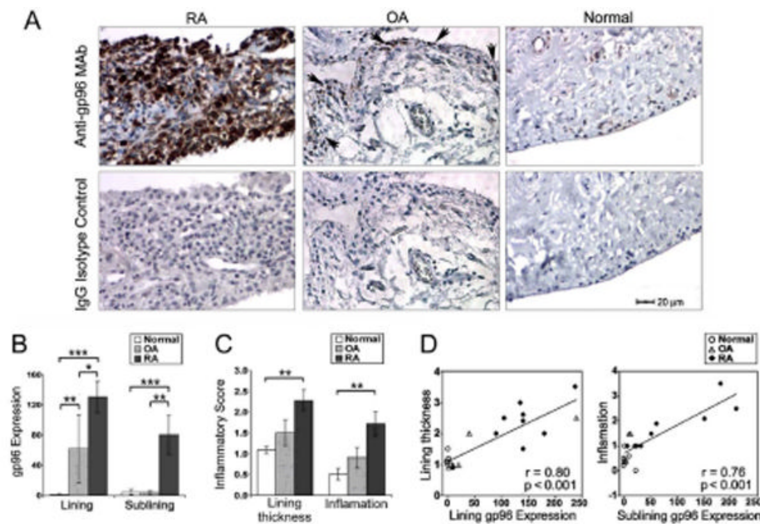

Figure 1. Gp96 is highly expressed in RA synovial tissue

RA (n = 9), osteoarthritis (OA, n = 5) or arthritis free control (normal, n = 6) synovial tissues were examined by immunohistochemisty employing rat monoclonal anti-gp96 antibody 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). * represents $p< 0.05$, ** $p< 0.01$, 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.

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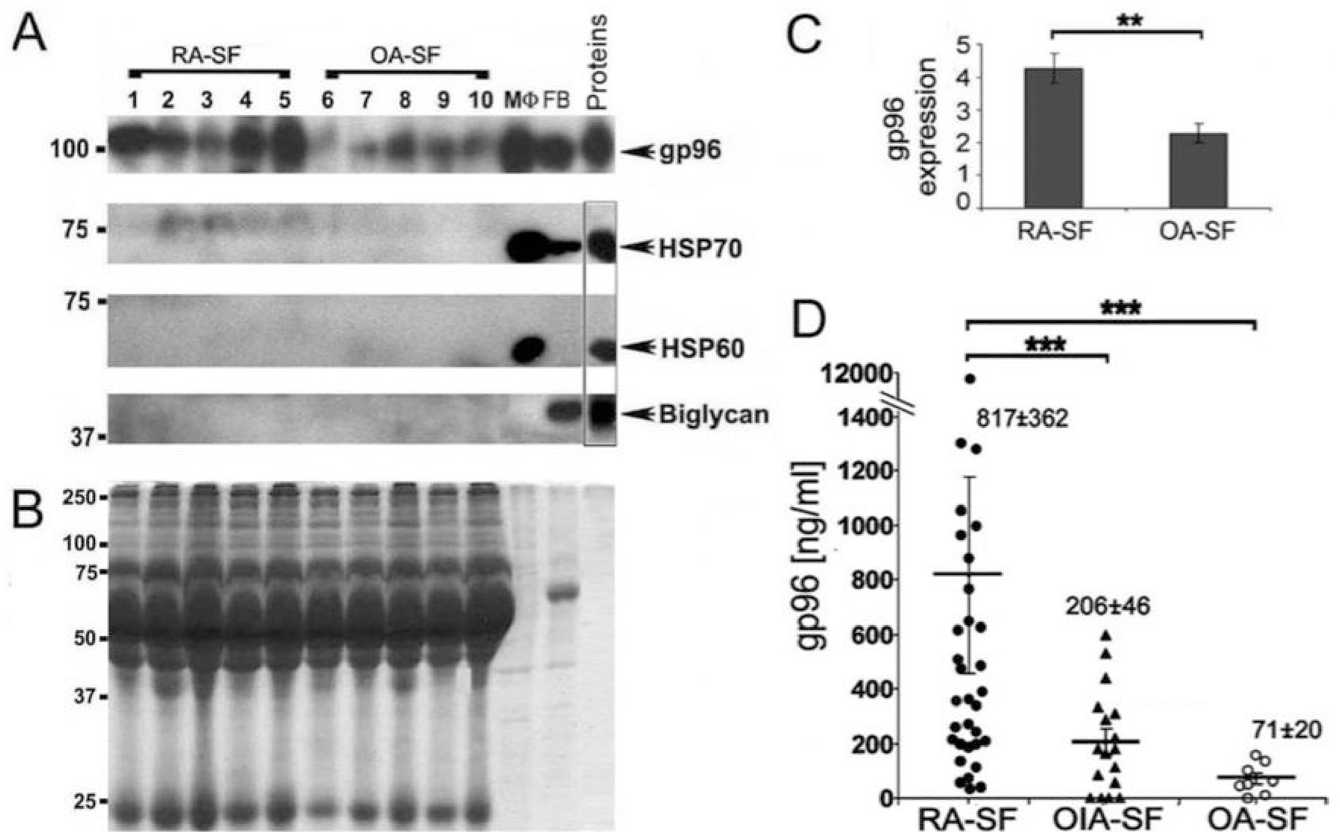

Figure 2. Gp96 is increased in RA synovial fluids by immunoblot analysis and gp-96 ELISA

(A) Immunoblot with monospecific antibodies to gp96, HSP60 and HSP70 and biglycan. Randomly selected synovial fluids (SFs) from patients with RA (samples 1-5) and osteoarthritis (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 (MΦs) and RA synovial fibroblast (FB) lysates (15μg), and recombinant proteins for each antibody (20-50 ng). Each synovial fluid was loaded equivalent to 4μl 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 synovial fluids, normalized to 4 μl of synovial fluid. (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. *** represents $p < 0.005$ between the indicated groups.

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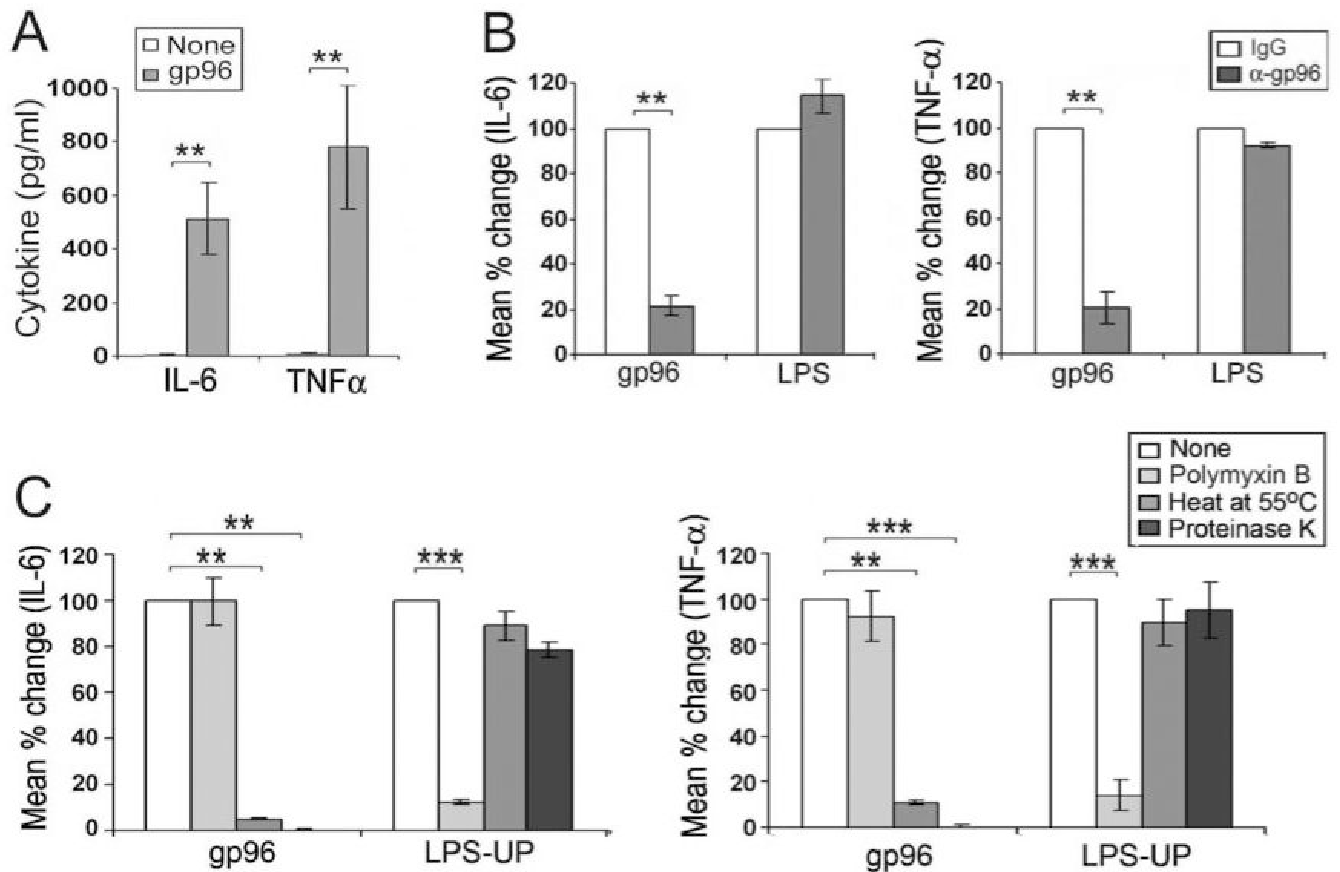

Figure 3. Gp96 activates macrophages

(A) *In vitro* differentiated control macrophages (n=11) were incubated with medium alone or with gp96 (10 μg/ml) for 4 hours. (B) The gp96 or LPS was pre-incubated with rabbit anti-gp96 antiserum at 1:100 or control rabbit IgG prior to addition to macrophages for 4 hours (n=3-4). (C) gp96 or ultra-pure LPS (LPS-UP, 10ng/ml) was incubated with macrophages in the presence of polymyxin B (1μg/ml), or treated by heating (55°C for 1 hour) or with proteinase K (50 μg/ml, 55°C for 45 min) prior to addition to macrophages for 4 hours (n=2). Results for panels Band C are presented as the % of IgG or medium alone control (none). The release of IL-6 and TNFα in the culture supernatants was quantified by ELISA. ** $p< 0.01$, *** $p<0.001$ compared between the indicated groups.

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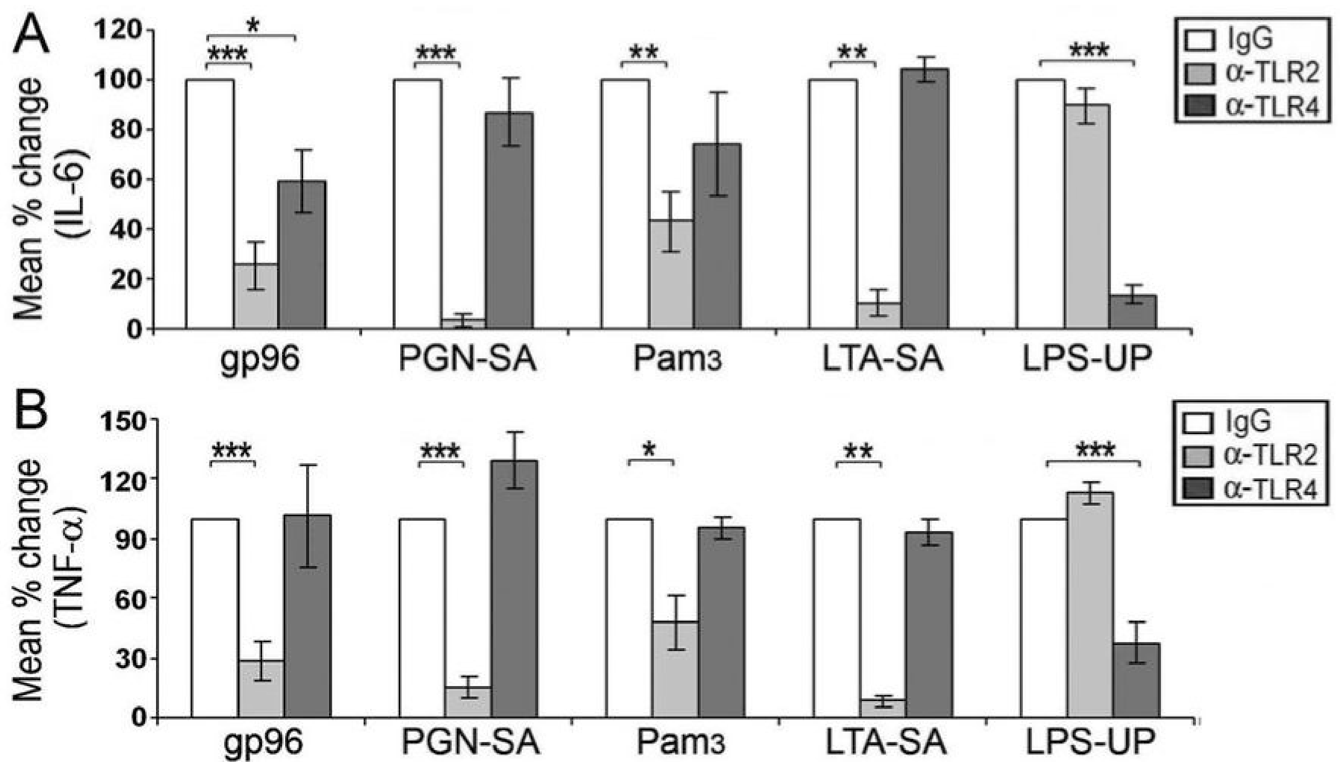

Figure 4. Gp96 activates macrophages primarily through TLR2

Macrophages were incubated with neutralizing antibodies to TLR2 and TLR4 (10 μg/ml) or isotype control IgG for 30 min prior to activation by gp96 (10 μg/ml, n=7), PGN-SA (20 μg/ml, n=4), Pam_3CSK4 (Pam3, 50ng/ml, n=5), LTA-SA (5μg/ml, n=3) and LPS-UP (10ng/ml, n=6) for 4 hours. The release of IL-6 (A) and TNFα (B) in the culture supernatants was quantified by ELISA. Results are presented as the % 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 TNFα (pg/ml) the 100% values are: gp96: 531± 131, PGN-SA: 528± 214, Pam3: 3310± 464, LTA-SA: 1392 ± 407, LPS-UP: 2542± 473. * represents p< 0.05, ** represents p< 0.01, *** p<0.001 compared with IgG controls.

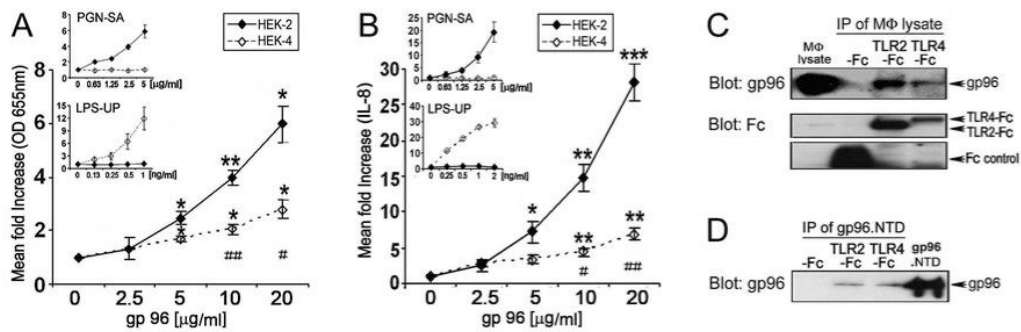

Figure 5. Gp96 activates HEK-TLR cell lines and interacts with extracellular domain of TLR2 and TLR4

(A-B) HEK-TLR2 and HEK-TLR4 cell lines were incubated with medium alone (0) or with increasing concentrations of gp96 (2.5-20 μg/ml) at 37°C for 24 hours. Activation of the cell lines was identified by activation of NF-κB-alkaline phosphatase promoter-reporter, which is quantified by 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 2-3 experiments performed in duplicate or triplicate, presented as fold increase compared to medium alone. * represents $p< 0.05$, ** represents $p< 0.01$, *** $p<0.001$ compared with medium alone. # represents $p< 0.05$, ## $p< 0.01$ comparing reactivity between HEK-TLR2 and HEK-TLR4 cell lines. (C-D) Protein G-agarose bound recombinant fusion proteins TLR2-Fc, TLR4-Fc and the Fc control were employed for interactive precipitation (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 antibodies to gp96. The presence of TLR2-Fc (87 kD), TLR4-Fc (93 kD) and Fc (30 kD) in the macrophage IPs were identified by probing the same blot employing an anti-human Fc antibody (C, lower panel).

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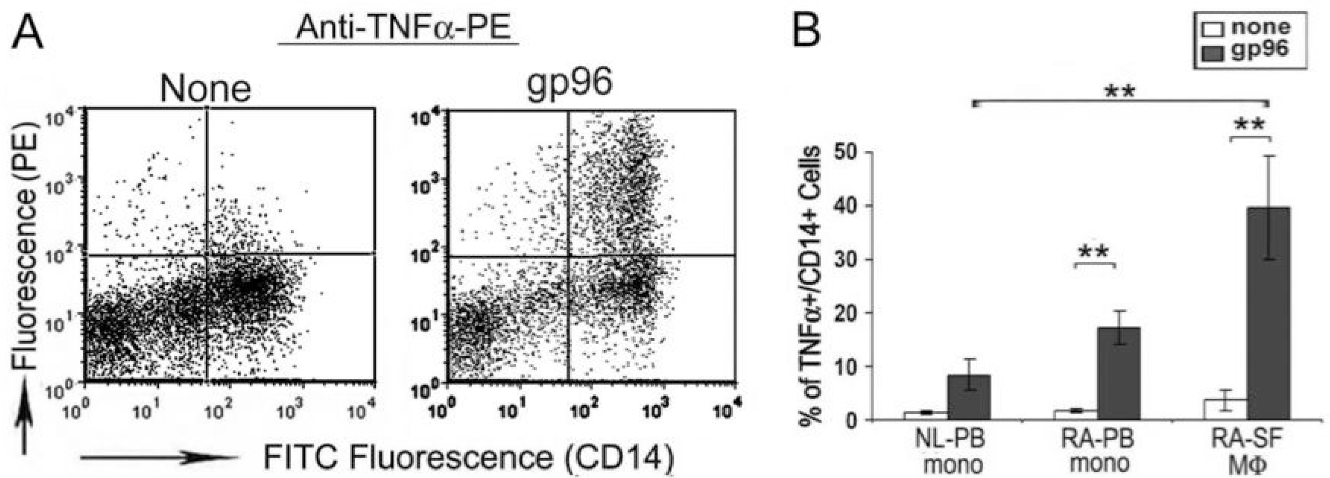

Figure 6. Gp96 induces TNFα in RA peripheral blood monocytes and synovial macrophages

Mononuclear cells isolated from peripheral blood (PB) of healthy donors (NL) and RA patients, and from RA synovial fluid (SF) were stimulated with medium only (none) or gp96 (50 μg) in the presence of brefeldin A (10 μg/ml) for over night. Two-color intracellular antibody staining was performed with FITC-labeled anti-CD14 and PE-labeled anti-TNFα or control IgG, which was analyzed by flow cytometry. (**A**) Representative histograms of mononuclear cells from RA synovial fluid with or without gp96 stimulation stained with PE-labeled anti-TNFα antibodies. (**B**) Summary of the induction of TNFα 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 (%) of double positive cells. * represents $p < 0.03$ and ** $p < 0.01$ between the indicated groups.

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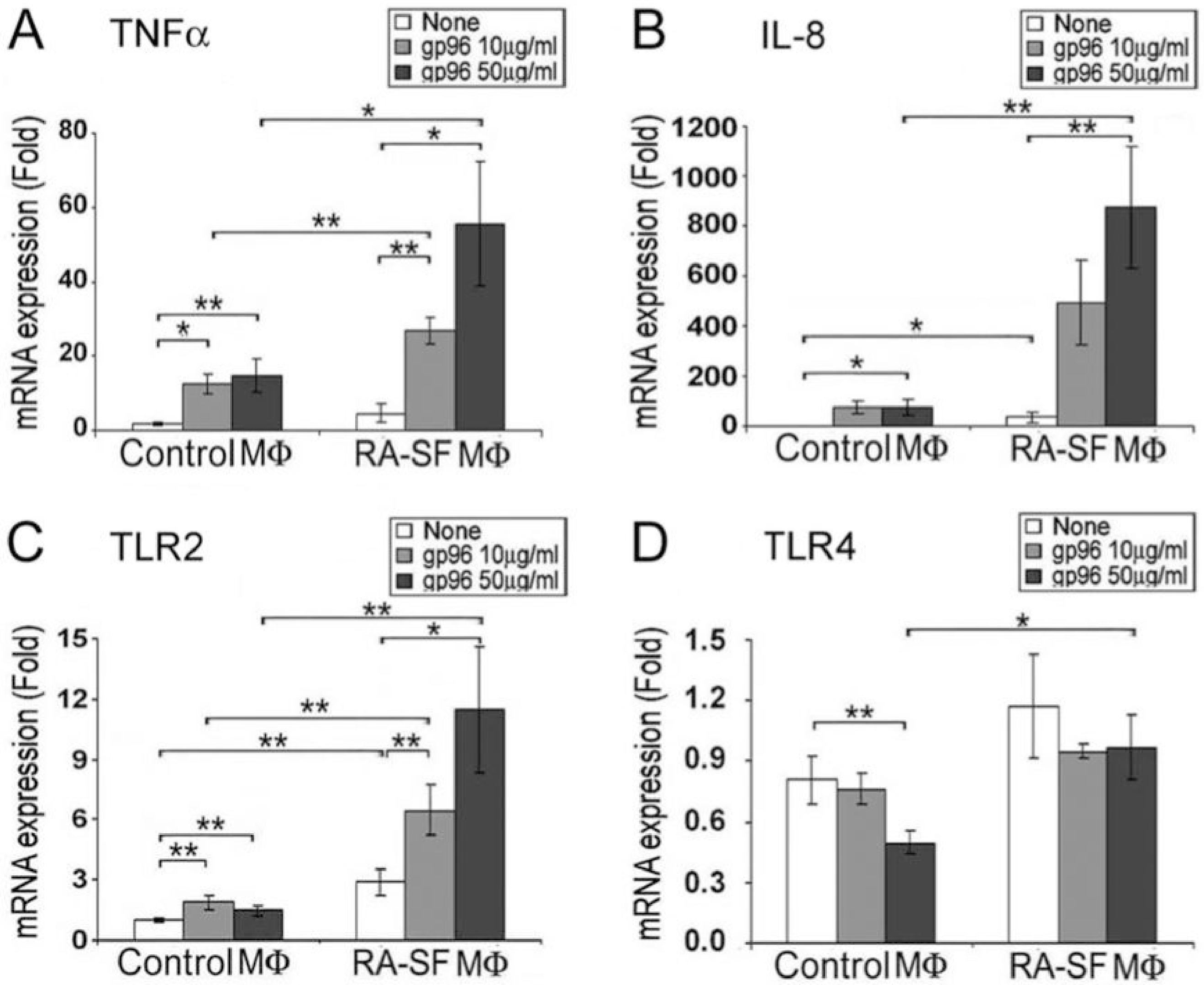

Figure 7. Increased TNFα, IL-8 and TLR2 gene expression in RA synovial fluid macrophages by gp96 stimulation

CD14+ macrophages (MΦs) purified from RA synovial fluid (n=5) by negative selection and in vitro differentiated control macrophages (n=7) were incubated with medium only (none) or gp96 at indicated dosages for 4 hours. TNFα (A), IL-8 (B), TLR2 (C) and TLR4 (D) gene expression were determined by qRT-PCR, presented as the fold of expression compared to medium alone for the control macrophages. * represents $p < 0.05$, and ** $p < 0.02$ between the indicated groups.

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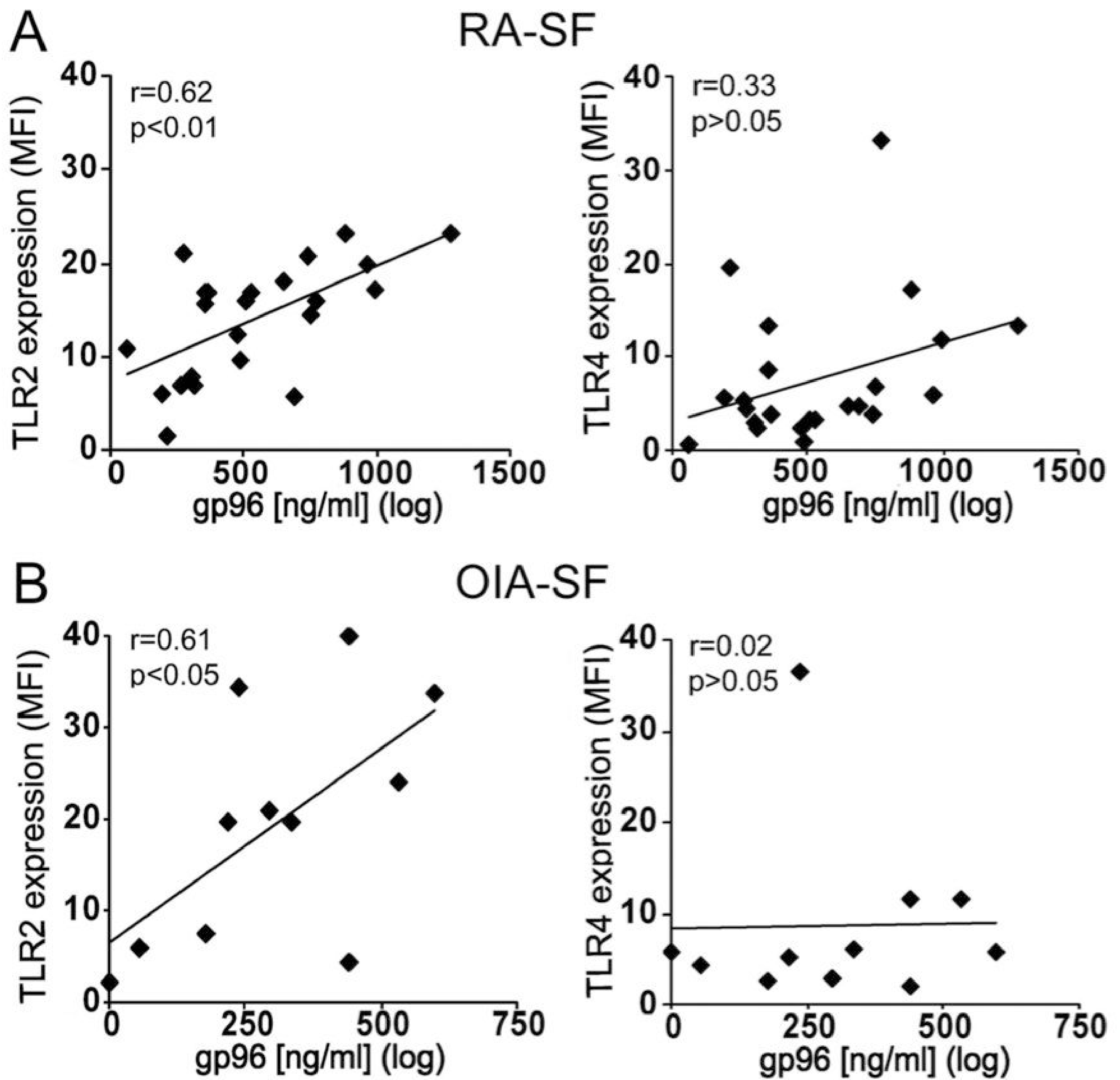

Figure 8. The concentration of gp96 in the synovial fluid correlates with TLR2 expression on macrophage surface

The levels of gp96 present in the fluids of patients with RA and with other forms of inflammatory arthritis (OIA) were analyzed for their relationship to the cell surface expression of TLR2 and TLR4 on macrophages isolated from the same synovial fluids. The gp96 concentration was determined by ELISA and the expression of TLR2 and TLR4 on the surface of macrophages is defined by MFI obtained by flow cytometry.