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Peptidoglycan Enhances IL-6 Production in Human Synovial Fibroblasts via TLR2 Receptor, Focal Adhesion Kinase, Akt, and AP-1- Dependent Pathway¹

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Peptidoglycan (PGN), the major component of the cell wall of Gram-positive bacteria, activates the innate immune system of the host and induces the release of cytokines and chemokines. We investigated the signaling pathway involved in IL-6 production stimulated by PGN in rheumatoid arthritis synovial fibroblasts. PGN caused concentration- and time-dependent increases in IL-6 production. PGN-mediated IL-6 production was attenuated by TLR2 small interfering RNA and nucleotide-binding oligomerization domain 2 small interfering RNA. Pretreatment with PI3K inhibitor (Ly294002 and wortmannin), Akt inhibitor, and AP-1 inhibitor (tanshinone IIA) also inhibited the potentiating action of PGN. PGN increased the focal adhesion kinase (FAK), PI3K, and Akt phosphorylation. Stimulation of rheumatoid arthritis synovial fibroblast cells with PGN increased the accumulation of phosphorylated c-Jun in the nucleus, AP-1-luciferase activity, and c-Jun binding to the AP-1 element on the IL-6 promoter. PGN mediated an increase in the accumulation of phosphorylated c-Jun in the nucleus, AP-1-luciferase activity, and c-Jun binding to AP-1 element was inhibited by Ly294002, Akt inhibitor, and FAK mutant. Our results suggest that PGN increased IL-6 production in human synovial fibroblasts via the TLR2 receptor/FAK/PI3K/Akt and AP-1 signaling pathway. *The Journal of Immunology*, 2009, 183: 2785–2792.

Rheumatoid arthritis (RA)³ is a chronic inflammatory disease characterized by robust infiltration of leukocytes into the synovium, resulting in hyperplasia of the synovial lining, progressive cartilage destruction, and erosion of the underlying bone (1). In response to the proinflammatory cytokines produced by macrophages, such as IL-1 β and TNF- α , RA synovial fibroblasts (RASf) produce chemokines that promote inflammation, neovascularization, and cartilage degradation via activation of matrix-degradation enzymes, such as matrix metalloproteinases (2).

Peptidoglycan (PGN), a cell wall component of Gram-positive bacteria, is an alternating β -linked *N*-acetylmuramyl and *N*-acetylglucosaminyl glycan whose residues are cross-linked by short peptides (3, 4). Like LPS as a cell wall component of Gram-negative bacteria, PGN induces most of the clinical manifestations of bacterial infections, including inflammation, fever, septic shock, etc.

(5). Most of these effects are due to the activation of macrophages and generation of proinflammatory cytokines, such as TNF- α , IL-6, and IL-8 (6–8). PGN binds CD14 and TLR2, which activates transcription factors and induces gene expression (9). Even though it is well known that bacterial products have multiple and various effects on the regulation of host defense and immune responses by macrophages, little is known about how PGN regulates induction of the IL-6 gene.

IL-6 is a multifunctional cytokine that plays a central role in both innate and acquired immune responses. It is the predominant mediator of the acute phase response, an innate immune mechanism which is triggered by infection and inflammation (10, 11). IL-6 also plays multiple roles during the subsequent development of acquired immunity against incoming pathogens, including regulation of the expressions of cytokine and chemokine, stimulation of Ab production by B cells, regulation of macrophage and dendritic cell differentiation, and the response of regulatory T cells to microbial infection (10, 11). In addition to these roles in pathogen-specific inflammation and immunity, IL-6 levels are elevated in chronic inflammatory conditions, such as RA (12, 13). Several consensus sequences, including those for NF- κ B, CREB, NF-IL-6, and AP-1 in the 5' promoter region of the IL-6 gene, have been identified as regulatory sequences that induce IL-6 in response to various stimuli (14, 15). AP-1 is commonly activated in response to inflammatory stimuli and has been implicated in cytokine expression and cellular immune responses (16, 17). Moreover, c-Jun/AP-1 has been implicated in human inflammatory disease (18), suggesting that expression of AP-1 in intestinal and inflammatory cells might be an important mediator of RA.

Focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase 2 involved in downstream signaling of integrins (19, 20), has recently been proposed as a mediator of inflammatory responses through the activation of MAPKs, including p38 MAPK and ERK pathways (21, 22). Although a role for FAK in IL-6 induction has been implied for some cell types, the signaling

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³ Abbreviations used in this paper: RA, rheumatoid arthritis; PGN, peptidoglycan; siRNA, small interference RNA; RASf, RA synovial fibroblasts; FAK, focal adhesion kinase; ODN, oligonucleotide; AS, antisense; MS, missense; LF2000, Lipofectamine 2000; NOD2, nucleotide-binding oligomerization.

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pathway for PGN in IL-6 production in synovial fibroblasts has not been extensively studied. In the present study, we explored the intracellular signaling pathway involved in PGN-induced IL-6 production in human synovial fibroblast cells. The results showed that PGN activates the TLR2 receptor and results in the activation of FAK/PI3K/Akt and AP-1 pathway, leading to up-regulation of IL-6 expression.

Materials and Methods

Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated HRP, rabbit polyclonal Abs specific for TLR2, TLR4, nucleotide-binding oligomerization domain 2 (NOD2), α -tubulin, FAK, p85, phosphorylated Akt, Akt, phosphorylated c-Jun, c-Jun, lamin B, and the small interfering RNAs (siRNAs) against FAK, TLR2, NOD2, and control for experiments using targeted siRNA transfection (each consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology. ON-TARGET smart pool FAK siRNA and ON-TARGET plus siCONTROL Nontargeting pool siRNA were purchased from Dharmacon. Rabbit polyclonal Abs specific for FAK phosphorylated at Tyr³⁹⁷, Tyr⁹²⁵, and Tyr⁵⁷⁷ were purchased from Cell Signaling Technology and Neuroscience. Ly294002, wortmannin, and Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-((R)-2-O-methyl-3-O-octadecylcarbonate)) were obtained from Calbiochem. The Tanshinone IIA was purchased from BIOMOL. An IL-6 enzyme immunoassay kit was purchased from Cayman Chemical. The AP-1 luciferase plasmid was purchased from Stratagene. The phosphorylation site mutant of FAK(Y397F) was a gift from Dr. J. A. Girault (Institut du Fer à Moulin, Moulin, France). The p85 α (Δ p85; deletion of 35 aa from residues 479–513 of p85) and Akt (Akt K179A) dominant-negative mutants were gifts from Dr. W. M. Fu (National Taiwan University, Taipei, Taiwan). The human IL-6 promoter construct pIL6-luc651(–651/+1), AP-1 site mutation (pIL6-luc651 Δ AP1), NF- κ B site mutation (pIL6-luc651 Δ NF- κ B), and C/EBP- β site mutation (pIL6-luc651 Δ C/EBP- β) were gifts from Dr. O. Eickelberg (Department of Medicine II, University of Giessen, Giessen, Germany). The pSV- β -galactosidase vector and luciferase assay kit were purchased from Promega. All other chemicals were obtained from Sigma-Aldrich.

Cell cultures

Human synovial fibroblasts were isolated using collagenase treatment from synovial tissues obtained from knee replacement surgeries of 10 patients with RA and three normal synovial tissues obtained at arthroscopy from trauma/joint derangement after approval by the local ethics committee. Patients with RA fulfilled the diagnostic criteria of the American College of Rheumatology (23). Fresh synovial tissues were minced and digested in a solution of collagenase and DNase. Isolated fibroblasts were filtered through 70- μ m nylon filters. The cells were grown on plastic cell culture dishes in 95% air-5% CO₂ with RPMI 1640 (Life Technologies) that was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (pH adjusted to 7.6). Fibroblasts from passages four to nine were used for the experiments.

Measurements of IL-6 production

Human synovial fibroblasts were cultured in 24-well culture plates. After reaching confluence, cells were treated with PGN and then incubated in a humidified incubator at 37°C for 24 h. For examination of the downstream signaling pathways involved in PGN treatment, cells were pretreated with various inhibitors for 30 min before PGN (30 μ g/ml) administration. After incubation, the medium was removed and stored at –80°C until assay. IL-6 in the medium was assayed using the IL-6 enzyme immunoassay kits according to the procedure described by the manufacturer.

mRNA analysis by RT-PCR

Total RNA was extracted from synovial fibroblasts using a TRIzol kit (MDBio). The reverse transcription reaction was performed using 2 μ g of total RNA that was reverse transcribed into cDNA using oligo(dT) primer, then amplified for 33 cycles using two oligonucleotide primers: IL-6, AAATGCCAGCCTGCTGACGAAG and AACAACAATCTGAGGTGCCATGCTAC; TLR2, TCT AAA GTC GAT CCG CGA CAT and TAC CCA GCT CGC TCA CTA CGT; and GAPDH, AAGCCATCAC CATCTTCCAG and AGGGGCCATCCACAGTCTTCT. Each PCR cycle was conducted for 30 s at 94°C, 30 s at 55°C, and 1 min at 68°C. PCR

products were then separated electrophoretically in a 2% agarose-DNA gel and stained with ethidium bromide.

Western blot analysis

The cellular lysates were prepared as described previously (24). Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human Abs against p85, phosphorylated Akt, Akt, or FAK (1/1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary Ab (1/1000) for 1 h at room temperature. The blots were visualized by ECL using Kodak X-OMAT LS film (Eastman Kodak). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics).

Oligonucleotide (ODN) transfection

RASF cells were cultured to confluence; the complete medium was replaced with Opti-MEM (Invitrogen) containing the antisense phosphorothioate ODNs (5 μ g/ml) that had been preincubated with 10 μ g/ml Lipofectamine 2000 (LF2000; Invitrogen) for 30 min. The cells were washed after 24 h of incubation at 37°C and washed again before the addition of medium containing PGN. All antisense ODNs were synthesized and then purified by HPLC (MDBio). The sequences used were as follows: c-Jun antisense (AS)-ODN, CGTTTCATCTTTGCAGT and missense (MS)-ODN, ACTGCAAAGATGGAAACG.

Synthesis of NF- κ B and AP-1 decoy ODNs

We used a phosphorothioate double-stranded decoy ODN carrying the NF- κ B-consensus sequence 5'-CCTTGAA GGGATTTCCCTCC-3'/3'-GGAACTTCCCTAAAGGGAGG-5'. The AP-1 decoy ODN sequence was 5'-TGCTGACTCATGTC-3'/3'-ACAGACTGAGTACAG-5'. The mutated (scrambled) form 5'-TTGCCGTACCTGACTTAGCC-3'/3'-AACGGCATGGACTGAATCCGG-5' was used as a control. ODN (5 μ M) was mixed with LF2000 (10 μ g/ml) for 25 min at room temperature, and the mixture was added to cells in serum-free medium. After 24 h of transient transfection, the cells were used for the following experiments.

Transfection and reporter gene assay

Human synovial fibroblasts were cotransfected with 0.8 μ g of AP-1-luciferase plasmid and 0.4 μ g of β -galactosidase expression vector. RASF cells were grown to 80% confluence in 12-well plates and were transfected on the following day by LF2000 (Invitrogen). DNA and LF2000 were pre-mixed for 20 min and then applied to the cells. After a 24-h transfection, the cells were incubated with the indicated agents. After a further 24-h incubation, the medium was removed and cells were washed once with cold PBS. To prepare lysates, 100 μ l of reporter lysis buffer (Promega) was added to each well and cells were scraped from the dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μ l) containing equal amounts of protein (20–30 μ g) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the cotransfected β -galactosidase expression vector.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was performed as described previously (24). DNA immunoprecipitated by anti-c-Jun Ab was purified. The DNA was then extracted with phenol-chloroform. The purified DNA pellet was subjected to PCR. PCR products were then resolved by 1.5% agarose gel electrophoresis and visualized by UV light.

The primers 5'-GAAGTACCTGACTTACATA-3' and 5'-TTGAGA CTCATGGGAAAATCC-3' were used to amplify across the human IL-6 promoter region (–312 to –39).

Statistics

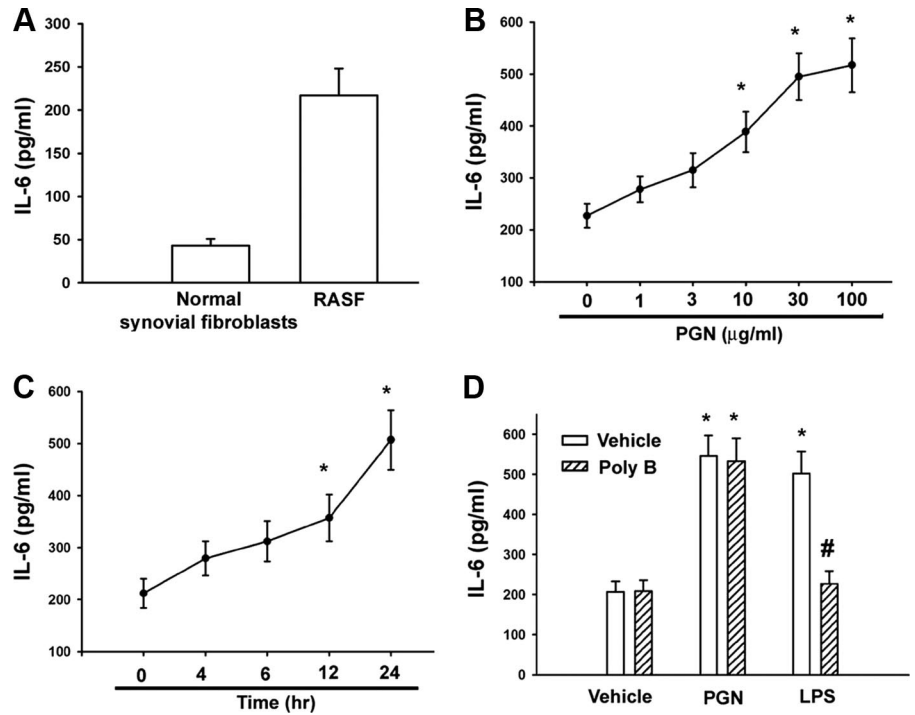
For statistical evaluation, the Mann-Whitney *U* test for non-Gaussian parameters and Student *t* test for Gaussian parameters (including Bonferroni correction) were used. The difference was significant if *p* < 0.05.

Results

PGN induces IL-6 production in human synovial fibroblasts

The typical pathology of RA includes chronic inflammation of the synovium, which is characterized by infiltrations of inflammatory

FIGURE 1. Concentration- and time-dependent increases in IL-6 production by PGN. *A*, Normal synovial fibroblasts ($n = 3$) and RASF ($n = 5$) were cultured for 48 h. Media were collected to measure IL-6. Human synovial fibroblasts were incubated with various concentrations of PGN for 24 h (*B*) or with PGN ($30 \mu\text{g/ml}$) for 4, 6, 12, or 24 h (*C*). Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. *, $p < 0.05$ as compared with basal level. *D*, RASF cells were pretreated with polymyxin B (Poly B; $1 \mu\text{M}$) for 30 min followed by stimulation with LPS ($1 \mu\text{M}$) or PGN ($30 \mu\text{g/ml}$) for 24 h. Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. *, $p < 0.05$ compared with basal level and #, $p < 0.05$ compared with LPS or PGN-treated group.



cells and synovial hyperplasia, especially fibroblast-like synoviocytes. Therefore, we decided to use human synovial fibroblasts to investigate the signaling pathways of PGN in the production of

IL-6, an inflammatory response gene. We found that the RASF expressed higher levels of IL-6 than normal synovial fibroblasts (Fig. 1*A*). Treatment of RASF with PGN ($1\text{--}100 \mu\text{g/ml}$) for 24 h

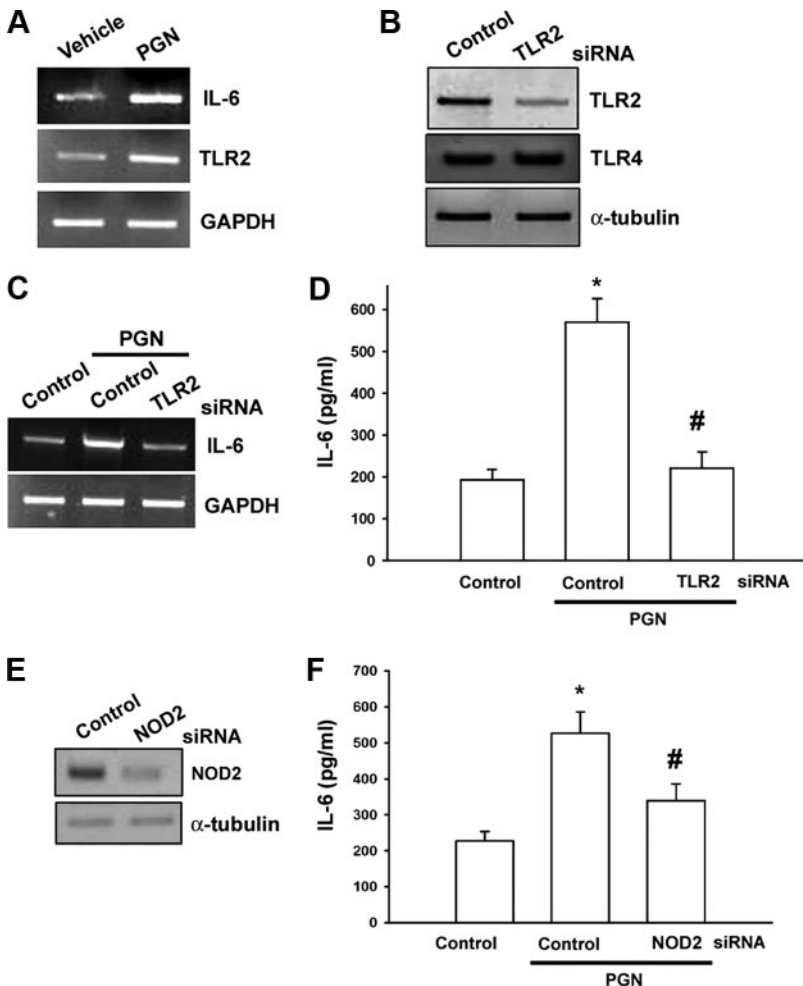
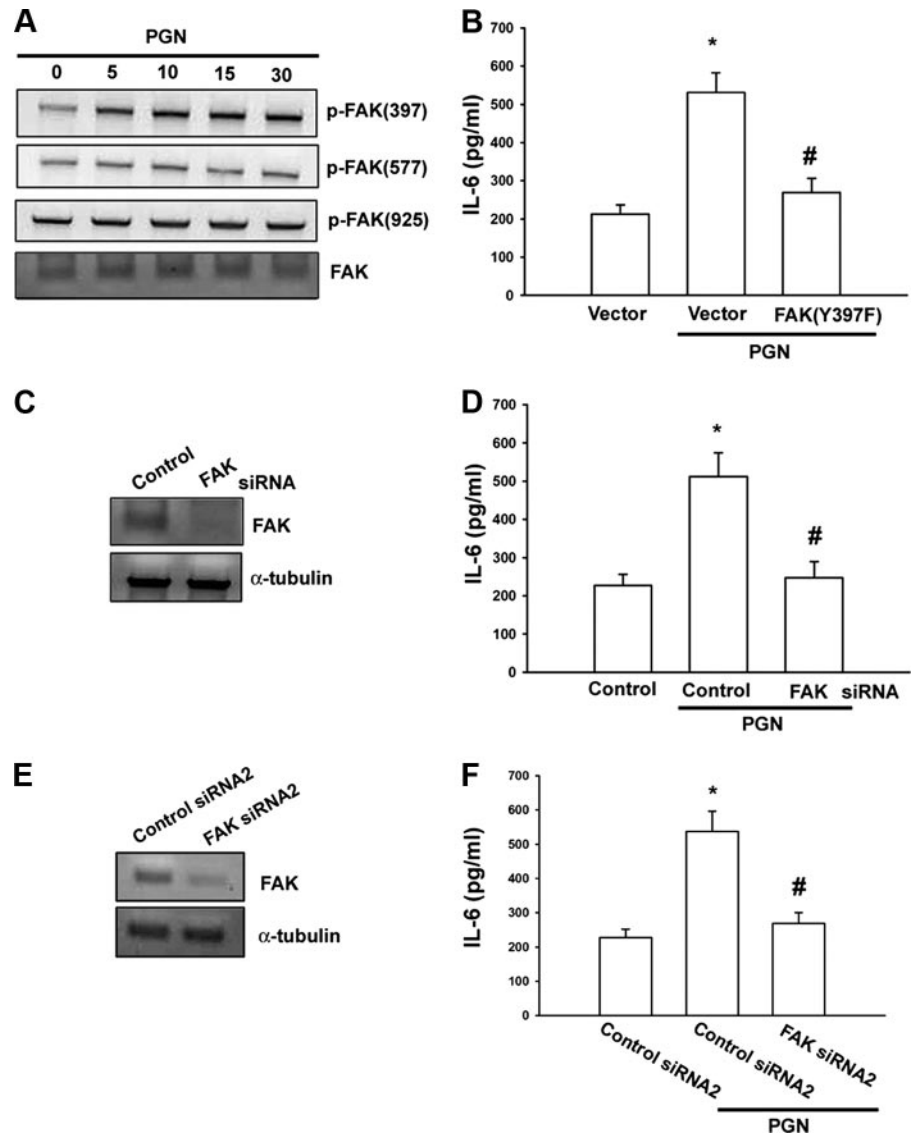


FIGURE 2. Involvement of TLR2 receptor in PGN-mediated IL-6 production in synovial fibroblasts. Total RNA was extracted from RASF cells and subjected to RT-PCR for IL-6 and TLR2 mRNAs using the respective primers. Note that RASF cells express IL-6 and TLR2 receptor mRNA, and IL-6 and TLR2 mRNA increased in response to PGN ($30 \mu\text{g/ml}$) application for 24 h (*A*). RASF cells were transfected with TLR2 or control siRNA for 24 h and the protein levels of TLR2 and TLR4 were determined by using Western blot analysis (*B*). RASF cells were transfected with TLR2 or control siRNA for 24 h followed by incubation with PGN ($30 \mu\text{g/ml}$) for 24 h to analyze the mRNA and protein expression, respectively. Total RNA and medium were collected and the expressions of IL-6 were analyzed by RT-PCR and ELISA (*C* and *D*). RASF cells were transfected with NOD2 or control siRNA for 24 h and the protein levels of NOD2 were determined by using Western blot analysis (*E*). RASF cells were transfected with NOD2 or control siRNA for 24 h and then stimulated with PGN ($30 \mu\text{g/ml}$) for 24 h. Media were collected to measure IL-6 (*F*). Results are representative of at least three independent experiments. *, $p < 0.05$ compared with control and #, $p < 0.05$ as compared with PGN-treated group.

FIGURE 3. FAK is involved in PGN-induced IL-6 production. **A**, Cells were incubated with PGN (30 $\mu\text{g/ml}$) for the indicated time intervals and cell lysates were then immunoblotted with an Ab specific for phosphor-FAK. **B**, RASF cells were transfected with FAK mutant or vector for 24 h and then stimulated with PGN (30 $\mu\text{g/ml}$) for 24 h. Media were collected to measure IL-6. **C**, RASF cells were transfected with FAK or control siRNA for 24 h and the protein levels of FAK were determined by using Western blot analysis. **D**, RASF cells were transfected with FAK or control siRNA for 24 h and then stimulated with PGN (30 $\mu\text{g/ml}$) for 24 h. Media were collected to measure IL-6. **E**, RASF cells were transfected with ON-TARGET smart pool FAK siRNA (FAK siRNA2) for 24 h and the protein levels of FAK were determined by using Western blot analysis. **F**, RASF cells were transfected with ON-TARGET smart pool FAK siRNA (FAK siRNA2) for 24 h and then stimulated with PGN (30 $\mu\text{g/ml}$) for 24 h. Media were collected to measure IL-6. *, $p < 0.05$ compared with control and #, $p < 0.05$ compared with PGN-treated group.



induced IL-6 production in a concentration-dependent manner (Fig. 1B), and this induction occurred in a time-dependent manner (Fig. 1C). After PGN (30 $\mu\text{g/ml}$) treatment for 24 h, the amount of IL-6 released had increased in RASF cells (Fig. 1C). To further confirm this stimulation-specific mediation by PGN without LPS contamination, we used polymyxin B, a LPS inhibitor. We found that polymyxin B (1 μM) completely inhibited LPS (1 μM)-induced IL-6 release. However, it had no effect on PGN (30 $\mu\text{g/ml}$)-induced IL-6 release in RASF cells (Fig. 1D).

Involvement of TLR2 receptor in PGN-mediated increase of IL-6 production

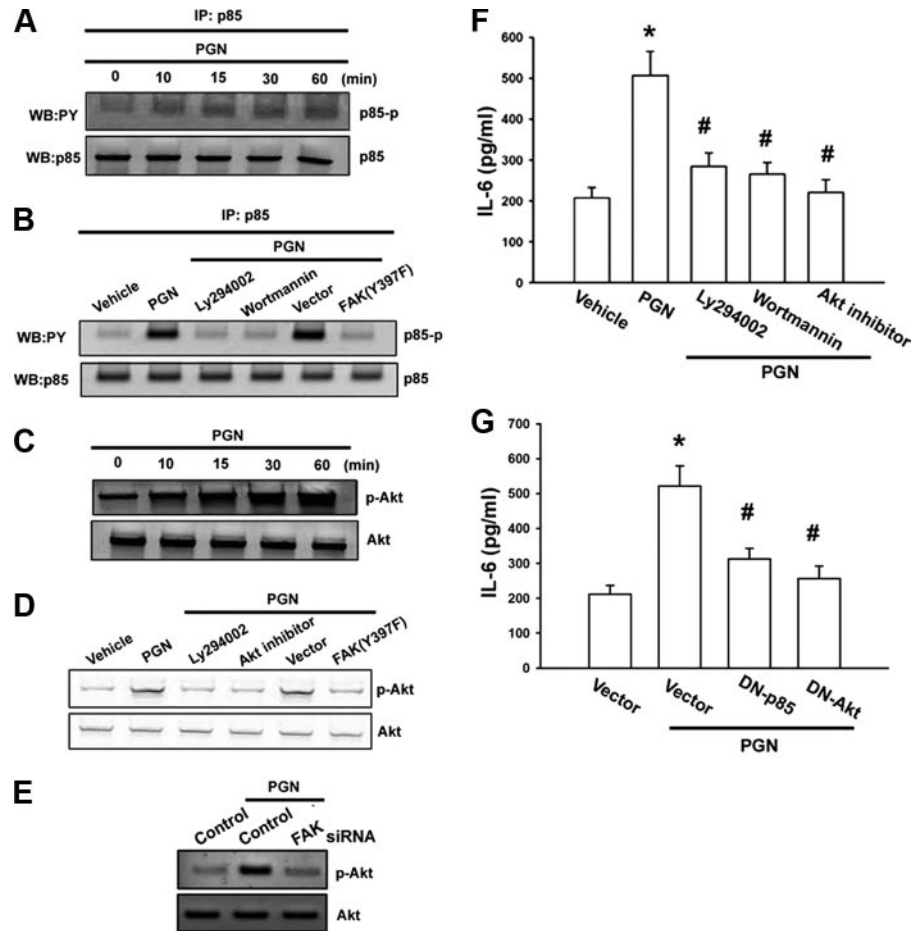
PGN exerts its effects through interaction with a specific TLR2 receptor (6). To investigate the role of the TLR2 receptor in the PGN-mediated increase of IL-6 production, we assessed the distribution of the TLR2 receptor in human synovial fibroblasts by RT-PCR analysis (Fig. 2A). The mRNA of the TLR2 receptor could be detected in human synovial fibroblasts (Fig. 2A). Upon PGN treatment for 24 h, the mRNA levels of IL-6 and TLR2 receptor were evidently increased (Fig. 2A). We next examined whether the TLR2 receptor is involved in the PGN-mediated increase of IL-6 release; specific inhibition of TLR2 receptor expression was accomplished with siRNA (Fig. 2B). The TLR2

siRNA did not affect the expression of TLR4 (Fig. 2B) and also did not affect the LPS-induced IL-6 production in human RASF cells (data not shown). By using RT-PCR and ELISA, we found that TLR2 receptor-specific siRNA but not control siRNA significantly blocked the PGN-mediated increase of IL-6 production in human RASF cells (Fig. 2, C and D). These results suggest that TLR2 may be involved in PGN-induced IL-6 expression and release in human synovial fibroblasts. It has been reported that PGN can also stimulate IL-6 expression through the intracellular receptor NOD2 pathway (25). We next examined whether the NOD2 pathway is involved in PGN-mediated IL-6 production. Transfection of RASF cells with NOD2 siRNA suppressed the NOD2 expression (Fig. 2E). In addition, NOD2 siRNA also reduced PGN-induced IL-6 production in human RASF cells (Fig. 2F). Therefore, the NOD2 signaling pathway is also involved in PGN-mediated IL-6 expression.

The signaling pathways of FAK, PI3K, and Akt are involved in the potentiating action of PGN

It has been reported that FAK is involved in the TLR2-mediated inflammatory response (26, 27). Phosphorylation of tyrosine 397 of FAK has been used as a marker of FAK activity. As shown in Fig. 3A, FAK phosphorylation increased in a time-dependent

FIGURE 4. PI3K and Akt are involved in PGN-mediated IL-6 production in synovial fibroblasts. Cells were incubated with PGN (30 $\mu\text{g/ml}$) for various time intervals (A) or pretreated with Ly294002 and wortmannin for 30 min or transfected with FAK mutant for 24 h (B) followed by stimulation with PGN for 60 min, and cell lysates were immunoprecipitated (IP) with anti-p85 Ab. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted (WB) with anti-phosphotyrosine (PY). RASF cells were incubated with PGN for the indicated time intervals (C) or pretreated with Ly294002 and Akt inhibitor for 30 min or transfected with FAK mutant for 24 h (D) followed by stimulation with PGN for 60 min, and Akt phosphorylation was determined by immunoblotting using phospho-Akt-specific Ab. RASF cells were transfected with FAK or control siRNA for 24 h followed by stimulation with PGN for 60 min, and Akt phosphorylation was determined by Western blot analysis (E). RASF cells were pretreated for 30 min with Ly294002, wortmannin, and Akt inhibitor or transfected with p85, Akt mutant, or vector (control) for 24 h followed by stimulation with PGN for 24 h (F and G). Media were collected for the measurement of IL-6. *, $p < 0.05$ compared with control and #, $p < 0.05$ compared with PGN-treated group.



manner in response to PGN stimulation, reaching the maximum between 10 and 30 min. However, PGN did not affect the phosphorylation of tyrosine 577 and 925 of FAK (Fig. 3A). Transfection of cells with FAK(Y397F) mutant reduced the PGN-mediated IL-6 production (Fig. 3B). We also used FAK siRNA to confirm the role of FAK in PGN-induced IL-6 expression. Transfection of cells with FAK siRNA reduced the expression of FAK (Fig. 3C). In addition, FAK siRNA also abolished PGN-mediated IL-6 production (Fig. 3D). To avoid the off-target effect of siRNA, we also assessed the role of FAK by using ON-TARGET smart pool FAK, which decreases nonspecific effects by chemical modification and pooling (28). Transfection of RASF cells with ON-TARGET smart pool FAK siRNA reduced the basal level of FAK (Fig. 3E). Similarly, inhibition of ON-TARGET smart pool FAK siRNA decreased PGN-induced IL-6 production (Fig. 3F). Therefore, FAK plays an important role in PGN-induced IL-6 expression. Phosphorylation of tyrosine 397 of FAK may provide a binding site for the Src homology 2 domain of the p85 subunit of PI3K (29). We next examined whether PGN stimulation also enhances the association of FAK with PI3K. Treatment of RASF cells with PGN led to a significant increase of phosphorylation of the p85 subunit of PI3K, as assessed by the measurement of phosphotyrosine from immunoprecipitated lysates using p85 (Fig. 4A). Pretreatment of cells with the PI3K inhibitor Ly294002 and wortmannin or transfection of cells with FAK(Y397F) mutant decreased PGN-increased p85 activity (Fig. 4B). Akt phosphorylation in response to PGN was then measured. As shown in Fig. 4C, treatment of RASF cells with PGN resulted in a time-dependent phosphorylation of Akt. Furthermore, the PGN-induced increase in Akt phosphorylation was markedly inhibited by the pretreatment of cells for 30 min

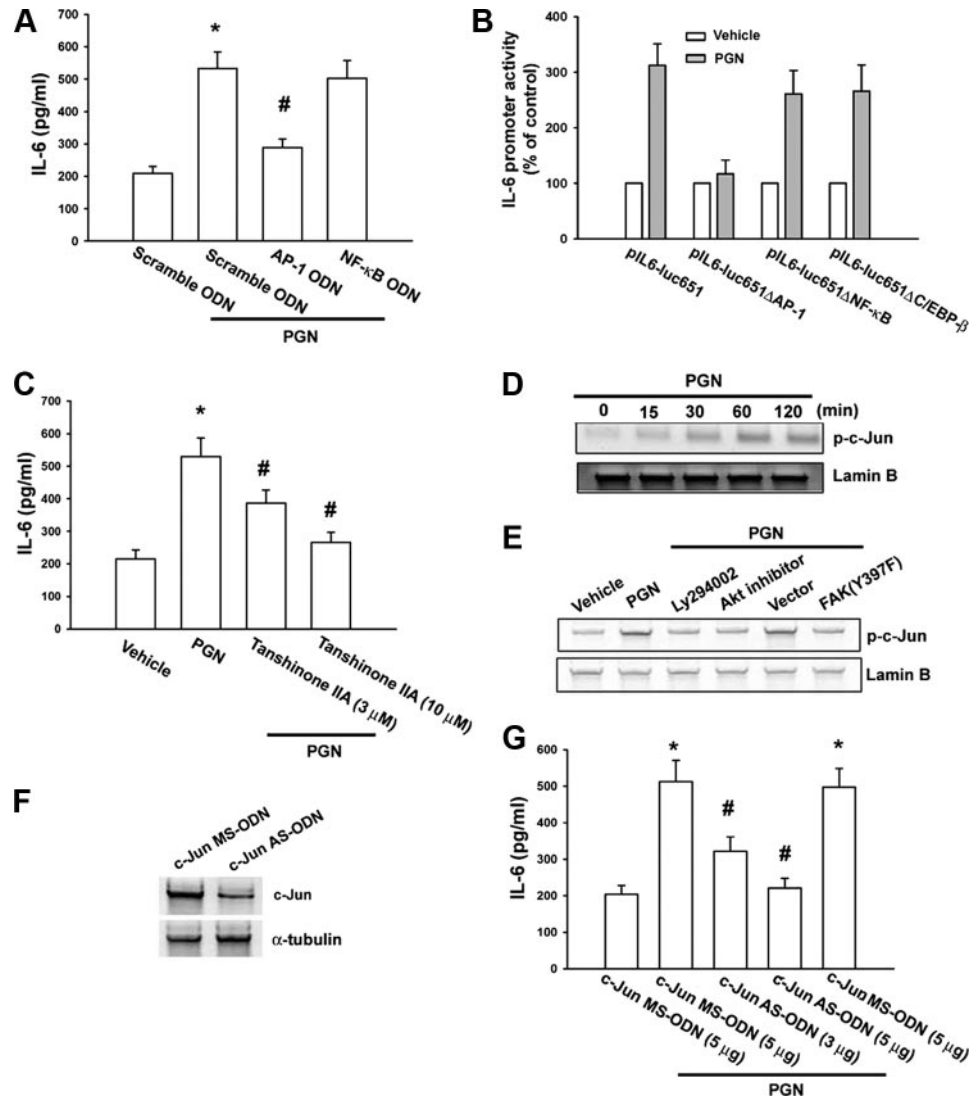
with Ly294002 and Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-((R)-2-O-methyl-3-O-octadecylcarbonate)) or transfection with the FAK(Y397F) mutant for 24 h (Fig. 4D). Transfection of cells with FAK siRNA also reduced PGN-increased Akt phosphorylation (Fig. 4E). In addition, pretreatment of RASF cells for 30 min with Ly294002, wortmannin, and Akt inhibitor or transfection with p85 and Akt mutant for 24 h markedly attenuated the PGN-induced IL-6 production (Fig. 4, F and G). Taken together, these results indicate that the FAK/PI3K/Akt pathway is involved in PGN-induced IL-6 production.

Involvement of AP-1 in PGN-induced IL-6 production

There are NF- κ B- and AP-1-binding sites on the IL-6 promoter region (14, 15). The increase of IL-6 production by PGN was antagonized by the *cis*-element decoy agonist AP-1-binding site (decoy AP-1 ODN) but not by the NF- κ B binding site (decoy NF- κ B ODN) or scrambled decoy (ODN) (Fig. 5A). The promoter region of human IL-6 contains three known *cis*-regulatory elements including AP-1, C/EBP- β , and NF- κ B binding sites (30). Three different IL-6 promoter constructs containing mutations at NF- κ B, AP-1, or C/EBP- β sites, respectively, were generated by site-directed mutagenesis. We found that PGN-stimulated luciferase activity was abolished by AP-1 binding site mutation, but not by NF- κ B or C/EBP- β site mutations (Fig. 5B). Therefore, the AP-1 binding site is more important than the NF- κ B or C/EBP- β site in PGN-induced IL-6 production.

The role of AP-1 was further established using the AP-1 inhibitor (tanshinone IIA) and showed that this inhibitor blocked the enhancement of IL-6 production induced by PGN. Fig. 5C shows that tanshinone IIA inhibited the enhancement of IL-6 production

FIGURE 5. AP-1 is involved in the potentiation of IL-6 production by PGN. *A*, RASF cells were transfected with NF- κ B ODN, AP-1 ODN, or scramble ODN before incubation with PGN for 24 h. Media were collected to measure IL-6. *B*, RASF cells were transfected with IL-6 luciferase plasmids before incubation with PGN for 24 h. Luciferase activity was then assayed. *C*, RASF cells were pretreated for 30 min with tanshinone IIA followed by stimulation with PGN for 24 h. Media were collected to measure IL-6. RASF cells were incubated with PGN for the indicated time intervals (*D*) or pretreated with Ly294002 and Akt inhibitor for 30 min or transfected with FAK mutant for 24 h (*E*), followed by stimulation with PGN for 120 min, and c-Jun phosphorylation in the nucleus was determined by immunoblotting using phospho-c-Jun-specific Ab. *F*, RASF cells were transfected with c-Jun AS-ODN or MS-ODN for 24 h and then stimulated with PGN (30 μ g/ml) for 24 h. Media were collected to measure IL-6. *, $p < 0.05$ compared with control and #, $p < 0.05$ compared with PGN-treated group.



induced by PGN. It has been reported that the AP-1 binding site between -283 and -276 was important for the activation of the IL-6 gene (14). AP-1 activation was further evaluated by analyzing the accumulation of phosphorylated c-Jun in the nucleus, as well as by the chromatin immunoprecipitation assay. Treatment of cells with PGN resulted in a marked accumulation of phosphorylated c-Jun in the nucleus (Fig. 5*D*). PI3K and Akt inhibitors or FAK(Y397F) mutant reduced PGN-induced accumulation of phosphorylated c-Jun in the nucleus (Fig. 5*E*). Transfection of cells with c-Jun AS-ODN but not MS-ODN suppressed the expression of c-Jun (Fig. 5*F*). PGN-induced IL-6 expression was also inhibited by c-Jun AS-ODN but not by MS-ODN (Fig. 5*G*). We next investigated whether c-Jun binds to the AP-1 element on the IL-6 promoter after PGN stimulation. The *in vivo* recruitment of c-Jun to the IL-6 promoter (-312 to -39) was assessed by the chromatin immunoprecipitation assay. *In vivo* binding of c-Jun to the AP-1 element of the IL-6 promoter occurred as early as 30 min and was sustained to 120 min after PGN stimulation (Fig. 6*A*). The binding of c-Jun to the AP-1 element by PGN was attenuated by the Ly294002, Akt inhibitor or FAK mutant (Fig. 6*B*). To further confirm that the AP-1 element is involved in the action of PGN-induced IL-6 expression, we performed transient transfection using the AP-1 promoter-luciferase constructs. Synovial fibroblasts incubated with PGN (30 μ g/ml) led to a 3.5-fold increase in AP-1

promoter activity. The increase of AP-1 activity by PGN was antagonized by Ly294002, wortmannin, and Akt inhibitor or p85, Akt, and FAK(Y397F) mutant or c-Jun AS-ODN (Fig. 6, *C* and *D*). Taken together, these data suggest that the activation of the FAK, PI3K, Akt, c-Jun, and AP-1 pathway is required for the PGN-induced increase of IL-6 in human RASF cells.

Discussion

PGN, the major component of the cell wall of Gram-positive bacteria, activates the innate immune system of the host and induces the release of cytokines and chemokines (4, 31). It has been reported that PGN induces the expression of inflammatory cytokines through activation of the transcription factor AP-1 in myeloma cells (32). In this study, we further identified IL-6 as a target protein for the PGN signaling pathway that regulates the cell inflammatory response. We also showed that potentiation of IL-6 by PGN requires activation of the TLR2 receptor, FAK, PI3K, Akt, and AP-1 signaling pathway. These findings suggest that PGN acts as an inducer of inflammatory cytokines such as IL-6 and enhances the inflammatory response in RA.

The cytoplasmic portion of TLRs shows high similarity to that of the IL-1 receptor family and is now called the Toll/IL-1R domain (32). Upon recognizing respective ligands, the Toll/IL-1R domain recruits MyD88/IL-1R-associated kinase/TNFR-associated

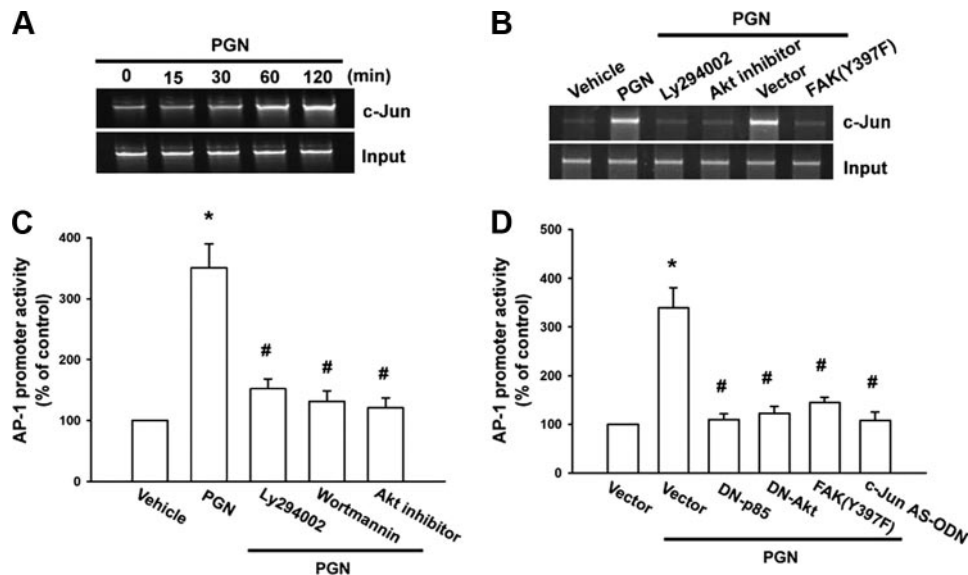


FIGURE 6. PGN increases c-Jun binding to AP-1 element on the human IL-6 promoter in synovial fibroblasts. RASF cells were treated with PGN for the indicated time intervals (A) or pretreated with Ly294002 and Akt inhibitor or transfected with FAK mutant (B) and then stimulated with PGN for 120 min, and the chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with anti-c-Jun. One percentage of the precipitated chromatin was assayed to verify equal loading (input). RASF cells were transfected with AP-1-luciferase expression vector and then pretreated with Ly294002, wortmannin, and Akt inhibitor or cotransfected with p85, Akt, and FAK mutant or c-Jun AS-ODN before incubation with PGN for 24 h (C and D). Luciferase activity was then assayed. *, $p < 0.05$ compared with control and #, $p < 0.05$ compared with PGN-treated group.

factor 6 and activates downstream signaling molecules such as MAPK and AP-1 (33). It has been reported that PGN binds TLR2, which activates transcription factors and induces gene expression (5, 6). In this study, we found that RASF cells expressed the TLR2 receptor by RT-PCR analysis. In addition, PGN increased the expression of IL-6 and TLR2. Furthermore, transfection with TLR2 siRNA antagonized the PGN-induced IL-6 production. These results suggest that TLR2 is an upstream receptor in PGN-induced IL-6 release.

FAK, a potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated signaling (34, 35). It has been shown that FAK is involved in the TLR2-mediated inflammatory response (26, 27). We demonstrated that PGN increased phosphorylation of tyrosine 397 but not 577 and 925 of FAK. Furthermore, the FAK(Y397F) mutant antagonized the PGN-mediated potentiation of IL-6 expression, suggesting that tyrosine 397 of FAK activation is an obligatory event in PGN-induced IL-6 production in human RASF cells. FAK contains tyrosine residues in motifs for binding to Src homology 2 domain. Phosphorylated tyrosine 397 of FAK has been shown to serve as a binding site for the Src homology 2 domain of the p85 subunit of PI3K (21). Phosphorylation of the p85 subunit is required for activation of the p110 catalytic subunit of PI3K. Pretreatment of RASF cells with the PI3K inhibitor Ly294002 or wortmannin antagonized the increase of IL-6 production by PGN. This was further confirmed by the result that the dominant-negative mutant of p85 inhibited the enhancement of IL-6 expression by PGN stimulation. The cytoplasmic serine kinase Akt was found to be activated by PGN stimulation in RASF cells. In addition, the Akt inhibitor or mutant also reduced the PGN-induced IL-6 expression in human RASF cells. Taken together, our results provide evidence that PGN increases IL-6 expression in human RASF cells via the FAK/PI3K/Akt signaling pathway.

Hormones and growth factors are known to regulate gene expression through AP-1 sites (35). It has been reported that PGN induces IL-6 secretion through the AP-1-dependent pathway (36). The AP-1 sequence binds to members of the Jun and Fos families

of transcription factors. These nuclear proteins interact with the AP-1 site as Jun homodimers or Jun-Fos heterodimers formed by protein dimerization through their leucine zipper motifs. The results of this study show that PGN induced c-Jun nuclear accumulation. The PI3K and Akt inhibitor or FAK mutant reduced the PGN-induced accumulation of phosphorylated c-Jun in the nucleus. In addition, c-Jun AS-ODN also abolished the PGN-induced IL-6 production in RASF cells. Therefore, the c-Jun activation is mediated by PGN-induced IL-6 expression. Furthermore, PGN increased the binding of c-Jun to the AP-1 element on the IL-6 promoter, as shown by chromatin immunoprecipitation assay. Binding of c-Jun to the AP-1 element was attenuated by Ly294002, Akt inhibitor, and FAK mutant. Using transient transfection with AP-1-luciferase as an indicator of AP-1 activity, we also found that PGN induced an increase in AP-1 activity. In addition, Ly294002, wortmannin, and Akt inhibitor or p85, Akt, and FAK mutant reduced PGN-induced AP-1 promoter activity. These results indicate that PGN might act through the FAK, PI3K, Akt, c-Jun, and AP-1 pathway to induce IL-6 activation in human RASF cells.

Patients with RA are at increased risk of developing infections and appear to be particularly susceptible to septic arthritis, osteomyelitis, and skin and soft tissue infections (37), which are mostly caused by *Staphylococcus aureus*. It has been shown that *S. aureus* and other Gram-positive bacteria are potent inducers of TNF- α secretion from macrophages (38). In response to TNF- α , RASF cells produce chemokines that promote inflammation neovascularization and cartilage degradation. To simply observe the effects of Gram-positive bacteria infection in RA patients, we used the major component of the cell wall of Gram-positive bacteria, PGN, to examine the proinflammatory cytokine (IL-6) in human RASF cells. We found that PGN increased IL-6 production and that the TLR2, FAK, Akt, and AP-1 signaling pathway may be involved in such production. Furthermore, the discovery of a PGN-mediated TLR2/AP-1-dependent pathway helps us to understand the mechanism of RA and may help us to develop more effective therapies in the future.

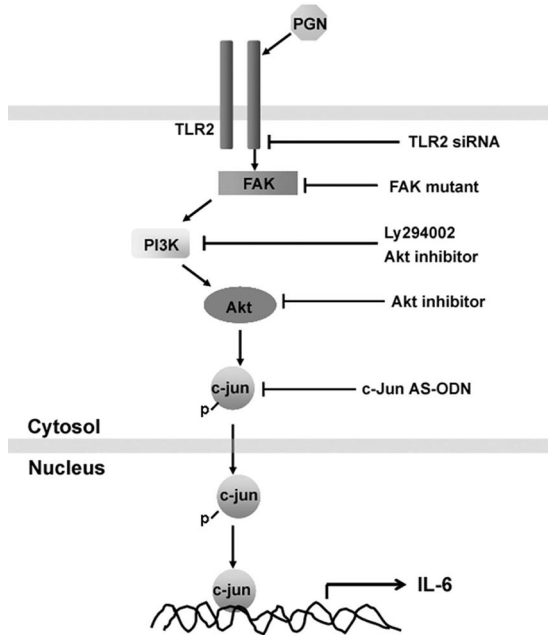


FIGURE 7. Schematic diagram of the signaling pathways involved in PGN-induced IL-6 production in synovial fibroblasts. PGN increases IL-6 expression by binding to the TLR2 receptor and activation of FAK, PI3K, Akt, which enhances binding of c-Jun to the AP-1 site, resulting in the transactivation of IL-6 expression.

In conclusion, we explored the signaling pathway involved in PGN-induced IL-6 production in human synovial fibroblasts. We found that PGN increases IL-6 production by binding to the TLR2 receptor and activating of FAK, PI3K, and Akt, which enhances binding of c-Jun to the AP-1 site and results in the transactivation of IL-6 production (Fig. 7).

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Disclosures

The authors have no financial conflict of interest.

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