박 사 학 위 논 문

Pim-1 Regulates NLRP3 Inflammasome-mediated Inflammatory Signals

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이 논문을 박사학위 논문으로 제출함

2022년 8월

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1. Introduction

Proviral integration moloney virus (Pim) kinase is a serine/threonine protein kinase that plays an important role in cell proliferation, survival, and movement regulation (1). Pim kinase is a pro-oncogene and has mainly been studied in tumors such as hematological malignancies, including vascular muscle, and in multiple other cell types (2), breast cells (3), and cardiomyocytes (4). There are three types of Pim kinase: Pim-1, Pim-2, and Pim-3 (5). They are located on chromosomes 17, X, and 15 (5). These Pim kinases are very similar to each other at the amino acid sequence (5), but vary slightly depending on the tissue distribution (6). Unlike other kinases, Pim kinase does not have a phosphorylation motif and a regulatory domain, and is configured to be activated consecutively when expressed (7,8). Thus, these proteins are regulated at the levels of transcription, translation, and proteolysis (9).

Recently, Pim-1 inhibition was shown to improve colitis by reducing the hyperactivity of macrophages and T helper (Th) 1 and Th17 immune responses in dextran sodium sulfate mouse models (10). Moreover, it has been reported that a *Pim-1*-targeting siRNA inhibits lipopolysaccharide (LPS)-induced up-regulation of interleukin (IL)-1β in RAW264.7 cells (11). Pim-1 was also reported to protect epithelial cells against damage caused by cigarette smoke and airway inflammation (12), and its inhibition was found to suppress the production of cytokines that cause allergic inflammation in the airway (13). Pim-1 is an important downstream signal of the signal transducer and activations of transcription (STAT) protein, and induces the

expression of various pro-inflammatory cytokiness (7). These findings suggest that Pim-1 kinase may be a potential therapeutic target for inflammatory regulation. However, the mechanisms underlying Pim functions, including inflammation regulation, remain insufficiently studied.

Inflammation is a complex biological response of the immune system. It can be by various factors such as damaged cells, toxic compounds, and pathogens (14). Toll-like receptors (TLRs) play an important role in the innate immune system by recognizing pathogen-related molecular patterns derived from various microorganisms (15). The involvement of innate immune receptor TLR in inflammatory diseases has been studied (16). TLRs are involved in chronic inflammation associated with rheumatoid diseases, including rheumatoid arthritis, gout, erythematouss, systemic lupus, and Lyme's disease (17). TLR4, in particular, is involved with several endogenous ligands that are known to activate innate immune system defects that collectively promote arthritis (18,19). TLR4 signaling involves the recruitment of specific adaptor molecules and occurs through mitogen-activated protein kinase (MAPKs), nuclear factor kappa B (NF-κB), and janus family tyrosine kinase (JAK)-STAT pathways (20-22). TLR signals are activated by pro-inflammatory cytokiness such as, tumor necrosis factor-alpha (TNF-α), IL-6, IL-1β, and cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) (23-25). Understanding the pathways associated with these factors and suppressing their excessive production can greatly help in the treatment of inflammatory diseases.

In the present study, I investigated the role of Pim-1 kinase in the LPS-mediated inflammatory signaling pathway. In addition, I demonstrated the anti-inflammatory effects and mechanism of action of

a novel PIM kinase inhibitor, KMU-11342 in THP-1, rheumatoid arthritis fibroblast-like synoviocyte (RA-FLS), and osteoclastogenesis.

2. Materials and Methods

2.1. Reagents:

The following reagents and antibodies were used in this study: LPS (*Escherichia coli* serotype 0111:B4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pim kinase inhibitors, PIM447 and AZD1208, were purchased from Selleck Chemicals (Houston, TX, USA). The transforming growth factor-β-activated kinase 1 (TAK1) inhibitor (5Z)-7-Oxozeaenol was purchased from Tocris Bioscience (Bristol, UK). Deoxyribonucleic acid (DNA) plasmid was used in the deletion mutant form pMX-dominant negative form of Pim-1 (Pim-1-DN) (aa81-313)-Flag and point mutant form pMX-Pim-1-K67M-Flag were provided by Dr. Nacksung Kim (Chonnam University, Gwangju, Korea). (2Z)-3((1H-imidazol-5yl)methylene)-5-(6-(cyclop-entylamino)pyrazine-2-yl)indolin-2-one (KMU-11342) was kindly supplied by Dr. Lee. JH (Keimyung University, Daegu, Korea; Figure 10A). Recombinant soluble receptor activator of NF-κB (sRANKL) were purchased from PeproTech (Rocky Hill, NJ, USA). Adenosine 5'-triphosphate disodium salt (ATP) and phorbol myristate acetate (PMA) were purchased from Santa Cruz Biotechnology and Sigma-Aldrich (St. Louis, MO, USA), respectively. Antibodies against TLR4, phospho(p)-IKKα/β, p-NF-κB p65, iNOS, Nod-like receptor family pyrin domain containing 3 (NLRP3), Caspase-1, speck-like protein containing a CARD (ASC), and were purchased from Cell Signaling Technology (Beverly, MD, USA). Anti-IL-1β antibody was purchased from Novus Biologicals (Centennial, CO, USA).

Antibodies against NFATc-1, p-c-Fos, JNK, and p-TAK1 were purchased from Cell Signaling (Beverly, MD, USA). Antibodies against p-p38, p38, ERK, Akt, MMP-9, and TAK1 were purchased from Santa Cruz. Biotechnology (Santa Cruz, CA, USA), Anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-rabbit IgG-HRP, anti-horse and anti-mouse IgG-horseradish peroxidase (HRP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Celecoxib, methotrexate, ibrutinib and tofacinib were purchased from Selleck Chemicals (Houston, TX, USA). XTT was purchased from Welgene Inc. (Gyeongsan, Korea).

2.2. Cell Culture and Osteoclast Differentiation:

All the cells used in this study were continuously cultured and used at the end of the experiment. THP-1 cell line, human monocytic leukemia cells, and the RAW264.7 cell line, murine macrophage cells, were used for the study. THP-1 cells were planted with an appropriate number of cells for each experiment and incubated for 24 h with 100 nM PMA diluted in the medium. THP-1 cell and RAW264.7 cell were purchased from the Korea Cell Line Bank (Seoul, Korea) and cultured in the Roswell Park Memorial Institute (RPMI1640; Welgene. Gyeongsan, Korea) and Dulbecco's Modified Eagle Medium (DMEM; GIBCO, BRL, Grand Island, NY, USA) media. A 1% anti bacterial antifungal solution (GIBCO, BRL, Grand Island, NY, USA) and 10% fetal bovine serum (FBS; GIBCO, BRL, Grand Island, NY, USA) containing penicillin and streptomycin were added to the culture medium. During incubation, the humidity was maintained at 95% and temperature at 37 °C with a

continuous supply of 5% CO_2. The cells were subcultured once or twice a week. To study osteoclast differentiation, RAW264.7 cells at 4 × 10^4 cells/well in 12 well plates were differentiated for 5 days with α-minimum essential medium (MEM; Welgene. Gyeongsan, Korea) media containing 50 ng/mL RANKL. The medium was changed every three days. The treatment group was pre-treatment with KMU-11342 at 0.01, 0.25, and 0.5 μM for 1 h and after RANKL treatment.

2.3. Small Interfering RNA and Transfection:

THP-1 cells were seeded on 12-well plates and transfected with Pim-1, Pim-2, Pim-3 siRNA (Life Technologies, Gaithersburg, MD, USA) and control siRNA using transfection reagent (Santa Cruz Biotechnology. Texas. USA). All transfections were carried out with Lipofectamine™ RNAiMAX (Life Technologies, Gaithersburg, MD, USA). THP-1 cells used were transfected with 30-45 nM siRNA per well. After 72 h, the cells were used for further experiments. RAW264.7 cells and BV2 cells were seeded on 12 well plates of 3 × 10^5 cells/well. Plasmid DNA was mixed with Lipofectamine 2000 (Life Technologies, Gaithersburg, MD, USA) reagent and added to the cells according to the manufacturer's protocol.

2.4. Isolation and Culture of Primary Human Rheumatoid Arthritis Fibroblast-like Synoviocytes:

RA-FLS were provided by the Division of Rheumatology, Department

of Internal Medicine (Chang-Nam Son, M.D.), Keimyung University, and primary cell separation was performed on tissues received from patients with osteoarthritis who sought to remove the tissues damaged during bone replacement surgery. Prior informed consent was obtained from all patients for the use of their tissues (Keimyung, University Dongsan Hospital [IRB No. 2020-11-031], approved by the Keimyung Academic Review Board). Fat tissue, ligaments, and cartilage were removed (by technicians at the institution) from tissues placed in a 100-mm dish containing cell-culture phosphate-buffered saline (PBS) and were cleaned with DMEM (Welgene, Gyeongsan, Korea). After crushing the osteoarthritic tissues into 2-3 mm pieces, 4 mg/mL type-1 collagenase (Worthington Biochemical, Freehold, NJ, USA) was added and the pieces were incubated under 5% CO_2 at 37 °C for 4 h. The isolated cells were centrifuged (1,200 rpm, 10 min) and resuspended in DMEM supplemented with 10% FBS, 2 mmol/L glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL) and rotated in a 75 cm^2 flask. One day later, the adherent cells were incubated at 5% CO_2 and 37 °C in DMEM containing 10% FBS, and the culture fluid was replace every three days. When 90% - 95% of the flask bottom was filled with fibroblast, the medium was diluted 1:3 with fresh culture medium, and the cells were cultivated serially and stored in nitrogen tank.

2.5. Kinase-Profiling Analysis:

To test the inhibition of inflammation-mediated kinase inhibitor KMU-11342, kinase-profiling was done at Eurofins Cerep S.A. (Celle-Lévescault, France). The kinase-profiling analysis was carried out

following the manufacturer's protocol. For individual kinases and kinase substrates, 1 μM/L compound and ATP at the Km value were used. Briefly, each kinase was tested in a reaction mixture containing 8 mM/L MOPS, pH 7.0, 0.2 M EDTA, 50 μM/L EAIYAAPFAKK, and 10 mM/L acetate; [Y-33P] ATP (specific activity and concentration if necessary) was used and the response to the added Mg/ATP mixture was measured. After incubation at room temperature for 40 min, phosphoric acid was added at a concentration of 0.5% to terminate the reaction, and 10 μL of the reaction mixture was spotted on a filter paper washed once in methanol and then with phosphoric acid, dried, and subject to scintillation counting for 4 min against the P30 filter mart.

2.6. Cytotoxicity Measurement:

Intracellular toxicity of the KMU-11342 synthetic compound was examined using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-*2H*-tetrazolium-5-carboxanilide (XTT) assay; 1 × 10^5 cells of THP-1 and RAW264.7 cells were plated in Sprinkle 96-well plates, treated with various concentrations of KMU-11342, and cultured in a culture vessel at 37 °C for 24 h. Subsequently, XTT solution was added to the culture solution and cell survival was measured on the following day; XTT was used at 0.5 mg/mL and reacted under the same culture conditions for 2 h, after which absorbance of the supernatant was measured at 450 nm.

2.7. Western Blotting Analysis:

Western blotting was performed to confirm the degree of expression

of inflammation-related signaling proteins in response to KMU-11342. RA-FLS and THP-1 cells were plated at 1 × 10^6 cells/well, 2 × 10^6 cells/well in 100 and 60 mm dishes, respectively. After culturing for 24 h, the cells were incubated for 1 h with KMU-11342 at 0.1, 0.5, and 1 μM/L and then stimulated for 6 h with LPS. Subsequently, total proteins were extracted from the processed samples by lysing the recovered cells in a solution RIPA buffer (Cell Signaling. Beverly, MD. USA). Protein concentrations were measured using a bicinchoninic acid (BCA) assay kit (Thermo Scientific, Wilmington, Denmark). The cell culture media supernatants were precipitated using trichloroacetic acid (TCA; protocol by Luis Sanchez 2001). Briefly, 1 volume of 100% TCA stock was added to 4 volumes of protein sample. The mixture was incubated for 10 min at 4 °C, spin downed, and the supernatnt was removed, leaving the protein pellet intact. The precipitated pellets were washed pellet with 200 μL cold acetone. The pellets were dried by placing tube in 95 °C heat block for 5-10 min to remove acetone. For SDS-PAGE, the sample was dissolved in 20 μL 2× or 4× sample buffer (with or without β-mercaptoethanol) and boiled for 10 min at 95 °C before loading sample onto a polyacrylamide gel. Equal amounts of protein were electrophoresed in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes, which were blocked 5% skim-milk for 1 h and then incubated for 24 h with appropriately diluted primary antibodies against specific target proteins. The membranes were next washed with Tris-buffered saline/Tween buffer and then incubated at room temperature for 1 h with HRP-conjugated anti-IgG secondary antibodies. Lastly, enhanced chemiluminescence was used to detect protein bands, and the signal strength was measured using a Chemi Image System (Fusion Fx7, Vilber Lourmat, Collégien, France). Protein

bands were quantified using Image J software program. β-actin was used as an internal control for Western blot analysis.

2.8. Immunoprecipitation:

For immunoprecipitation, cells were harvested, washed with cold PBS, and protein were extracted with a lysis buffer (containing NP-40 0.5%, 0.5 M Tris (pH7.4), 5 M NaCl, 0.5 M EDTA, and 0.5 M $MgCl_2$). Concentration of the whole cell lysates were measured, and all samples were incubated overnight with primary antibody at 4 °C. The cell lysates were then incubated with protein G-agarose (Santa Cruz, CA, USA) for 2 h. The beads were washed in lysis buffer and boiled for 10 min. following which confirmation was done by Western blotting.

2.9. RNA Isolation and Quantitative Real Time PCR:

Total mRNA was extracted from THP-1 cells after Pim-1 was knockdown with siRNA. *Pim-1*, *Pim-2*, and *Pim-3* knockdown was confirmed using real-time qPCR. Additionally, pro-inflammatory cytokine and chemokine-related gene expression were analyzed at the mRNA level by using real-time qPCR. THP-1 cells were cultured by plating the same number of cells in 6-well dishes and then were treated for 1 h with KMU-11342 at 0.1, 0.5, and 1 μM/mL, after which the cells were stimulated with LPS for 6 h. Total RNA from each sample was extracted according to the manufacturer's protocol by using TRIzol solution (Invitrogen, San Diego, CA, USA), and 1 μg of the extracted (and quantified) RNA was reverse-transcribed using deoxynucleoside

triphosphate (dNTP), buffer, dichithotritol, RNase inhibitor, and SuperScript II reverse transcriptase. The synthesized cDNA was used for RT-PCR performed using specific primers and the results were analyzed. The primers used in the study are listed in Table 1.

2.10. Immunofluorescence Staining:

RA-FLS and THP-1 cells (1×10^3) were cultured in 8-chamber glass slides for 24 h, treated for 1 h with KMU-11342 at 0.1, 0.5, and 1 μM/L, and then stimulated with LPS for 6 h. The cells were washed with PBS, fixed with 4% formaldehyde, permeabilized using 0.2% Triton X-100 in PBS, and incubated for 1 h with bovine serum albumin (BSA) to block nonspecific binding. Subsequently, the cells were incubated with primary antibodies at 4 °C for 24 h and then with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Thermo Scientific, Wilmington, Denmark) and 4'-6-diamidino-2-phenylindole (DAPI) for nuclear staining (Vector Laboratories, Burlingame, CA, USA), after which the stained cells were examined under a fluorescence microscope.

2.11. Tartrate-resistant Acid Phosphatase Staining (TRAP) and F-actin Ring Staining:

RAW264.7 cells were plated in a 12-well culture dish and stimulated with 50 ng/mL RANKL or pre-treatment KMU-11342 (0.01, 0.1, and 0.25 μM/mL) for 1 h. After five days of culture, cells were stained for TRAP using a leukocyte acid phosphatase kit (TaKaRa. Bio. Kyto,

Japan) according to the manufacture's protocol. Cultured cells were fixed in fixation solution for 20 min at room temperature, and washed with distilled water. Cells with three or more nuclei were counted as multinuclear osteoclast when viewed microscopically.

For actin ring staining, RAW264.7 cell were plated in a 12-well culture dish and stimulated with 50 ng/mL RANKL for 6 days. The staining was performed using Phalloidin CruzFluorTM488 Conjugate (Santa Cruz, CA, USA). Cells were washed with PBS, and fixed in 4% neutral buffered formalin for 20 min at room temperature. Cell permeabilization was done for 10 min with 0.1% Triton X-100 in PBS. Blocking was done with 1% BSA for 1 h at room temperature and incubated with stain antibody (1:5000 dilution) for 30 min. The cells were then washed thrice with PBS and photographed using a fluorescence microscope.

2.12. Statistical Analysis:

Differences between the groups were analyzed using student's two tailed *t*-test. Analysis of the experimental results was conducted through the GraphPad Prism 5.0 program (GraphPad Software, San Diego, CA, USA). One way analysis of variance was performed for comparison between each group, and significance between control and experimental groups was analyzed at $p < 0.05$ level using Tukey's multiple composition test (* $p < 0.05$, ** $p < 0.01$, # $p < 0.001$).

3. Results

3.1. The Role of Pim-1 in LPS-mediated Inflammation:

3.1.1. Pim Kinase Inhibitors Attenuate LPS-induced NLRP3 inflammasome Activation in THP-1 Cells:

To determine whether Pim plays an important role in LPS-mediated inflammation in THP-1 cells, I used two Pim kinase inhibitors (PIM447 and AZD1208). Both inhibitors suppressed LPS-induced up-regulation of pro-IL-1β, IL-6, and TNF-α in THP-1 cells (Figure 1A,B). In addition, they also inhibited LPS-induced phosphorylation of NF-κB p65 at Ser536 and NLRP3 protein up-regulation (Figure 1C-E).

3.1.2. LPS-induced Pim-1 Activation and Up-regulation of Pro-inflammatory Cytokines:

Next, I investigated Pim-1 protein expression in LPS-mediated inflammation in THP-1 cells. In the present study, THP-1 cells were exposed to different concentrations of LPS for 1, 3, 6, 15, and 24 h, then the expression levels of Pim-1 and pro-IL-1β were detected by Western blotting. The results showed that treatment with LPS increase the expression levels of Pim-1 and pro-IL-1β in a dose-and time-dependent manner (Figure 2A,B). In addition, THP-1 cells were exposed to different concentrations of LPS for 6 h, then the mRNA expression levels of Pim-1 and pro-inflammatory cytokines were detected by real-time qPCR. As shown in Figure 2C-F, treatment with LPS increased the mRNA expression levels of Pim-1, IL-1β, TNF-α, and

IL-6 in a dose-dependent manner. Previous study demonstrated that Pim-1 phosphorylates the pro-apoptotic protein protein B-cell lymphoma 2-associated agonist of cell death (BAD) (26). Therefore, I used p-BAD (Ser112) as a Pim-1 substrate. As shown in Figure 2A,B, as Pim-1 was up-regulated by LPS, the phosphorylation of Bad was also increased.

3.1.3. Pim-1 Knockdown Attenuates LPS-mediated Up-regulation of Pro-inflammatory Cytokines in THP-1 Cells:

To determine whether Pim-1 regulates pro-inflammatory cytokine, Pim-1 siRNA was used. There was no noticeable change in external morphology in THP-1 cells when transfected with either control siRNA or Pim-1 siRNA, with or without LPS treatment under the phase-contrast microscope (Figure 3A). Pim-1 siRNA transfection significantly down-regulated the mRNA expression level of Pim-1 (Figure 3B). Moreover, Pim-1 siRNA transfection significantly inhibited LPS-mediated up-regulation of Pim-1 mRNA expression in THP-1 cells (Figure 3C). Pim-1 siRNA transfection also suppressed LPS-mediated up-regulation of Pim-1 protein expression in THP-1 cells (Figure 3D,E). Next I investigated the effect of Pim-1 knockdown on pro-inflammatory cytokines. As shown in Figure 3F-I, Pim-1 siRNA transfection attenuated LPS-mediated up-regulation of the expression levels of pro-IL-1β, TNF-α, and IL-6 in THP-1 cells. Next, to demonstrate other isotypes of Pim-1, Pim-2 and Pim-3 affect LPS-induced pro-inflammatory cytokines, siRNAs of Pim-2 and Pim-3 were used. Both Pim-2 and Pim-3 siRNA transfections significantly inhibited LPS-mediated up-regulation of mRNA expression levels of Pim-2 and Pim-3 in THP-1 cells (Figure 3L,M). Furthermore, both knockdowns of Pim-2 and Pim-3 down-regulated LPS-mediated up-regulation of the

expression levels of pro-IL-1β, IL-6, and TNF-α in THP-1 cells (Figure 3J,K). These results suggest that Pim kinases regulate pro-inflammatory cytokines involved in the LPS-mediated inflammatory mechanism.

3.1.4. Pim-1 Knockdown Attenuates LPS-mediated Up-regulation of iNOS and COX-2 Expression in THP-1 Cells:

I investigated the relationship between Pim-1 and the enzymes iNOS and COX-2, which induce inflammatory reactions. Pim-1 siRNA transfection inhibited LPS-induced up-regulation of iNOS and COX-2 expressions (Figure 4A-C). Moreover, Pim-1 siRNA transfection suppressed LPS-induced up-regulation of COX-2 protein expressions in immunofluorescence analysis (Figure 4D).

3.1.5. Pim-1 Knockdown Attenuates LPS-mediated Activation of MAPKs and NF-κB in THP-1 Cells:

LPS induced the phosphorylation of IKKα/β, NF-κB p65, ERK, JNK, and p38 in THP-1 cells, but Pim-1 siRNA transfection inhibited their LPS-mediated phosphorylation (Figure 5A,B). Pim-1 siRNA transfection also suppresses the nuclear translocation of NF-κB p65 in THP-1 cells under fluorescence microscopy (Figure 5C).

3.1.6. Pim-1 Knockdown Attenuates LPS-mediated NLRP3 Inflammasome activation in THP-1 Cells:

I investigated the role of Pim-1 on the activation of NLRP3 inflammasome, which is an important mechanism in inflammatory responses and is regulated by the intrinsic immune system. As shown in Figure 6A, as Pim-1 was up-regulated by LPS, the expression levels

of pro-IL-1β, NLRP3, pro-caspase-1, and ASC were also up-regulated. Pim-1 siRNA transfection inhibited the secretion of pro-IL-1β, IL-1β, and caspase-1 into the cell culture media and the expression levels of NLRP3, pro-IL-1β, and pro-caspase-1 in THP-1 cells co-stimulated with LPS and ATP (Figure 6B). Additionally, Pim-1 siRNA transfection significantly attenuated the up-regulation of NLRP3 and IL-1β mRNA levels induced by LPS and co-treatment with LPS and ATP (Figure 6D,E). Moreover, Pim-1 siRNA transfection inhibited the up-regulation of NLRP3, pro-caspase-1, caspase1, pro-IL-1β, and p-Bad (Ser112) protein levels in RAW264.7 cells (Figure 6C). However, caspase-11, which promotes NLRP3 inflammasome activation and increases pro-IL-1 β levels (27,28), was not affected by Pim-1 siRNA transfection (Figure 6C).

3.1.7. Pim-1 Phosphorylates TAK1 at Ser412 in LPS-mediated Inflammatory in THP-1 Cells:

Because TAK1 plays an important role in TLR4/MyD88 signaling, I examined the correlation between Pim-1 and TAK1 in LPS-mediated inflammatory signals. First, Pim-1 knockdown and Pim kinase inhibitor did not change the expression levels of TLR4 and MyD88 proteins (Figure 7A,B). Pim-1 siRNA transfection suppressed LPS-mediated up-regulation of p-TAK1 (Ser412), Pim-1, p-Bad (Ser112), and pro-IL-1 β protein levels in THP-1 cells (Figure 7C). In addition, (5Z)-7-Oxozaenol, a TAK1 inhibitor, suppressed LPS-induced up-regulation of Pim-1, p-Bad (Ser112), and pro-IL-1β protein levels in THP-1 cells (Figure 7D). The Pim kinase inhibitors suppressed LPS-induced up-regulation of p-TAK1 (Ser412), p-Bad (Ser112), and pro-IL-1β protein levels in THP-1 cells (Figure 7E). These results

indicated that Pim-1 and TAK1 are correlated in LPS-mediated inflammatory signals.

3.1.8. Pim-1 Kinase Activity Modulates the Interaction between Pim-1 and TAK1:

Immunoprecipitation was performed to further investigate the interactions between Pim-1 and TAK1. LPS treatment induced the interaction between endogenous Pim-1 and TAK1 in THP-1 cells (Figure 8A). Moreover, both Pim-1 siRNA transfection and (5Z)-7-Oxozaenol inhibited LPS-stimulated the interaction between endogenous Pim-1 and TAK1, along with down-regulation of Pim-1 in THP-1 cells (Figure 8B,C). In addition, Pim-1-DN also suppressed LPS-induced the interaction between endogenous Pim-1 and TAK1, along with down-regulation of pro-IL-1β in RAW264.7 cells (Figure 8D). Interestingly, Pim-1-K67M attenuated LPS-induced the interaction between endogenous Pim-1 and TAK1, along with down-regulation of pro-IL-1β in BV2 cells (Figure 8E). These results indicated that Pim-1 kinase activity plays an important role in the interaction with TAK1 and the LPS-mediated inflammatory signals.

3.1.9. Pim-1 Knockdown Attenuates LPS-mediated Activation of JAK/STAT Pathway in THP-1 Cells:

Next, I investigated whether Pim-1 plays an important role in LPS-induced activation of JAK/STAT pathway. Pim-1 siRNA transfection inhibited LPS-mediated up-regulation of p-JAK1 (Tyr 1034/1035) and p-STAT (Tyr705) in THP-1 cells (Figure 9A). Moreover, Pim447, AZD1208, and (5Z)-7-Oxozaenol inhibited LPS-stimulated up-regulation of p-JAK1 (Tyr 1034/1035) and p-STAT

(Tyr705) in THP-1 cells (Figure 9B,C). These result indicated that Pim-1 is a upstream modulator of JAK1 and STAT3.

3.2. The Anti-Inflammatory Effect of a Novel Pim Kinase Inhibitor, KMU-11342:

3.2.1. Chemical Structure of KMU-11342 and its Effects on the Activities of Pim Kinases:

The chemical structure of KMU-11342 is presented in Figure 10A. To evaluate that KMU-11342 has inhibitory effect against Pim kinase, the Pim kinase inhibitory activity of the compound was investigated. The kinase analysis revealed that KMU-11342 inhibited Pim-1, Pim-2, and Pim-3 activities to -3, 0, and 4, respectively (Table 2). These results showed that KMU-11342 has strong inhibitory activities against Pim kinases.

3.2.2. KMU-11342 Inhibitis the Production of TLR4-mediated Chemokines in Human RA-FLS:

To determine the toxicity of KMU-11342 in THP-1 cells, XTT assay was performed. As shown in Figure 10B, KMU-11342 had no cytotoxicity up a concentration of 1 μM. Moreover, KMU-11342 significantly suppressed LPS-stimulated up-regulation of chemokine mRNA levelss (CXCL10, CCL2/MCP-1, CCL3/MCP-1α, and CCL4/MCP-1β) in Human RA-FLS (Figure 10C-F).

3.2.3. KMU-11342 Inhibitied of LPS-induced Pro-inflammatory Cytokine in RA-FLS and THP-1 Cells:

Cytokines are important factors in mediating inflammation of infectious microorganisms, such as bacteria and viruses, and many mechanisms of innate immunity. Therefore, I next investigated whether KMU-11342 affects expression levels of crucial inflammatory cytokines such as IL-1β, TNF-α, and IL-6 in LPS-treated RA-FLS and THP-1 cells. Notably, pretreatment of cells with KMU-11342 of 0.5 and 1 μM inhibited LPS-induced up-regulation of mRNAs and Proteins of proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6 in RA-FLS and THP-1 cells (Figure 11A-C,E-G).

3.2.4. KMU-11342 Inhibitied of COX-2 and iNOS Expression in RA-FLS and THP-1 Cells:

COX-2 and iNOS play important roles in inflammation. When RA-FLS and THP-1 cells were treated with LPS, the expression levels of iNOS and COX-2 were increased in both mRNA and protein levels, but KMU-11342 (0.5 and 1 μM) suppressed the both expression levels (Figure. 12). However, the protein expression level of COX-1 did not suppressed by KMU-11342 (Figure 12C). These results indicated that KMU-11342 has a selective inhibitory effect on COX-2.

3.2.5. KMU-11342 Inhibitied of LPS-induced NF-κB in RA- FLS and THP-1 Cells:

In inflammatory reactions, NF-κB is known to regulate the expression of iNOS, COX-2, and various pro-inflammatory cytokines. In order to determine whether NF-κB, an important transcription factor, is regulated by KMU-11342, I investigated the phosphorylation of IKKα/β and NF-κB p65. LPS remarkably increased the phosphorylation of IKKα/β and NF-κB p65 in RA-FLS and THP-1 cells, but the phosphorylation of

IKKα/β and NF-κB p65 were decreased after treatment of KMU-11342 (0.5 and 1 μM concentrations) (Figure 13A,D). In addition, immunofluorescence staining revealed that KMU-11342 inhibits the translocation of NF-κB p65 from cytosol to nucleus in RA-FLS and THP-1 cells (Figure 13C,F). These findings showed that KMU-11342 inhibits NF-κB-mediated inflammatory reactions.

3.2.6. KMU-11342 Inhibited of LPS-induced TAK1 and MAPKs in THP-1 Cells:

I investigated whether KMU-11342 inhibits MAPKs, which are upstream signals of the pro-inflammatory cytokines, COX-2, and iNOS. As shown in Figure 14A,B, KMU-11342 suppressed LPS-stimulated activation of JNK and TAK1 but not ERK and p38 in THP-1 cells.

3.2.7. KMU-11342 Inhibited LPS-induced NLRP3 Inflammasome Activation in THP-1 Cells:

The activation of NLRP3 inflammasome, a protein complex, plays an important role in production, maturation and secretion of the pro-inflammatory cytokine IL-1β. Pretreatment of THP-1 cells with 0.5 μM KMU-11342 suppressed LPS-induced up-regulation of NLRP3 inflammasome. However, protein expression levels of ASC and pro-caspse-1 were not inhibited. Next, I investigated whether KMU-11342 inhibits activation and maturation of IL-1β under ATP stimulation. As shown in Figure 14E, in THP-1 cells, KMU-11342 inhibited production and maturation of IL-1β and activation of caspase-1, which are induced by co-treatment with LPS and ATP. Therefore, these results revealed that KMU-11342 inhibits NLRP3 inflammasome activation.

3.2.8. KMU-11342 Inhibits LPS-Stimulated Inflmmation in THP-1 Cells:

Next, comparing several drugs (celecoxib, ibrutinib, methotrexate, tofacinib) currently used as anti-inflammatory agents (Figure 15A), KMU-11342 is appearsed to have good potential as an anti-inflammatory agent, as it inhibits IL-1β factors, which are cytokines more or more than them. KMU-11342 inhibited phosphorylation of the Pim-1 substrate Bad. This suggests that KMU-11342 inhibits PIM kinases.

3.3. The Inhibitory Effect of KMU-11342 on Osteoclastogenesis:

3.3.1. KMU-11342 Inhibition of RANKL induced Osteoclast Differentiation and F-Actin Ring Formation:

As rheumatoid arthritis persists, bones and cartilage are eventually destroyed. I investigated whether KMU-11342 inhibits osteoclast differentiation, which is responsible for bone absorption and F-actin ring formation of osteoclasts, an important indicator of bone absorption. Osteoclast differentiation was generated from RAW264.7 cells in the presence of RANKL (50 ng/mL) for five days. Immunofluorescence analysis revealed that KMU-11342 strongly suppressed the formation of TRAP-positive polynuclear osteoclasts and the degree of F-actin ring formation of osteoclasts in a dose-dependent manner (Figure 17A). Moreover, KMU-11342 treatment significantly inhibited counting numbers of TRAP-positive osteoclasts in a dose-dependent manner (Figure 17C). Moreover, KMU-11342 had no cytotoxicity up a concentration of 0.5 μM in RAW264.7 cells (Figure 17B).

3.3.2. KMU-11342 inhibited RANKL-stimulated up-regulation of Genetic Factors involved in Osteoclast Differentiation:

Next, I investigated whether KMU-11342 suppresses representative osteoclast differentiation factors. As shown in Figure 18A-E, KMU-11342 inhibited RANKL-stimulated up-regulation of various osteoclast differentiation factors, such cathepsin K, TRAP, osteogenic phosphatase (OSCAR), nuclear factor of activated T cells c1 (NFATc1), and MMP-9. Moreover, KMU-11342 inhibited RANKL-stimulated c-Fos and NFATc1 protein levels, important transcription factors involved in bone cell differentiation and production (Figure 18F-I). Additionally, the expression level of MMP-9 protein was also inhibited by KMU-11342 treatment (Figure 18H).

3.3.3. KMU-11342 Inhibits RANKL-induced activation of TAK1/ MAPKs/NF-κB Signal Pathway in Osteoclast Differentiation:

To determine the mechanism by which KMU-11342 inhibits osteoclast differentiation, I investigated the activation of TAK1, Akt, and MAPKs (ERK, JNK, and p38), which are major components of RANKL-induced osteoclast differentiation. As shown in Figure 19A,B, KMU-11342 strongly inhibited RANKL-induced phosphorylation of IKKα/β and NF-κB. Moreover, immunofluorescence analysis showed that KMU-11342 also suppresses the nuclear translocation of NF-κB p65 in RAW264.7 cells under fluorescence microscopy (Figure 19C). In particular, KMU-11342 inhibited RANKL-induced phosphorylation of TAK1, Akt, ERK, JNK, and p38 in RAW264.7 cells. These result suggested that KMU-11342 has inhibitory effect on osteoclast differentiation through inhibiting TAK1, Akt, ERK, JNK, and p38.

Table 1. Primer Sequences

Primers		Sequences (5'→ 3')
Human primer		
Pim-1	Forward	GCT CGG TCT ACT CAG GCA TC
	Reverse	CGG GCA TCT GAC AAG AGA GG
Pim-2	Forward	GCC TCA CAG ATC GAC TCC AG
	Reverse	GAA GCA GGG CAC CAG AAC C
Pim-3	Forward	ACC GCG ACA TTA AGG ACG AAA
	Reverse	ACA CAC CAT ATC GTA GAG AAG CA
NLRP3	Forward	GAG GCA ACA CTC TCG GAG AC
	Reverse	TCT GGC TGG AGG TCA GAA GT
CXCL10	Forward	GTG CGA TTC AAG GAG TAC CTC
	Reverse	TGA TGG CCT TCG ATT CTG GAT T
MCP-1	Forward	CAG CCA GAT GCA ATC AAT GCC
	Reverse	TGG AAT CCT GAA CCC ACT TCT
MIP-1α	Forward	AGT TCT CTG CAT CAC TTG CTG
	Reverse	CGG CTT CGC TTG GTT AGG AA
MIP-1β	Forward	CTG TGC TGA TCC CAG TGA ATC
	Reverse	TCA GTT CAG TTC CAG GTC ATA CA
iNOS	Forward	CTG TCT GGT TCC TAC GTC ACC
	Reverse	CCC ACG TTA CAT GGG AGG ATA
COX-2	Forward	ATC ACA GGC TTC CAT TGA CC
	Reverse	TAT CAT CTA GTC CGG AGG GG
IL-1β	Forward	CCT TGG GCC TCA AGG AAA A
	Reverse	CTC CAG CTG TAG AGT GGG CTT A
TNF-α	Forward	GGA GAA GGG TGA CCG ACT CA
	Reverse	CTG CCC AGA CTC GGC AA
IL-6	Forward	ATG GCA CAG TAT CTG GAG GAG
	Reverse	TAA GCT GGA CTC ACT CTC GGA
β-actin	Forward	AAT CTG GCA CCA CAC CTT CTA

	Reverse	ATA GCA CAG CCt GGA TAG CAA
Mouse primer		
Cathepsin K	Forward	CAG CAG AAC GGA GGC ATT GA
	Reverse	CCT TTG CCG TGG CGT TAT AC
TRAP	Forward	AAG GCG AGA GAT TCT TTC CCT G
	Reverse	ACT GGG GAC AAT TCA CTA GAG C
NFATc1	Forward	GGG TCA GTG TGA CCG AAC AT
	Reverse	GGA AGT CAG AAG TGG GTG GA
MMP-9	Forward	GCC CTG GAA CTC ACA GCA CA
	Reverse	TTG GAA ACT CAC ACG CCA GAA G
OSCAR	Forward	AGG GAA ACC TCA TCC GTT TG
	Reverse	GAG CCG GAA ATA AGG CAC AG
β-actin	Forward	ATT TCT GAA TGG CCC AGG T
	Reverse	CTG CCT CAA CAC CTC AAC C

Table 2. Pim Kinase Inhibitory Activities of KMU-11342

KMU-11342 1 μM	
Kinase	**Activity (% control)***
Pim-1(h)	-3
Pim-2(h)	0
Pim-3(h)	4

*Values were obtained from the kinase Profiler TM project of Eurofins.

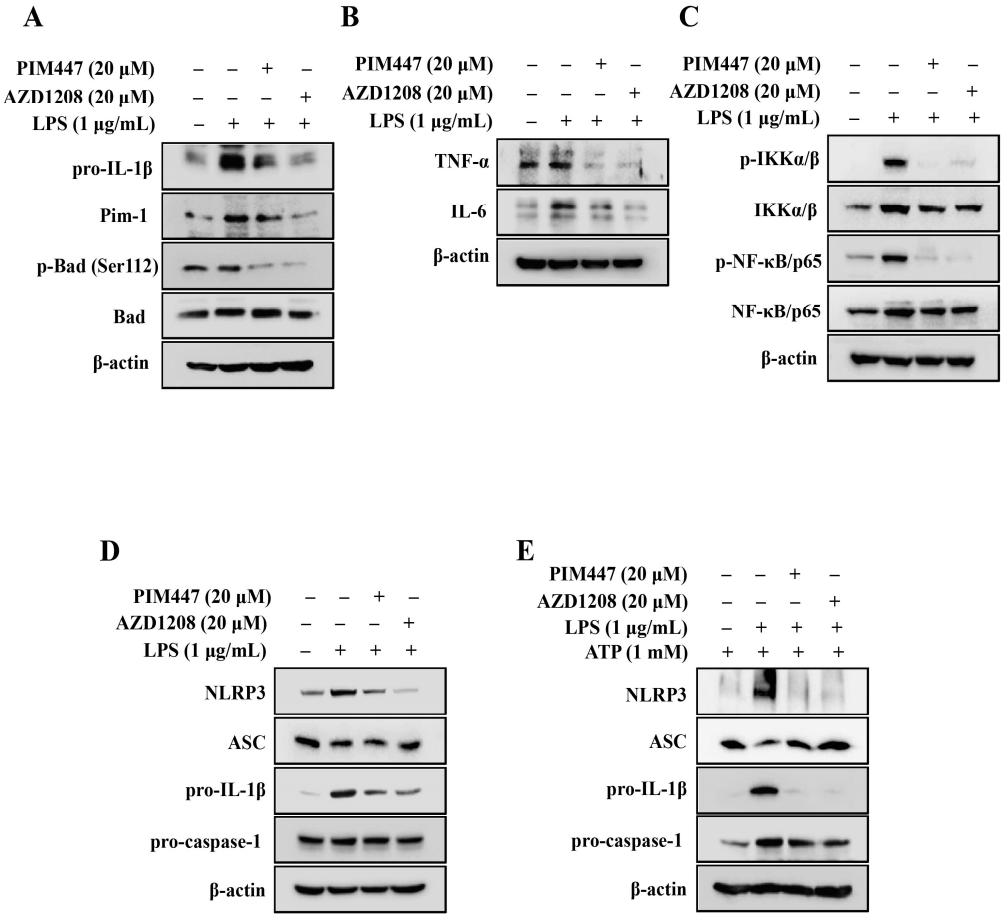


Figure 1. The anti-inflammatory effect of Pim kinase inhibitors on LPS-mediated inflammation in THP-1 cells. THP-1 cells were differentiated into macrophages using 100 nM PMA for 24 h, then the cells were stimulated with LPS (1 μg/mL) for 6 h after pre-treatment with PIM447 (20 μM) and AZD1208 (20 μM) for 1 h. (A) Whole cell lysates were isolated and used to measure the protein expression levels of pro-IL-1β, Pim-1, p-Bad (Ser112), and Bad by Western blotting. (B) Whole cell lysates were isolated and used to measure the protein expression levels of TNF-α and IL-6 by Western

blotting. (C) Whole cell lysates were isolated and used to measure the protein expression levels of p-IKKα/β, IKKα/β, p-NF-κB p65, and NF-κB p65 by Western blotting. (D) Whole cell lysates were isolated and used to measure the protein expression levels of NLRP3, ASC, pro-IL-1β, and pro-caspase-1 by Western blotting. (E) Cells were differentiated into macrophages using 100 nM PMA for 24 h, then the cells were stimulated with LPS (1 μg/mL) with/without ATP (1mM) for 6 h after pretreatment with PIM447 (20 μM) and AZD1208 (20 μM) for 1 h. Whole cell lysates were isolated and used to measure the protein expression levels of NLRP3, ASC, pro-IL-1β, and pro-caspase-1 by Western blotting.

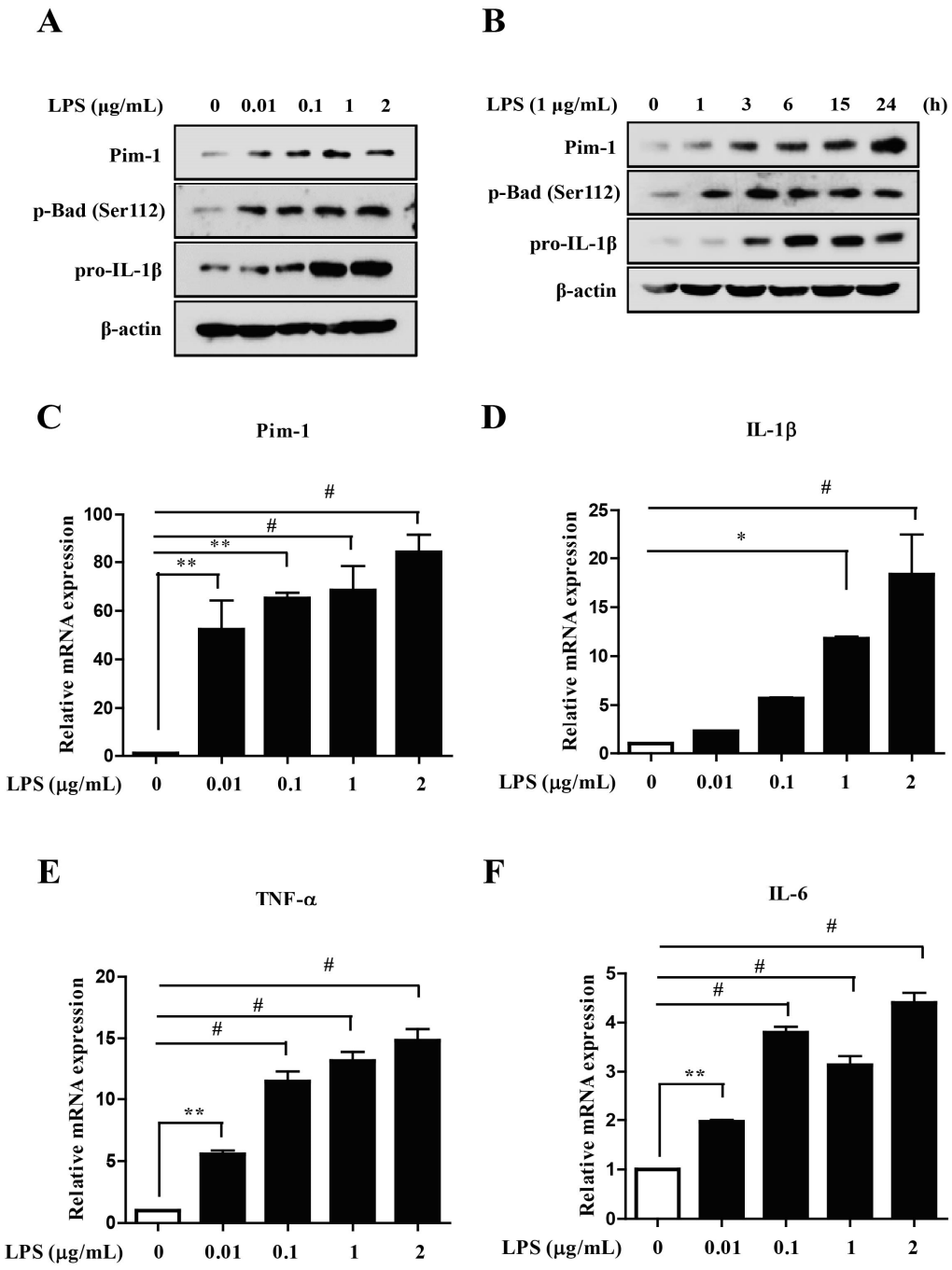
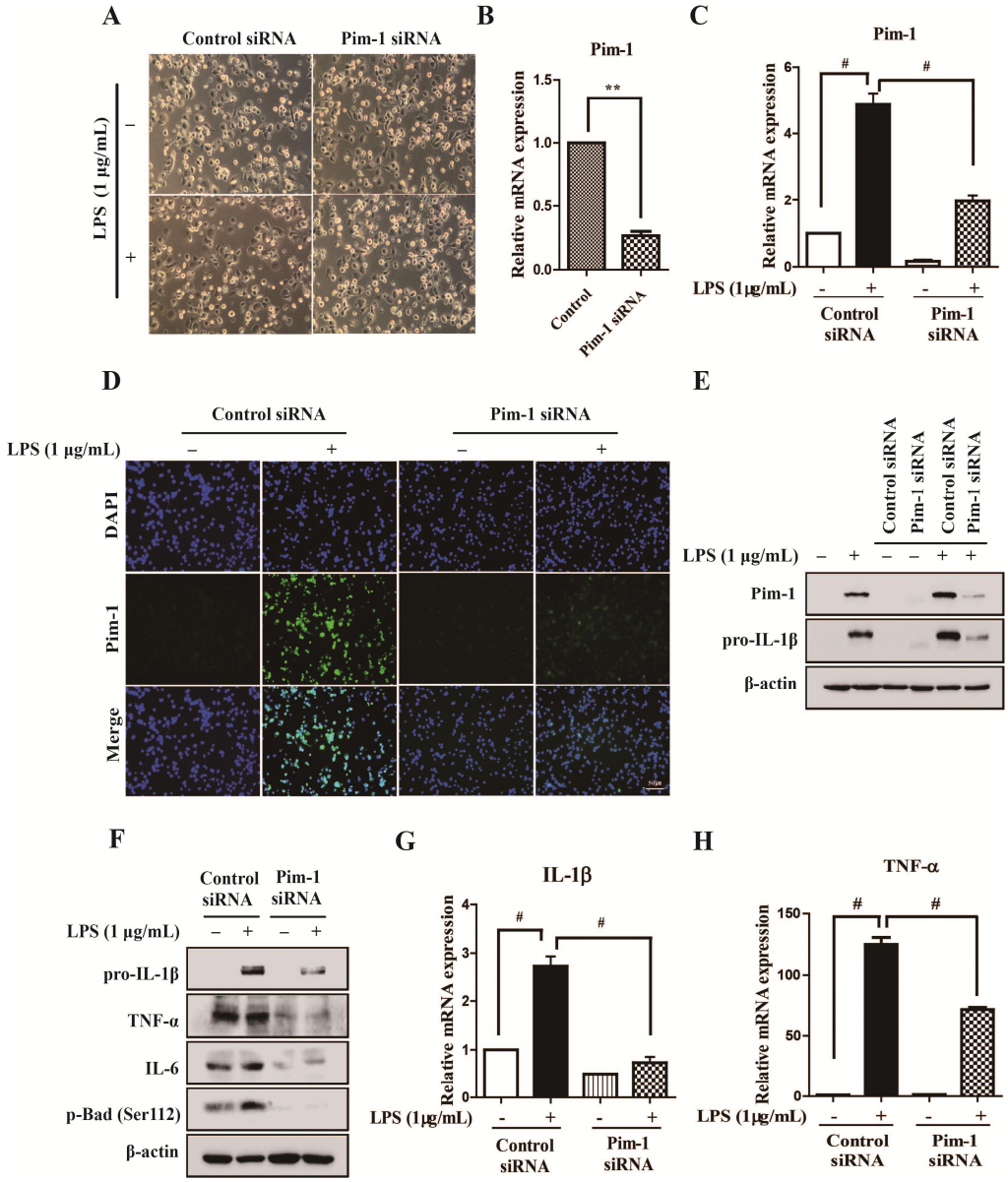


Figure 2. The expression levels of Pim-1 and pro-infflammatory cytokine in LPS-stimulated THP-1 cells. (A) THP-1 cells

were stimulated with LPS (0.01, 0.1, 1, and 2 μg/mL) for 6 h. Whole cell lysates were isolated and used to measure the protein expression levels of Pim-1, p-Bad, and pro-IL-1β by Western blotting. (B) Cells were stimulated with LPS (1 μg/mL) for the indicated time points. Whole cell lysates were isolated and used to measure the protein expression levels of Pim-1, p-Bad, and pro-IL-1β by Western blotting. (C-F) Cells were stimulated with LPS (0.01, 0.1, 1, and 2 μg/mL) for 6 h. Total RNA was extracted, and used to evaluate the mRNA expression levels of Pim-1, IL-1β, TNF-α, and IL-6 by real-time qPCR (* $p < 0.05$, ** $p < 0.01$, # $p < 0.001$).



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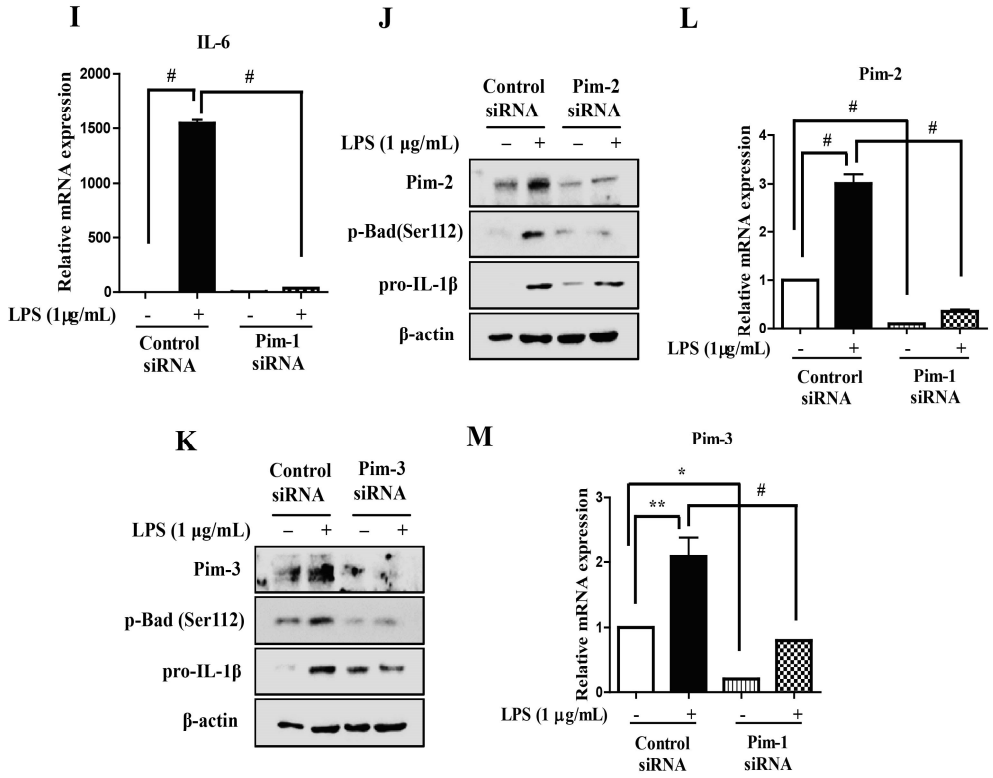

Figure 3. The effect of Pim-1 knockdown in LPS-mediated inflammatory signals of THP-1 cells. THP-1 cells transfected with control siRNA or Pim-1 siRNA for 72 h and then stimulated with LPS (1 μg/mL) for 6 h. (A) The effects of Pim-1 siRNA on cellular morphological changes were observed by microscopy (×200). (B,C) Total RNA was extracted, and used to evaluate the mRNA expression levels of Pim-1 by real-time qPCR. (D) Cells were stained with antibodies to Pim-1 (green) and DAPI (blue) and captured at ×200 using fluorescence microscope (scale bar = 50 μm). (E) Whole cell lysates were isolated and used to measure the protein expression levels of Pim-1 and

pro-IL-1β by Western blotting. (F) Whole cell lysates were isolated and used to measure the protein expression levels of pro-IL-1β, IL-6, TNF-α, and p-Bad (Ser112) by Western blotting. (G-I,L,M) Total RNA was extracted, and used to evaluate the mRNA expression levels of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6), Pim-1, Pim-2, and Pim-3, respectively (* $p < 0.05$, ** $p < 0.01$, # $p < 0.001$). (J,K) Whole cell lysates were isolated and used to measure the protein expression levels of Pim-2 and Pim-3 by Western blotting, respectively.

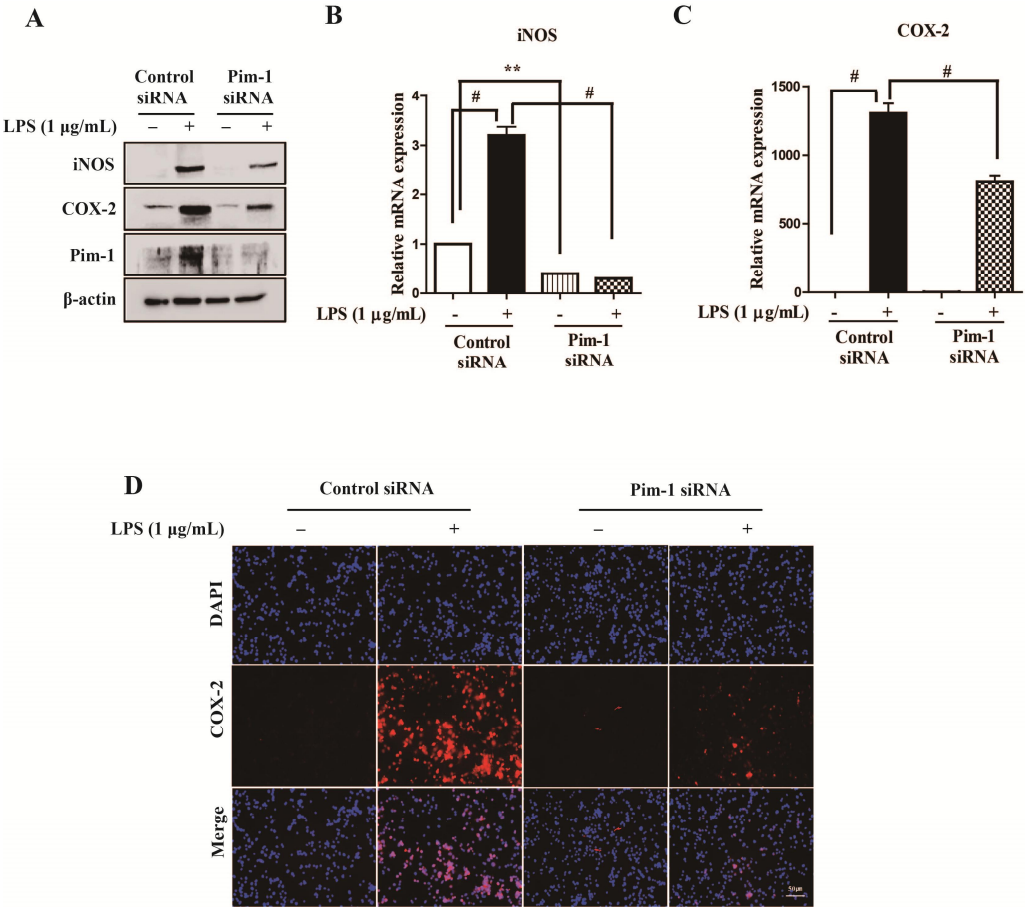

Figure 4. The effect of Pim-1 knockdown in LPS-induced iNOS and COX-2 in THP-1 cells. (A) Whole cell lysates were isolated and used to measure the protein expression levels of iNOS and COX-2 by Western blotting. (B,C) Total RNA was extracted, and used to evaluate the mRNA expression levels of iNOS and COX-2, respectively (** $p < 0.01$, # $p < 0.001$). (D) Cells were stained with antibodies to COX-2 (red) and DAPI (blue) and captured at ×200 using fluorescence microscope (scale bar = 50 μm).

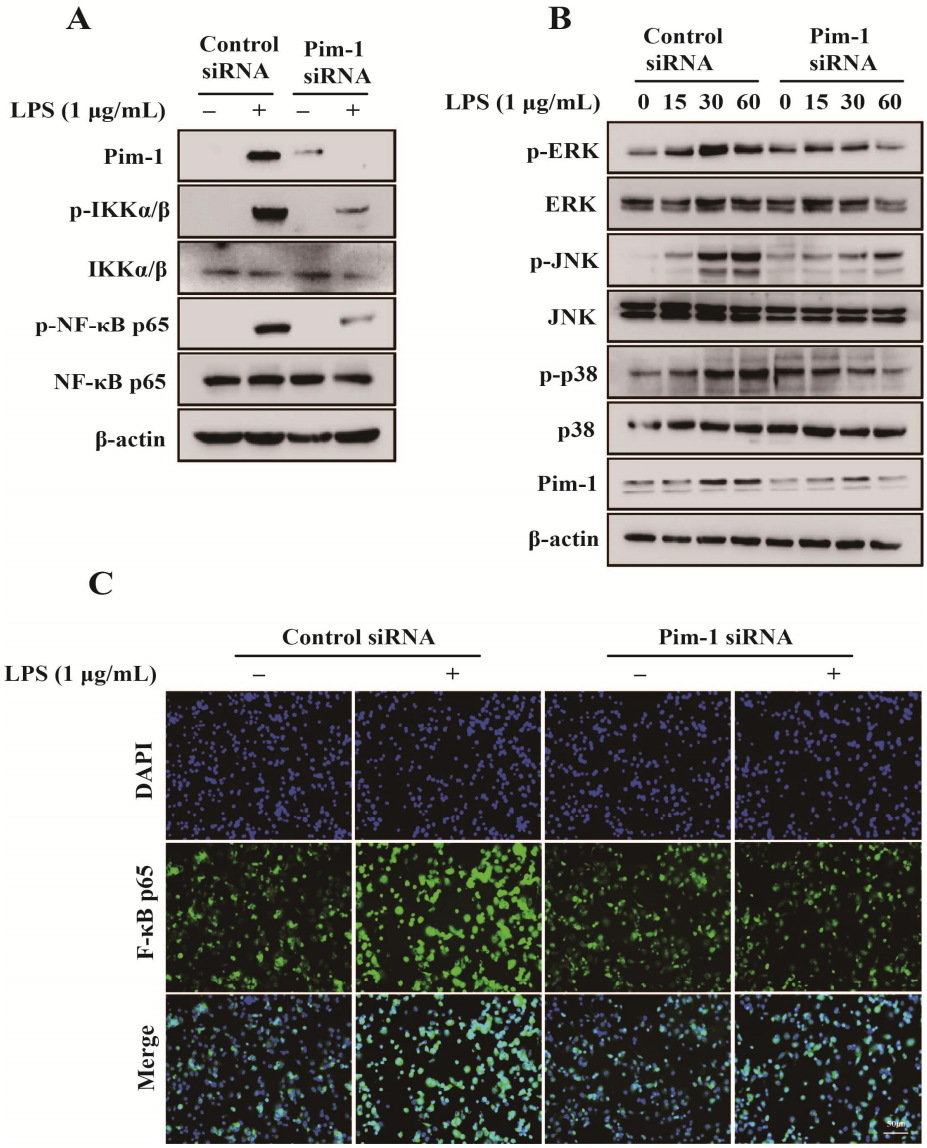

Figure 5. The effect of Pim-1 knockdown in LPS-mediated activation of NF-κB p65 and MAPKs expression in THP-1 cells. THP-1 cells transfected with control siRNA or Pim-1 siRNA for 72 h and then stimulated with LPS (1 μg/mL) for 6 h. (A) Whole cell lysates were isolated and used to measure the protein expression levels of p-IKKα/β and p-NF-κB p65

by Western blotting. (B) Cells were stimulated with LPS (1 μg/mL) for the indicated time points. Whole cell lysates were isolated and used to measure the protein expression levels of p-ERK, ERK, p-JNK, JNK, p-p38, and p38 by Western blot analysis. (C) Cells were stained with antibodies to NF-κB p65 (green) and DAPI (blue) and captured at ×200 using fluorescence microscope (scale bar = 50 μm).

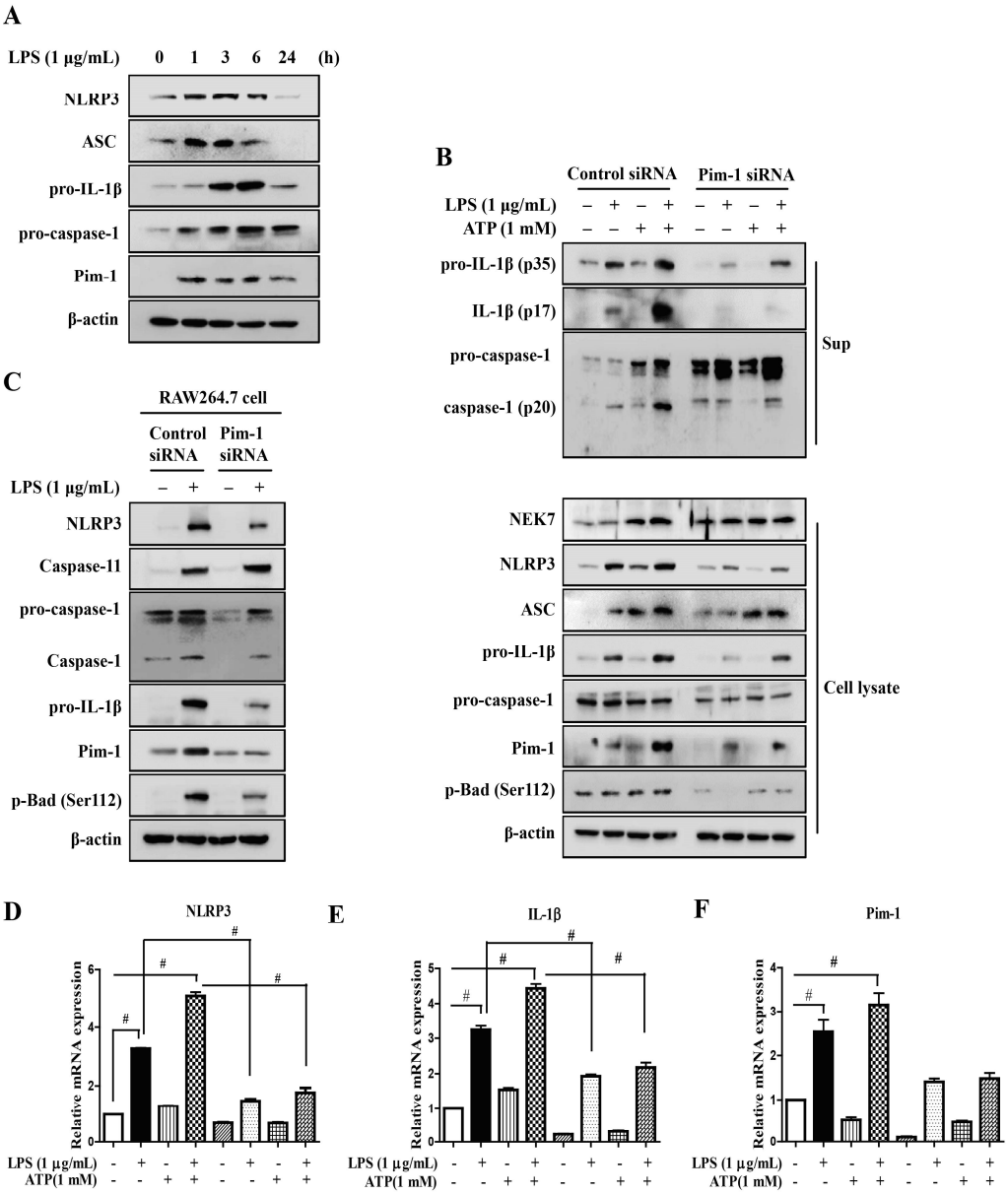

Figure 6. The effect of Pim-1 knockdown in LPS-mediated activation of NLRP3 inflammasome in THP-1 cells. (A) Cells were stimulated with LPS (1 μg/mL) for the indicated time points. Whole cell lysates were isolated and used to measure the

protein expression levels of NLRP3, ASC, pro-IL-1β, and Caspase-1 by Western blotting. (B) Cells were stimulated with LPS (1 μg/mL) and/or ATP (1 mM) for 6 h. Cell lysate (Lysate) and media supernatant (Sup) were isolated and used to measure the protein expression levels of pro-IL-1β, IL-1β, pro-caspase-1, and caspase-1 for the Sup as well as NLRP3, ASC, pro-caspase-1, and pro-IL-1β for the Lysate by Western blot analysis. (C) RAW264.7 cells transfected with control siRNA or Pim-1 siRNA for 72 h and then stimulated with LPS (500 ng/mL) for 6 h. Whole cell lysates were isolated and used to measure the protein expression levels of NLRP3, caspase-11, pro-IL-1β, and caspase-1 by Western blotting. (D,E,F) Total RNA was extracted, and used to evaluate the mRNA expression levels of NLRP3, IL-1β, and Pim-1, respectively. (# $p < 0.001$).

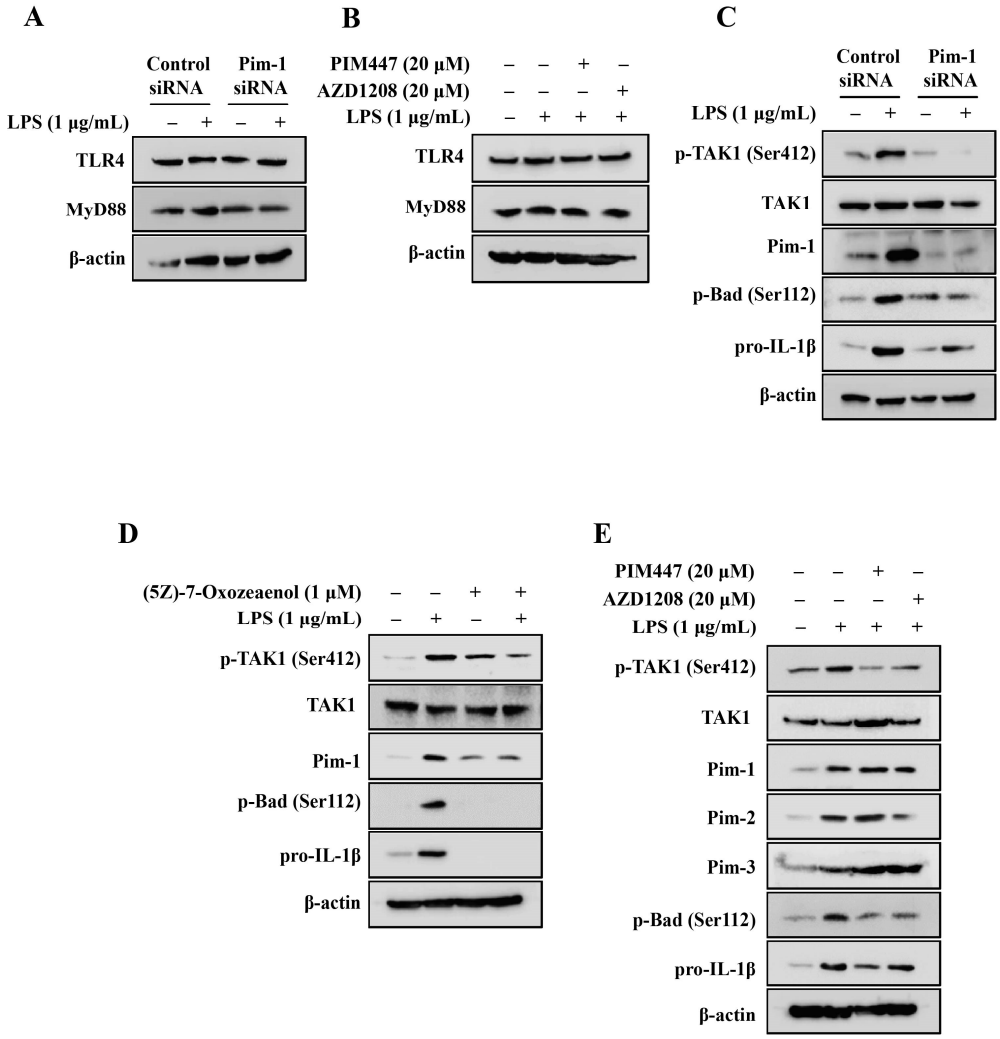

Figure 7. The effect of Pim-1 knockdown in LPS-mediated activation of TAK1 expression in THP-1 cells. (A) Whole cell lysates were isolated and used to measure the protein expression levels of TLR4 and MyD88 by Western blotting. (B) Cells were stimulated with LPS (1 μg/mL) for 6 h after pre-treatment with PIM447 (20 μM) and AZD1208 (20 μM) for 1 h. Whole cell lysates were isolated and used to measure the protein expression levels of TLR4 and MyD88

by Western blotting. (C-E) Cells were transfected with control siRNA or Pim-1 siRNA, and pre-treatment with TAK1 inhibitor (5Z)-7-Oxozeaenol, (1 μM), or with PIM447 (20 μM) and AZD1208 (20 μM) for 1 h before LPS (1 μg/mL) stimulation. Whole cell lysates were isolated and used to measure the protein expression levels of p-TAK1, TAK1 and pro-IL-1β by Western blotting.

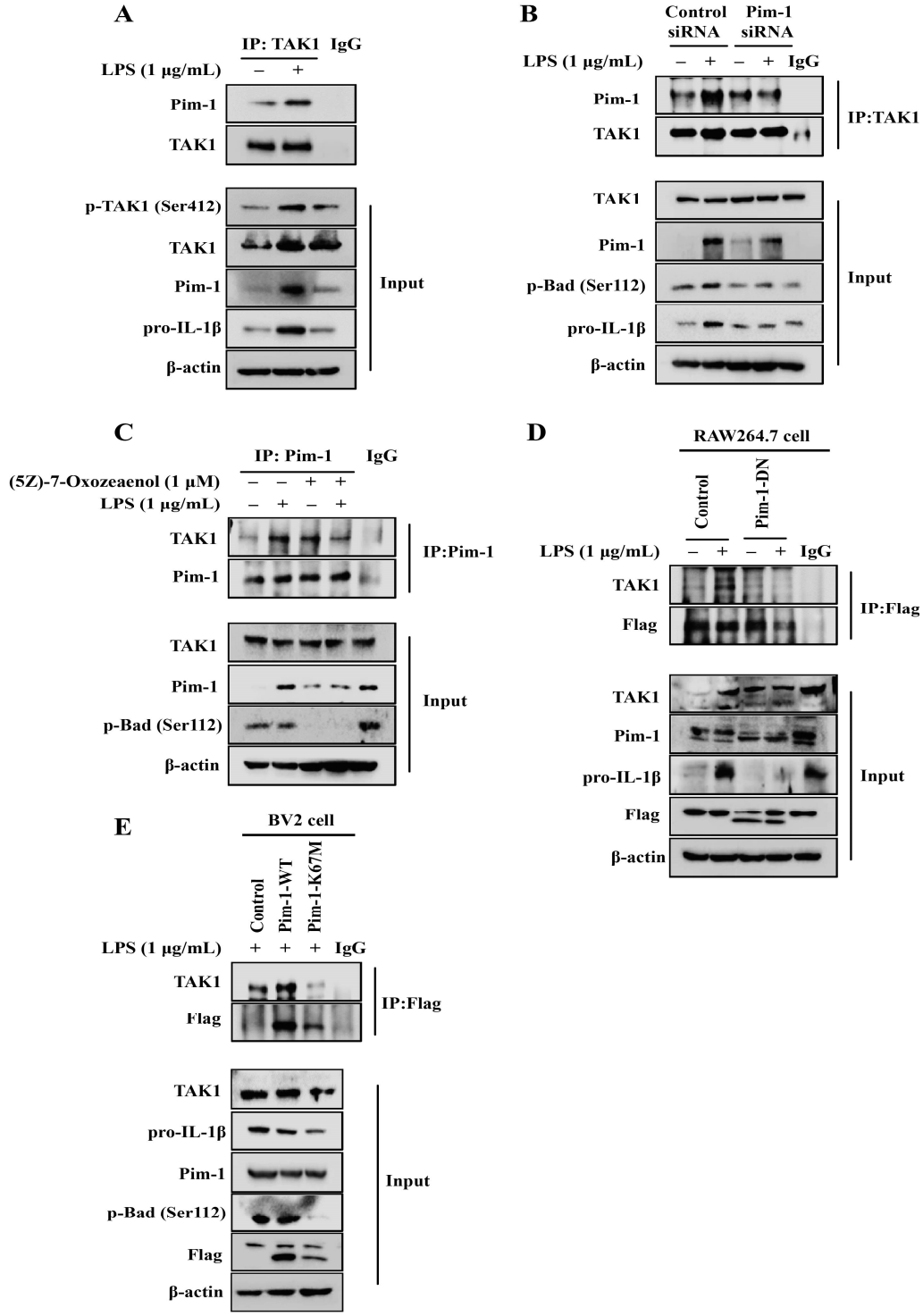

Figure 8. Interaction of Pim-1 with TAK1 protein in LPS-induced THP-1 cells. (A) THP-1 cells were stimulated with LPS (1 μg/mL) for 6 h, and cell lysates were subjected to immunoprecipitation with TAK1, then the protein expression levels of Pim-1 and TAK1 were detected by Western blotting. (B) Cells were transfected with control siRNA or Pim-1 siRNA, and Whole cell lysates were subjected to immunoprecipitation with TAK1, then the protein expression levels of Pim-1 and TAK1 were detected by Western blotting. (C) Cells were pre-treatment with 1 μM of TAK1 inhibitor (5Z)-7-Oxozeaenol for 1 h before LPS (1 μg/mL) stimulation. Whole cell lysates were subjected to immunoprecipitation with Pim-1, then the protein expression levels of TAK1 and Pim-1 were detected by Western blotting. (D,E) Raw264.7 and BV2 cells were transiently transfected with plasmids expressing pMX-IRES-EGFP-Flag empty vector (control) or pMX-Pim-1 DN, pMX-pim-1 WT, and pMX-Pim-1 K67M (mutant forms). The cell lysates were subjected to immunoprecipitation with anti-Flag. then the protein expression levels of TAK1 and Flag were detected by Western blotting.

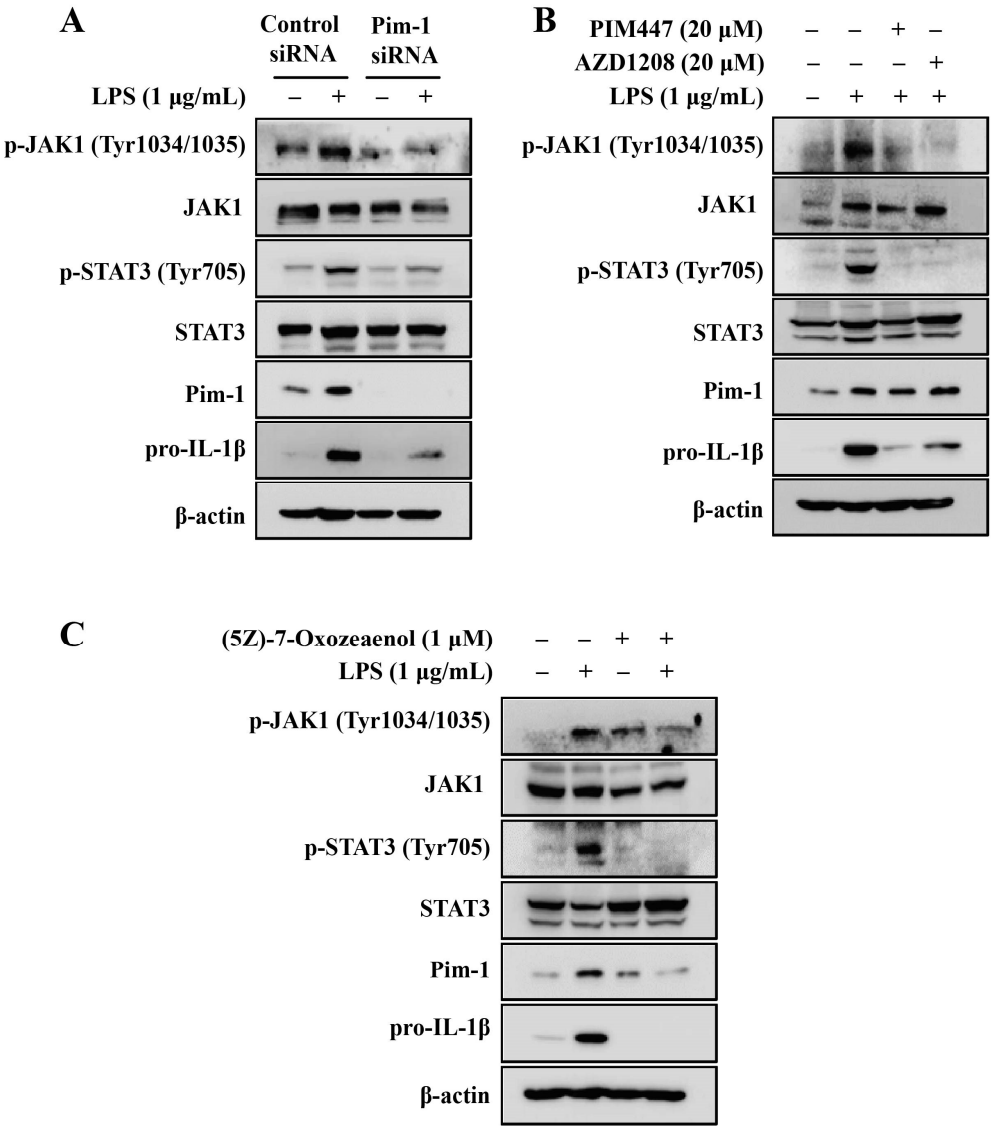

Figure 9. The effect of Pim-1 knockdown in LPS-induced JAK/STAT pathway in THP-1 cells. (A-C) Cells were transfected with control siRNA or Pim-1 siRNA, and pre-treatment with TAK1 inhibitor (5Z)-7-Oxozeaenol, (1 μM), or with PIM447 (20 μM) and AZD1208 (20 μM) for 1 h before LPS (1 μg/mL) stimulation. Whole cell lysates were isolated and used to

measure the protein expression levels of p-JAK1, JAK1, p-STAT3, STAT3, and pro-IL-1β by Western blotting.

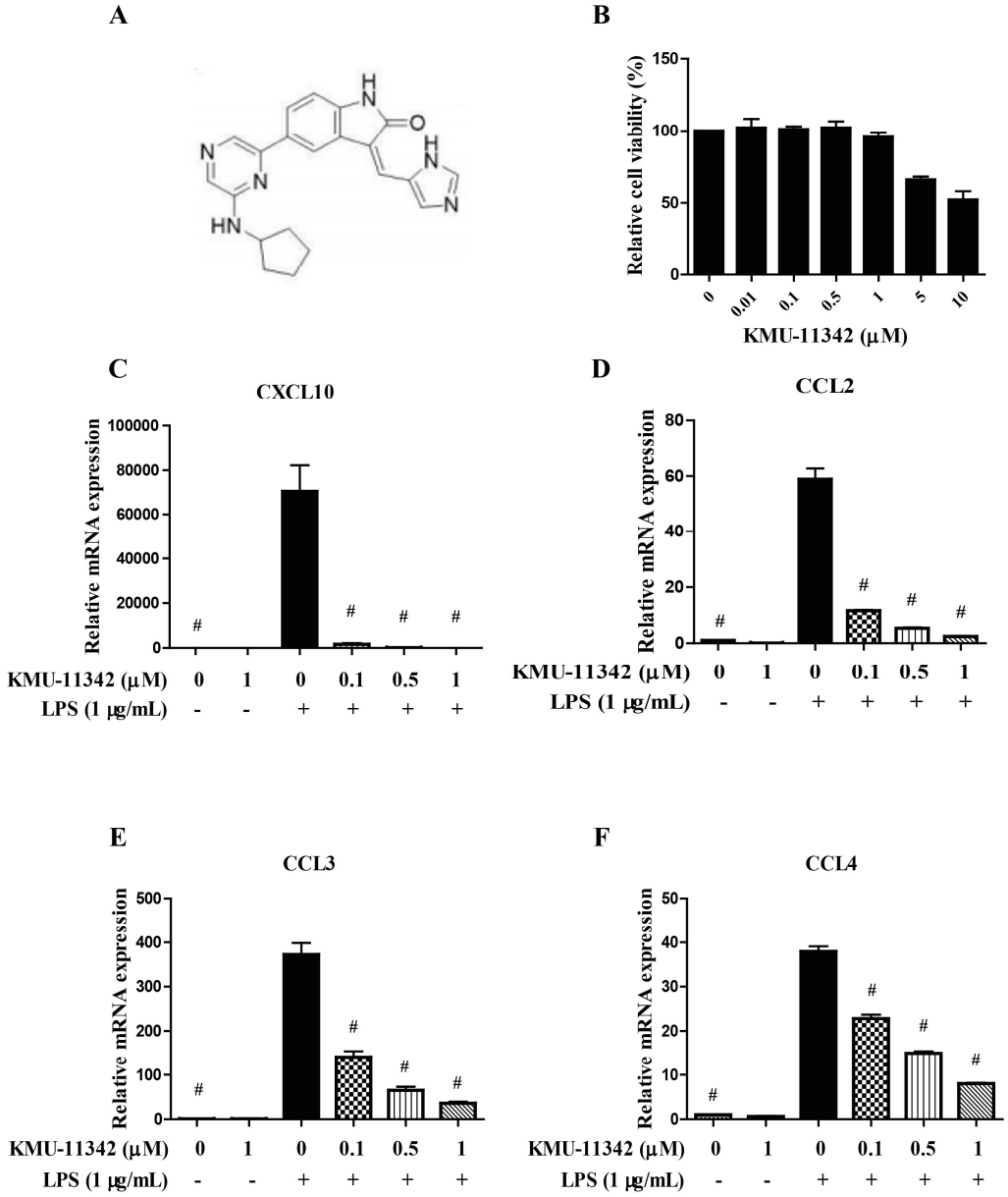

Figure 10. Chemical structure of KMU-11342 and its suppression of chemokines expression in human RA-FLS. (A) Chemical structure of KMU-11342. (B) Cytotoxic effect of KMU-11342 in THP-1 cells was performed by XTT assay. Cell were

differentiated into macrophages for 24 h using PMA (100 nM), then the cells were treated with different doses of KMU-11342 (0.01, 0.1, 0.5, 1, 5, and 10 μM) for 24 h. (C-F) RA-FLS Cells were treated with LPS (1 μg/mL) for 6 h after pre-treatment with different doses of KMU-11342 (0.1, 0.5, and 1 μM) for 1 h. Total RNA was extracted, and used to evaluate the mRNA expression levels of CXCL10, CCL2, CCL3, and CCL4, respectively. # $p < 0.001$ compared with the LPS only group.

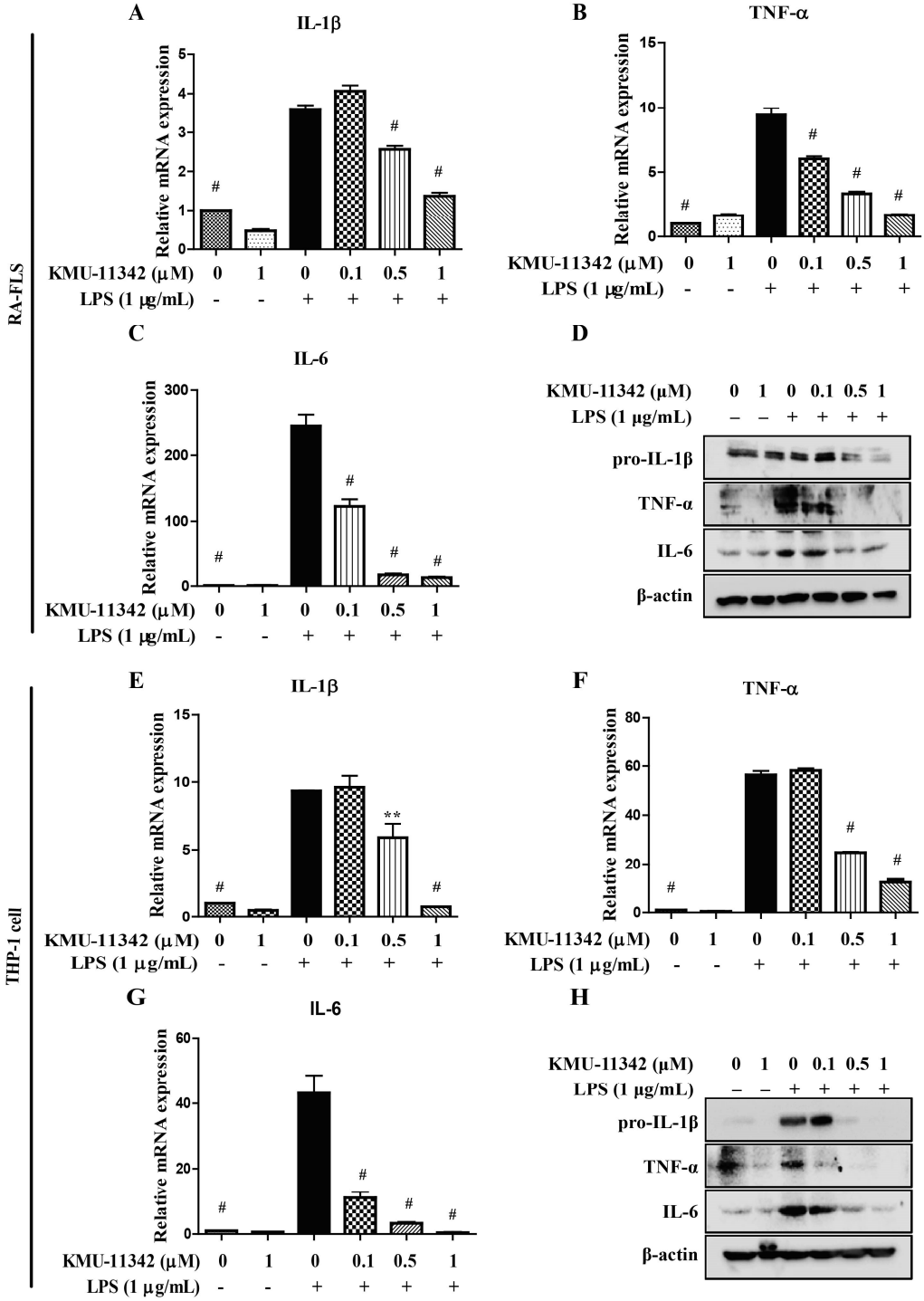

Figure 11. Inhibitory efeect of KMU-11342 on LPS-induced up-regulation of IL-1β, TNF-α, and IL-6 in RA-FLS and THP-1 cells. RA-FLS and THP-1 cells were pre-treatment with the indicated concentrations of KMU-1134 (0.1, 0.5, and 1 μM) for 1 h and stimulated with LPS (1 μg/mL) for 6 h. (A-C) In RA-FLS; total RNA was extracted, and used to evaluate the mRNA expression levels of IL-1β, TNF-α and IL-6, respectively # $p < 0.001$ compared with LPS alone treated group. (D) Whole cell lysates were isolated and used to measure the protein expression levels of IL-1β, TNF-α and IL-6 by Western blotting. (E-G) In THP-1 cells; Total RNA was extracted, and used to evaluate the mRNA expression levels of IL-1β, TNF-α and IL-6, respectively ** $p < 0.01$, # $p < 0.001$ compared with LPS alone treated group. (H) Whole cell lysates were isolated and used to measure the protein expression levels of IL-1β, TNF-α and IL-6 by Western blotting.

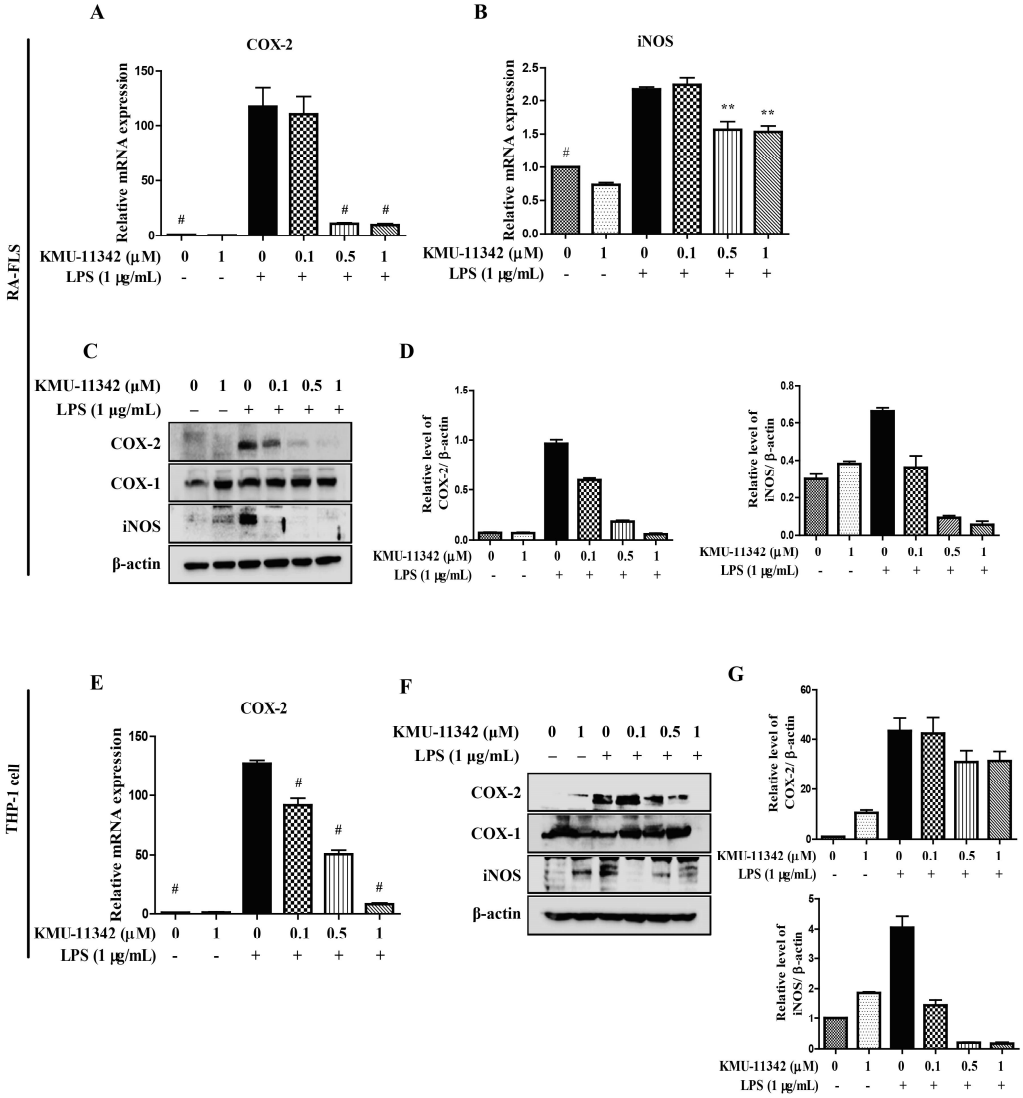

Figure 12. Inhibitory effect of KMU-11342 on LPS-induced up-regulation of COX-2 and iNOS expression in RA-FLS and THP-1 cells. (A) In RA-FLS; total RNA was extracted, and used to evaluate the mRNA expression levels of COX-2 and iNOS, respectively ** $p<0.01$, # $p < 0.001$ compared with LPS alone treated group. (C) Whole cell lysates were isolated

and used to measure the protein expression levels of COX-1, COX-2 and iNOS by Western blotting. (D) Image-J software was used to analyze the relative optical density of the COX-2 and iNOS band in the Lysate. (E) In THP-1 cells; total RNA was extracted, and used to evaluate the mRNA expression levels of COX-2, respectively. # $p < 0.001$ compared with LPS alone treated group. (F) Whole cell lysates were isolated and used to measure the protein expression levels of COX-1, COX-2 and iNOS by Western blotting. (G) Image-J software was used to analyze the relative optical density of the COX-2 and iNOS band in the Lysate.

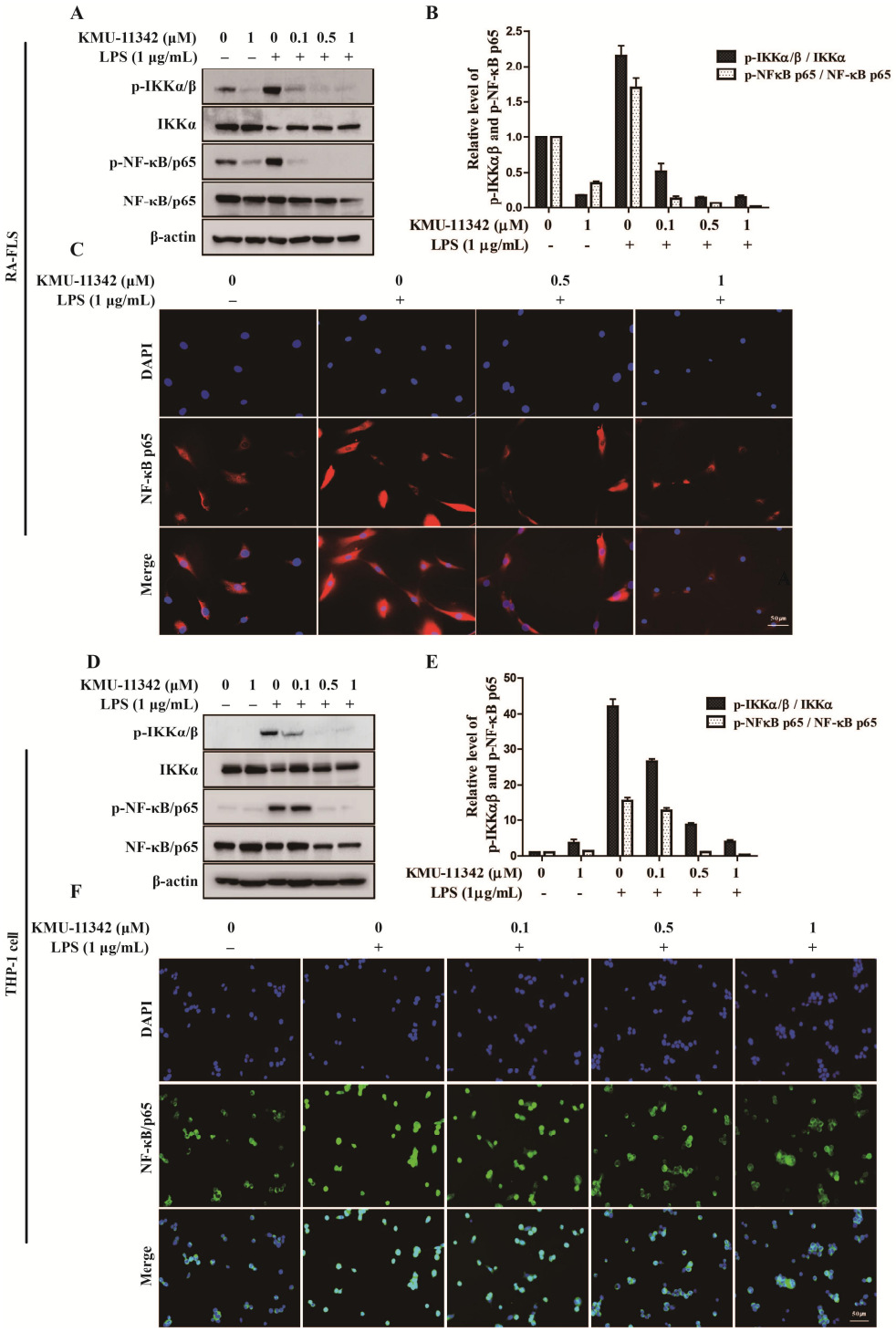

Figure 13. Inhibitory effect of KMU-11342 on activation of NF-κB signals in RA-FLS and THP-1 cells. RA-FLS and THP-1 cells pre-treatment with KMU-11342 for 1 h and stimulated with LPS (1 μg/mL) for 6 h. (A) In RA-FLS; whole cell lysates were isolated and used to measure the protein expression levels of p-IKKα/β, IKKα, p-NF-κB p65 and NF-κB p65 by Western blotting. (B) Image-J software was used to analyze the relative optical density of the p-IKKα/β, IKKα, p-NF-κB p65 and NF-κB p65 band in the Lysate (C) Cells were stained with antibodies to NF-κB p65 (red) and DAPI (blue) and captured at ×200 using fluorescence microscope. (scale bar = 50 μm). (D) In THP-1 cells; whole cell lysates were isolated and used to measure the protein expression levels of p-IKKα/β, IKKα, p-NF-κB p65 and NF-κB p65 by Western blotting. (E) Image-J software was used to analyze the relative optical density of the p-IKKα/β, IKKα, p-NF-κB p65 and NF-κB p65 band in the Lysate (F) Cells were stained with antibodies to NF-κB p65 (green) and DAPI (blue) and captured at ×200 using fluorescence microscope. (scale bar = 50 μm).

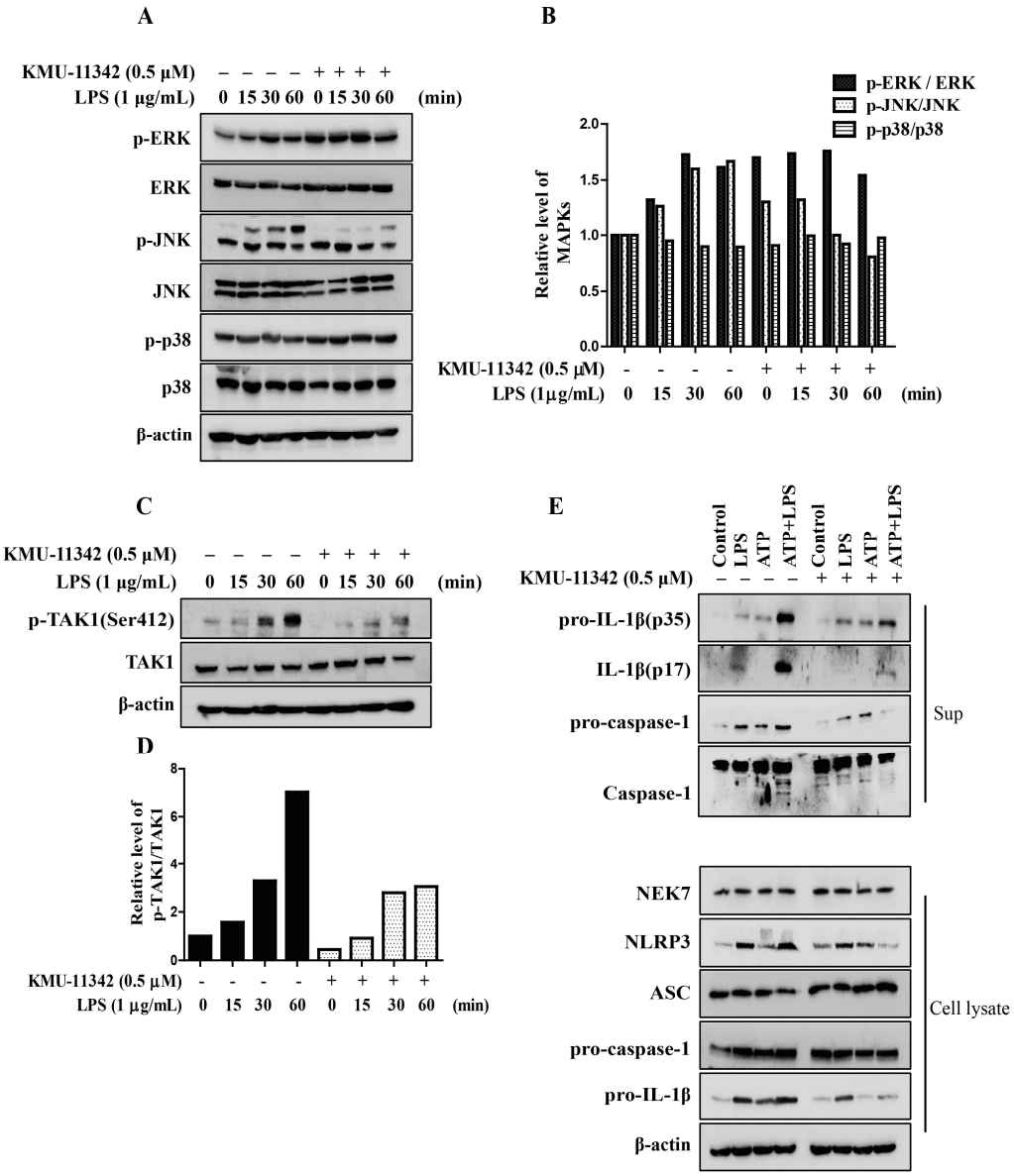

Figure 14. Inhibitory effect of KMU-11342 on LPS-induced activation of TAK1, and MAPKs and NLRP3 inflammasome in THP-1 cells. (A) Cells were stimulated with LPS (1 μg/mL) for the indicated time points. Whole cell lysates were isolated and used to measure the protein expression levels of p-ERK,

ERK, p-JNK, JNK, p-p38, and p38 by Western blot analysis. (B) Image-J software was used to analyze the relative optical density of the p-ERK, ERK, p-JNK, JNK, p-p38, p38, and β-actin band in the Lysate. (C) Cells were stimulated with LPS (1 μg/mL) for the indicated time points. Whole cell lysates were isolated and used to measure the protein expression levels of p-TAK1 and TAK1 by Western blot analysis. (D) Image-J software was used to analyze the relative optical density of the p-TAK1 and TAK1 band in the Lysate. (E) Cells were stimulated with LPS (1 μg/mL) and/or ATP (1 mM) for 6 h. Cell lysate (Lysate) and media supernatant (Sup) were isolated and used to measure the protein expression levels of pro-IL-1β, IL-1β, pro-caspase-1, and caspase-1 for the Sup as well as NLRP3, ASC, pro-caspase-1, and pro-IL-1β for the Lysate by Western blot analysis.

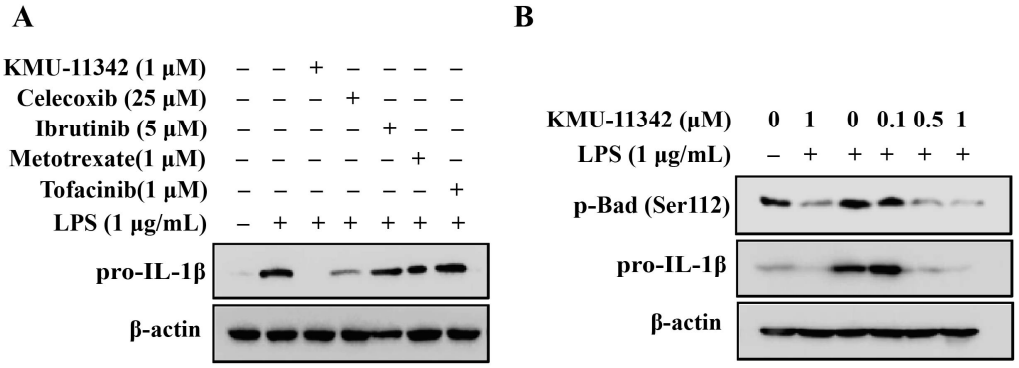

Figure 15. Anti-inflammatory potentiality of KMU-11342 in THP-1 cells. (A) Cells were differentiated into macrophages for 24 h using PMA (100 nM), and treated with LPS (1 μg/mL) for 6 h after pre-treatment with KMU-11342 (1 μM), celecoxib (25 μM), ibrutinib (5 μM), methotrexate (1 μM) and tofacinib (1 μM) for 1 h. Whole cell lysates were isolated and used to measure the protein expression levels of pro-IL-1β by Western blot analysis. (B) Cells were pre-treatment with KMU-11342 (0, 0.1, 0.5, and 1 μM) for 1 h and stimulated with LPS (1 μg/mL) for 6 h. Whole cell lysates were isolated and used to measure the protein expression levels of pro-Bad and pro-IL-1β by Western blot analysis.

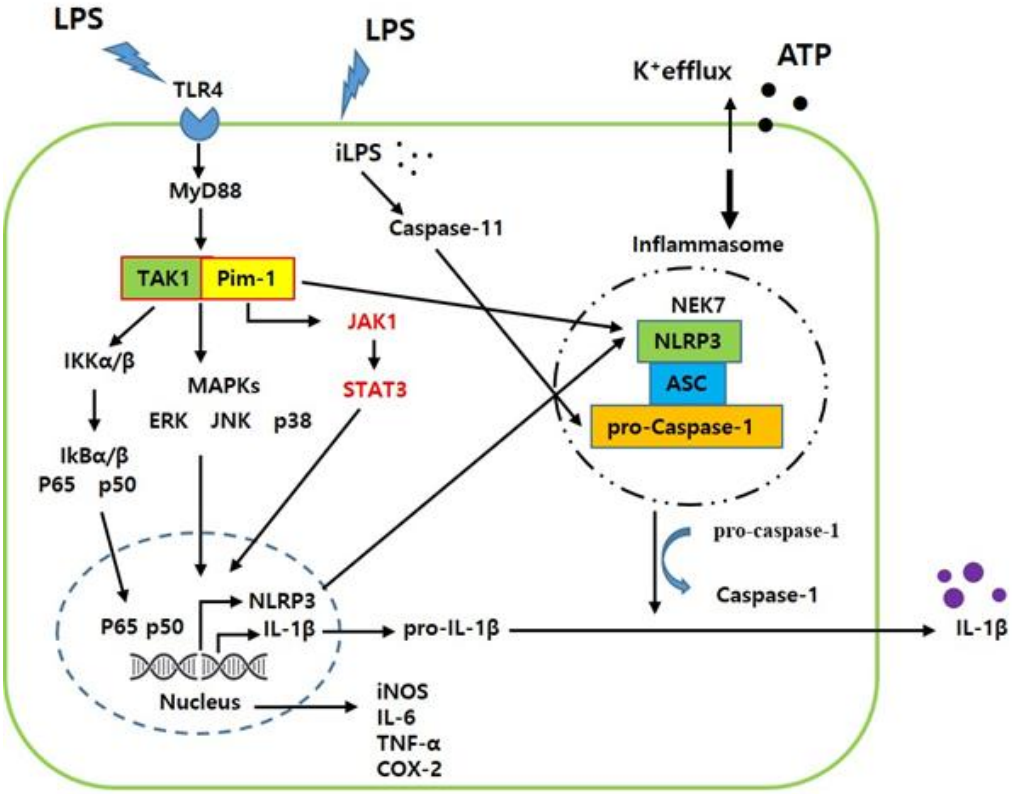

Figure 16. Scheme of Pim-1 regulation in LPS-induced inflammatory signal pathway. Pim-1 is activated by interaction with TAK1 via TLR4 and is involved in activation signal models for classical NLRP3 inflammatory activation by either TLR4 or caspase-11. In response to LPS stimulation, Pim-1 interacts with TAK1 and results in its phosphorylation. Pim-1 regulates downstream NLRP3 and pro-IL-1β expression through NF-κB activation. Abbreviations: LPS, lipopolysaccharide; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; TAK1, transforming growth factor-β-activated kinase 1; IKK, inhibitor NF-κB kinase; MAPKs, mitogen-activated protein kinases; NF-κB,

nuclear factor-κB; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2 TNF-α, tumor necrosis factor-α; L-1β, interleukin-1β; NLRP3, NOD-like receptor family, pyrin domain containing 3; ASC, apoptosis-associated speck-like protein containing a CARD.

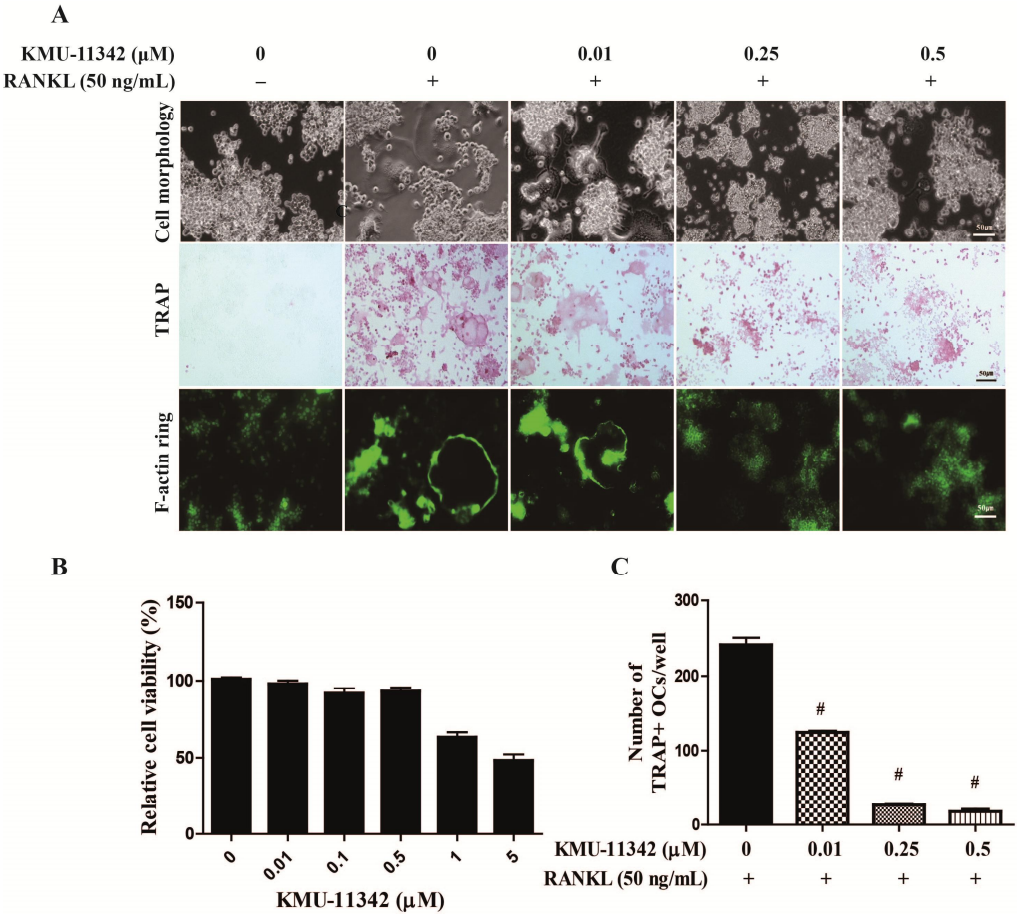

Figure 17. Inhibitory effect of KMU-11342 on RANKL-induced osteoclast differentiation in RAW264.7 cells. (A) Cells were pre-treatment with various concentrations of KMU-11342 (0.01, 0.25, and 0.5 μM) for 1 h, and made to undergo RANKL (50 ng/mL)-induced differentiation for 5 days. Each microscopic photograph depicts osteoclast differentiation cell morphology, stained with TRAP-stained osteclasts, and stained with rhodamine palloidin to show F-actin ring formation. (B) Cytotoxic effect of KMU-11342 in RAW264.7 cells was performed by XTT assay. Cells were treated with

different doses of KMU-11342 (0.01, 0.1, 0.5, 1, and 5 μM) for 24 h. (C) The number of TRAP-positive multinuclear cells were counted. # $p < 0.001$ compared with RANKL only treated group.

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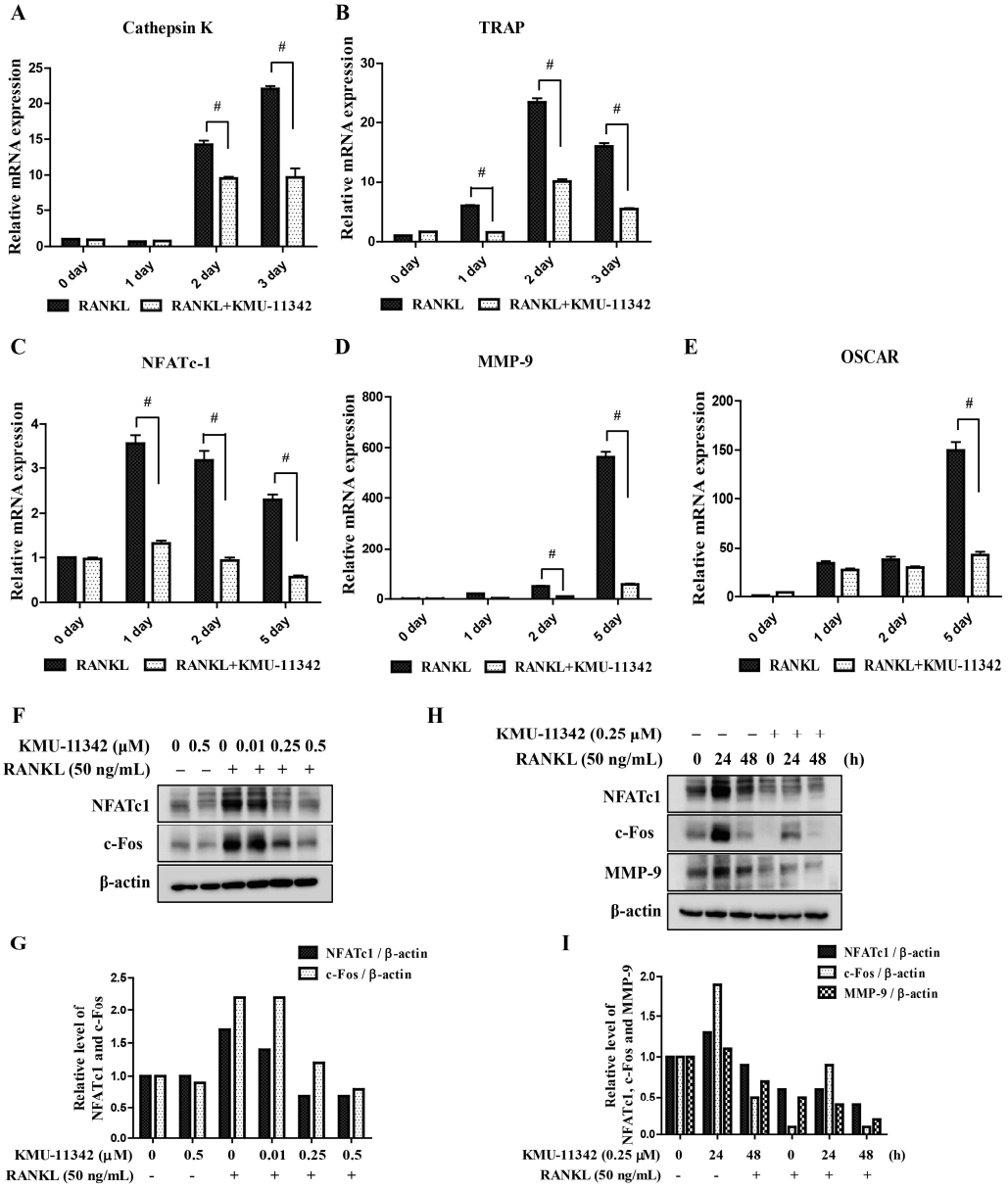

Figure 18. Inhibitory effect of KMU-11342 on osteoclastogenesis factors in RANKL-induced osteoclast differentiation of RAW264.7 cell. (A-E) RAW264.7 cells were treated KMU-11342 (0.25 μM) for 1 h and then stimulated with RANKL (50 ng/mL) for 1

to 5 days. Total RNA was extracted, and used to evaluate the mRNA expression levels of cathepsin K, TARP, NFATc-1, MMP-9, and OSCAR, respectively. # $p < 0.001$ compared with RANKL only treated group. (F) Cells were treated various concentration of KMU-11342 (0, 0.01, 0.25, and 0.5 μM) for 1 h and stimulated with RANKL (50 ng/mL) for 6 h. Whole cell lysates were isolated and used to measure the protein expression levels of NFAT-c1 and c-Fos by Western blot analysis. (G) Image-J software was used to analyze the relative optical density of the NFAT-c1 and c-Fos band in the Lysate. (H) Cells were treated various concentration of KMU-11342 (0.25 μM) for 1 h and stimulated with RANKL (50 ng/mL) for 24 to 48 h. Whole cell lysates were isolated and used to measure the protein expression levels of NFAT-c1, c-Fos, and MMP-9 by Western blot analysis. (I) Image-J software was used to analyze the relative optical density of the NFAT-c1, c-Fos, and MMP-9 band in the Lysate.

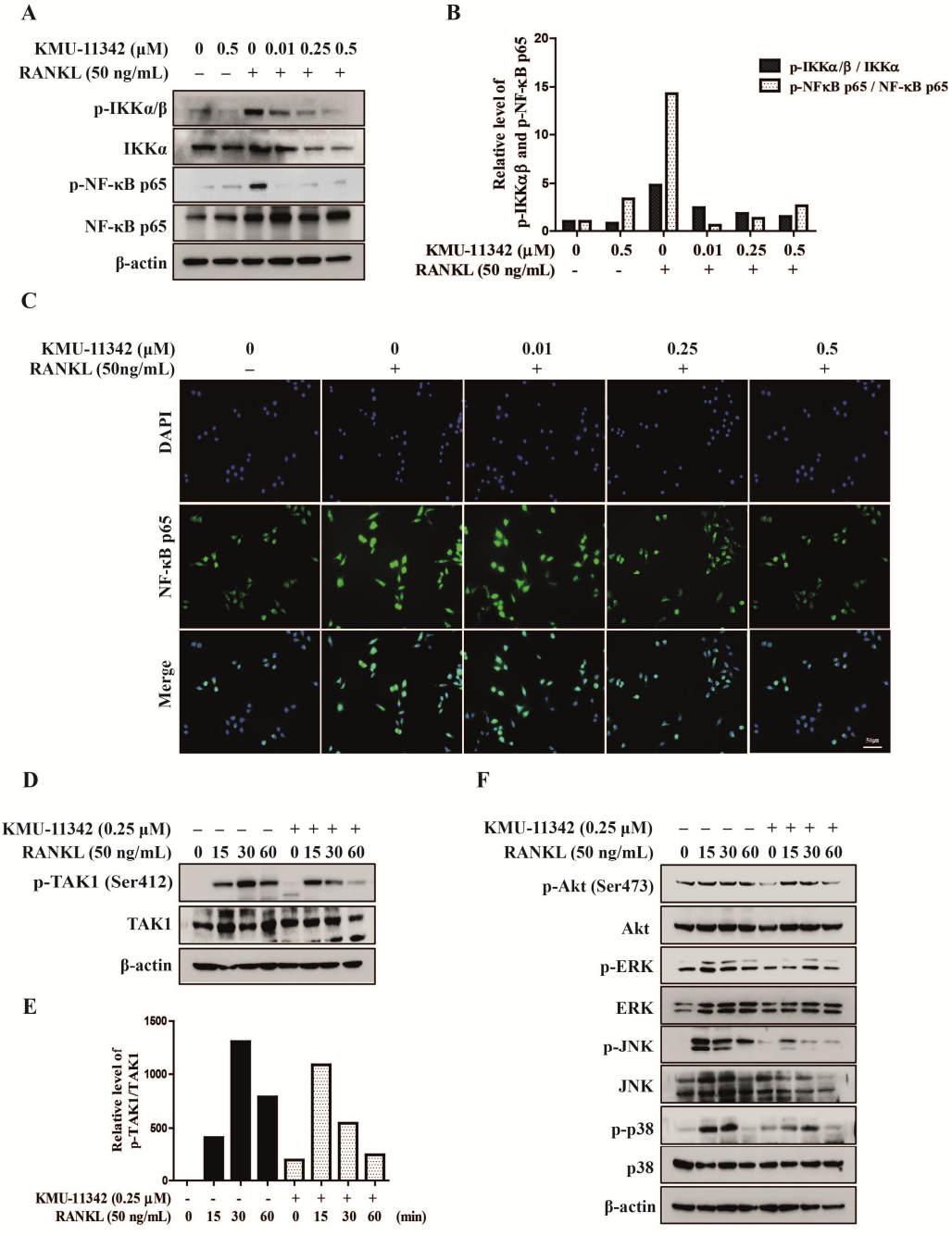

Figure 19. Inhibitory effect of KMU-11342 on RANKL-induced activation of NF-κB, TAK1, and MAPKs in RAW264.7 cell. translocation

and TAK1/MAPKs phosphorylations in RANKL-induced osteoclast differentiation in RAW264.7 cell. (A) Whole cell lysates were isolated and used to measure the protein expression levels of p-IKKα/β, IKKα, p-NF-κB p65 and NF-κB p65 by Western blotting. (B) Image-J software was used to analyze the relative optical density of the p-IKKα/β, IKKα, p-NF-κB p65 and NF-κB p65 band in the Lysate (C) Cells were stained with antibodies to the NF-κB p65 (green) and DAPI (blue) and captured at ×200 using fluorescence microscope. (scale bar = 50 μm). (D) Whole cell lysates were isolated and used to measure the protein expression levels of p-TAK1 and TAK1 by Western blotting. (E) Image-J software was used to analyze the relative optical density of the p-TAK1 and TAK1 band in the Lysate (F) Cells were stimulated with RANKL (50 ng/mL) for the indicated time points. Whole cell lysates were isolated and used to measure the protein expression levels of p-Akt, Akt, p-ERK, ERK, p-JNK, JNK, p-p38, and p38 by Western blot analysis.

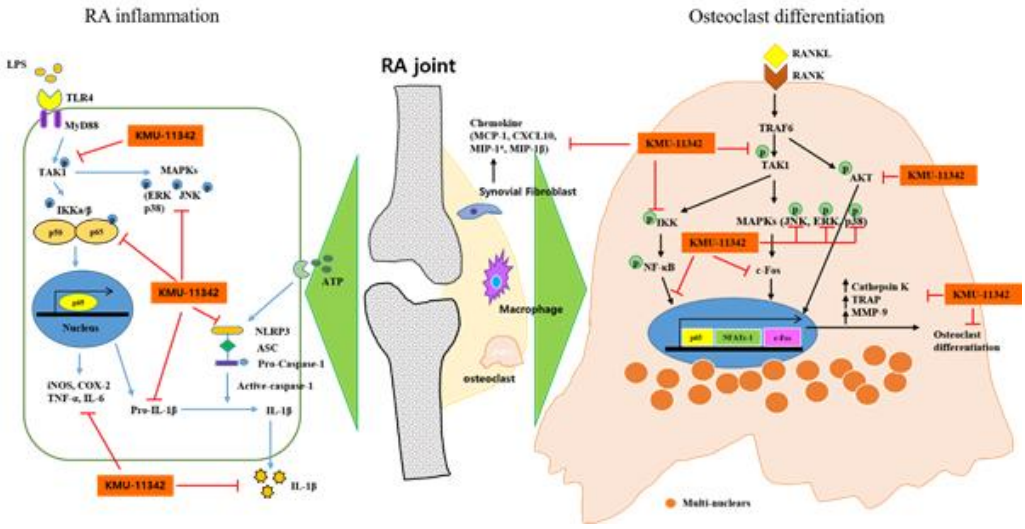

Figure 20. Mechanistic scheme summarizing the anti-inflammatory effects of KMU-11342. LPS, lipopolysaccharide; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; TAK1, transforming growth factor-β-activate kinase 1; IKK, inhibitor NF-κB kinase; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; NLRP3, NOD-like receptor family, pyrin domain containing 3; ASC, apoptosis-associated speck-like protein containing a CARD. Receptor activator of nuclear factor kappa-B ligand; RANKL, receptor activator of nuclear factor κ B; RANK, tartrate-resistant acid phosphatase; TRAP, matrix metalloproteinase-9; MMP-9,

4. Discussion

Pim-1 kinase is a serine/threonine kinase a proto-oncogene encoding (29). Pim-1 gene encodes two proteins, 34 kD and 44 kD, initiated by AUG and CUG codons, respectively (30). The Pim-1 gene is located in chromosome 17 in humans and chromosome 6 in mice (8). Pim-1 has mainly been studied in the field of cancer biology, the contexts of cell cycle regulation, survival, cell growth, and senescence. For example, Pim-1 plays a role in cell cycle progression by binding and phosphorylating the phosphatase Cdc25A, a positive G1-specific cell cycle regulator (31). Pim-1 is involved in cell survival as it inactivates Bad by through phosphorylation of pro-apoptotic Bad at Ser112 (26). In addition, inhibition of Pim-1 stimulates phosphorylation and activates the mTORC1 regulator adenosin monophosphate-dependent protein kinase (AMPK) to block rapamycin-sensitive mammalian targets of mTORC1. Therefore Pim-1 is involved in metabolism and cell growth (32). Moreover, Pim-1 is also involved in cell sensations; Pim-1 binds directly to the chromoshadow domain of heterochromatin protein (HP)1α and phosphorylates HP1 to alter the structure or noise of chromatin (33). Recently, more studies focusing on the Pim-1 functions associated with inflammation are being carried out. Recent reports revealed that inhibition of Pim-1 in LPS-induced acute lung injury (ALI) reduced cytokine and p65 upregulation (34). It is also known that Pim-1 regulates activities of transcription factors such as NFATc1 and NF-κB (35-37). NFATc1 and NF-κB play an important role in inflammatory responses, and the former plays an important role in regulating Th1, and Th2 patterns in cytokine production (38,39). Moreover, Pim-1 was

up-regulated in RA synovium and RA-FLS, and an inhibitor of Pim kinase significantly decreased the proliferation, migration, and MMP secretion in RA-FLS in vitro (40). These findings suggest that Pim-1 kinase could be a potential a therapeutic target for the regulation of inflammation. Therefore, investigating the mechanisms and functions of Pim kinase for controlling inflammation is likely to facilitate improvement in prognosis of patients with inflammatory diseases. However, mechanism by which Pim-1 regulates LPS-induced inflammation and its association with NLRP3 inflammasome is unclear.

In the present study, the potential of Pim-1 as a target for inflammation regulation in THP-1 cells was evaluated, and the mechanism of suppression of inflammation and osteoclastogenesis by a potent Pim kinase inhibitor was investigated.

First, the potential of Pim kinase as a target for regulating inflammation was confirmed using the Pim kinase inhibitors, Pim447 and AZD1208. As shown in Figure 1, these Pim kinase inhibitors suppressed LPS-induced activation of NLRP3 inflammasome in THP-1 cells. Further, they suppressed LPS-induced up-regulation of pro-inflammatory cytokines, IL-6, and TNF-α phosphorylation of NF-κB p65 in THP-1 cells. These results demonstrated that Pim-1 plays an important role in LPS-mediated inflammatory signaling pathway. A cytokine is a small secretory protein produced to regulate and influence immune responses and is an important indicator of inflammatory responses (41). Among the various cytokines, IL-1β, IL-6, and TNF-α are known as the representative pro-inflammatory cytokines involved in the LPS-mediated inflammatory signaling pathway (42). These pro-inflammatory cytokiness are mainly produced by activated phagocytes cells and are responsible for inflammatory responses (43). In

this study, the expression of LPS-induced pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α was confirmed. As shown in Figure 2, LPS increased Pim-1, IL-1β, IL-6, and TNF-α expression along with phosphorylation of Bad at Ser112 in THP-1 cells. This suggests that Pim-1 plays an important role in the LPS-mediated inflammatory signaling mechanism. Pim-1 siRNA was used to investigate the mechanisms underlying these effects of Pim-1, which is most prominently related to inflammation among the Pim kinases.

LPS induction and activation of signal transmission pathways in macrophage cells induce transcriptional activation of iNOS and COX-2 as inflammation-mediated promoter enzyme factors (44). Pim-1 knockdown by siRNA reduced mRNA and protein expression iNOS and COX-2. Therefore, it was confirmed that Pim-1 regulates LPS-mediated inflammation involving iNOS and COX-2. Next, I confirmed whether Pim-1 regulates NF-κB in the LPS-mediated inflammatory signaling pathway. The transcription factor NF-κB regulates genes related to inflammatory responses at various levels (45) and induces anti-inflammatory genes regulating cytokines, chemokines and adhesive molecules essential for innate and adaptive immune responses (46). An IKK complex comprising of the subunits IKKα, IKKβ and IKKɣ regulates NF-κB-suppressing IκB activity by and serves as a hub for NF-κB activation (47). As shown in Figure 5, Pim-1 siRNA suppressed p-IKKα/β and p-NF-κB p65. In addition, Pim-1 siRNA inhibited the translocation of NF-κB p65 from the cytoplasm to the nucleus in LPS-treated THP-1 cells. Moreover, MAPK plays an important role in the production and downstream signaling of pro-inflammatory cytokiness (48). MAPK transmits external signals to the nucleus to induce the expression of gene regulatory factors, such as NF-κB (49). Pim-1

siRNA inhibited the phosphorylation of ERK, JNK and p38 in the MAPKs pathway. As a result, Pim-1 regulates LPS-mediated inflammation in the upstream NF-κB p65 and MAPKs pathways.

It has been reported that phosphorylation of RelA/p65 at Ser276 is activated when Pim-1 is stimulated by TNF-α (37). Interestingly, in this study, the phosphorylation of NF-κB p65 at Ser536 was activated by LPS stimulation. The Ser536 of the p65 subunit responds to inflammatory stimuli and exhibits the most potent phosphorylation sites, which are highly conserved in other species, and potentially plays a role in NF-κB activity regulation (46). Unlike the phosphorylation of Ser276 of p65, which is an essential contributor to NF-κB activation both endogenous and externally, the functional contribution of Ser536 to IKK-mediated phosphorylation remains unknown (50). Therefore, further study is warranted.

Next, I identified the effects of Pim-1 on the activity of inflammasomes, a complex of inflammatory molecules, during the LPS-mediated inflammatory signaling pathway. The activation of NLRP3 inflammasome, have been extensively studied in the research field of maturation and secretion of IL-1β, and is involved in initial responses to inflammation (51). NLRP3 inflammasome causes proteolytic cleavage of pro-caspase-1 into active caspase-1, which converts the cytokine precursor pro-IL-1β into mature and biologically active IL-1β (52). In the present study, Pim-1 downregulation suppressed NLRP3, pro-IL-1β, caspase-1 activation, and caspase-1 activation dependent-secretion of IL-1β. Caspase-11 is independent of the traditional LPS receptor TLR4, and indirectly activates NLRP3 inflammasomes through intracellular LPS and bacteria (53). However, Pim-1 downregulation does not affect the activation of caspase-11, non-traditional inflammatory complex

(Figure 6).

In this study, Pim-1 downregulation did not alter TLR4 expression levels and adapter protein MyD88 in LPS-mediated inflammatory signaling pathway. TAK1 acts as a signaling medium as a major factor promoting inflammatory pathways, triggering LPS-mediated TLR4 to initiate downstream signaling cascades via TAK1 (54).

TAK1 was originally identified as a mitogen-activated protein kinase (MAP3K) that is activated by the transforming growth factor-β (TGF-β), but was later characterized as a major regulator of inflammation and immune signals mediated by cytokines, TLR, and T and B cell receptors (55,56). Additionally, TAK1 activates the IκB kinase complex, and phosphorylates NF-κB to activate the NF-κB pathway (57). In this study, the interrelation between TAK1 and Pim-1 in the LPS-mediated inflammatory signal pathway was comfirmed. As shown in Figure 7 , Pim-1 downregulation suppressed TAK1 phosphorylation and the TAK1 inhibitor, (5Z)-7-Oxozeaenol decreased Pim-1 expression levels in the LPS-mediated inflammatory signaling pathway. These results demonstrated that TAK1 and Pim-1 influence each other in this pathway. Pim-1 kinase has been shown to interact with numerous proteins participating in various signaling pathways (8,58). The kinase domain of Pim-1 is important for the interactions of TAK1 and Pim-1 bonds in the overexpressed states. (36). In the present study, endogenous Pim-1 interacted with TAK1 in control cells (Figure 8). Moreover, LPS treatment further increased the binding of Pim-1 and TAK1. When the kinase domain was inactivated using the mutant forms, Pim-1 DN or Pim-K67M, the binding of Pim-1 and TAK1 was inhibited. These result suggested that kinase domain of Pim-1 plays an important role in interactions with TAK-1.

Finally, I identified the effects of Pim-1 on the JAK/STAT pathway, known as the inflammatory signaling pathway. The JAK/STAT pathway is induced by various cytokines, interferons, growth factors and related molecules such as growth hormones, and leptin, and is a signaling mechanism that allows extracellular factors to control gene expression (22). Pim-1 transcription can be activated by ILs, which is then transferred to the nucleus through two groups of proteins called JAK/STAT (29). In addition, phosphorylation of STAT3 also induces Pim-1 expression in patients with pulmonary hypertension in humans (59). However, previous studies have reported that Pim-1 down-regulates the JAK/STAT pathway (60). In this study, Pim-1 downregulation inhibited LPS-induced phosphorylation of JAK1 and STAT3 in THP-1 cells. These result suggested that Pim-1 is closely related to the JAK/STAT pathway to regulate its expression and function.

Taken together, Pim-1 induces generation of pro-IL-1β via NF-κB activation through the TLR4 signaling, which is the priming stage, in the LPS-induced inflammatory phase. Additionally, Pim-1 seems to induce inflammation by activating NLRP3 inflammasome, the second activating stage signal, to secrete mature IL-1β and caspase-1. Further, the interaction of Pim-1 with TAK1 appears to play crucial role in the LPS-mediated inflammatory signaling pathway.

Next, using KMU-11342, which has a novel PIM kinase inhibitory effect, its potential as treatment agent for rheumatoid arthritis(RA), and its anti-inflammatory mechanism was confirmed. RA is a systemic inflammatory joint disease, and its pathophysiology includes RA-Fibroblast-like-synoviocytes (FLS) activation, osteoclastogenesis, and inflammation of synovial membrane (61). FLS play a crucial role in producing cytokines that perpetuate inflammation and proteolytic

enzymes that contribute to cartilage destruction (62). The TLRs are expressed by cells within the RA joints and various endogenous TLR ligands are present within the joint of patients with RA (63). Therefore, in this study, RA-FLS was used to determine whether LPS-mediated inflammatory mechanisms were inhibited. Chemokines regulate the immune system, act as a major medium for inflammatory reactions, and are mainly induced by infection (64). Pro-inflammatory chemokines expressed in TLRs signaling play an important role in the pathology of RA and are an important medium derived from RA-FLS in response to cytokines and TLR ligands (62,63). In particular, I confirmed that the following chemokines CXCL10, CCL2, CCL3, and CCL4, are highly expressed in RA (65). In RA-FLS, KMU-11342 inhibited LPS/TLR-mediated up-regulation of chemokine (Figure 10). Cytokines promote autoimmuneity at each stage of a rheumatoid arthritis pathogenesis, maintain chronic inflammatory synovitis, and promot the destruction of adjacent joint tissues (66). Therfore, I tested important cytokines that mediate the initiation of inflammation and innate immune responses. In this study, KMU-11342 suppressed LPS-mediated up-regulation of IL-1 β, IL-6, and TNF-α, in both RA-FLS and THP-1 cells. These results suggest that KMU-11342 exhibits anti-inflammatory effects.

Many studies have revealed that the synovial tissues in patients with RA express COX-2 (67-69). Moreover, recent studies have demonstrated that specific reductive enzymes (COX-2 and iNOS), cytokines, and their products are involved in chronic inflammatory diseases (70-72). In this study, KMU-11342 inhibited LPS-mediated up-regulation of COX-2 and iNOS.

TLRs activate a downstream signaling system with the transcription factor NF-κB (73). The NF-κB pathway is one of the explainable

signaling pathways associated with arthritis is the nuclear factor-Kappa B pathway (74). NF-κB is a nuclear transcription factor that regulates the expression of various infectious mediators, including inductive enzymes such as COX-2, and iNOS, TNF-α, IL-1β, IL-6, chemokines and cytokines such as cell-attached molecules (75). KMU-11342 inhibited LPS-mediated IKKα/β phosphorylation, which activated NF-κB inhibitor IκBα in RA-FLS and THP-1 cells and prevents phosphorylation and nuclear translocation of NF-κB p65. These results suggested that KMU-11342 inhibits effect of NF-κB activity by inhibiting NF-κB p65 phosphorylation and its migration from cytoplasm to nucleus. Interestingly, KMU-11342 inhibited NF-κB p65 and IKK/α/β phosphorylation in RA-FLS cells that were not LPS-treated (Figure 13). These results suggest that KMU-113542 can be a therapeutic option for RA treatment.

Furthermore, MAPKs are associated with major regulators of pro-inflammatory cytokiness (e.g. IL-1, IL-12, IL-23, and TNF-α) production, and three of MAPKs(ERK, JNK, and p38) appear to be expressed in the synovial tissues of patients with RA or osteoarthritis (76). TAK1, or MAP3K7, a major signaling mediator involved in innate and acquired immune responses, has been shown to incorporate inflammatory signals mediated by cytokines, TLR, and T and B cell receptors (55,77). In this study, KMU-11342 inhibited phosphorylation of MAPKs and TAK1. This indicates that KMU-11342 is a candidate anti-inflammatory agent, that inhibits the major regulators of inflammatory responses.

NLRP3 inflammasome activation induces pro-caspase-1 activation, which induces maturation and secretetion of IL-1, an pro-inflammatory cytokines. (78). NLRP3 inflammasomes are known to be involved in the

pathogenesis of gout arthritis (79) and are expressed in synovial myeloid and endothelial cells in patients with RA (80). KMU-11342 also inhibited NLRP3 protein expression along with pro-IL-1β. It also prevented the release of mature IL-1β through the inhibition of the NLRP3 inflammasome signaling pathway.

Recently, numerous anti-inflammatory drugs that targets various inflammatory signaling pathways are being developed (81). For example, methotrexate has been an effective drug widely used as a treatment agent for RA since the 1980s. pro-inflammatory cytokiness blockings agents, such as TNF-α inhibitors (adalimumab, etanercept, certolizumab, infliximab, and golimumab), IL-1 antagonists (anakinra), and IL-6 inhibititors (toxylizumab) have also been used (82). Furthermore, tofacitinib is a small molecule inhibitor of JAK1, JAK3, and JAK2, and is the first kinase inhibitor used in the United States (83). However, long-term use of biological drugs for in RA treatment increases the relative risk of cancer and infection, and causes drug reactions and various other problems inherent with in drug refractive and chronic drug use. Due to these hudles, drug development for RA treatment is warranted. As shown in Figure 15A, KMU-11342 had superior anti-inflammatory efficiency compared to celecoxib, ibrutinib, metotrexate, and tofacinib. Although additional studies are required, these results indicates that it is a potential candidate drug for RA treatment.

RA, as mentioned earlier, features massive alluvial proliferation and minimal infiltration of inflammatory cells, followed by cartilage and bone destruction eventually if chronic inflammation persists (84). Bone homeostasis is maintained by balancing bone formation by osteoblasts and bone destruction by osteoclasts (85). However, if this balance is

disturbed, when the etiology persists, as in RA, many pro-inflammatory cytokiness and chemokines express essential factors for osteoclast differentiation, leading to osteoclast formation (86). Osteoclasts are usually generated from precursor cells of the monocyte-macrophage system. The binding between RANK and its ligand RANKL is essential for bone cell generation (87). After RANKL-RANK binding transcription factors necessary for osteoclast formation are expressed through NF-receptor-associated factor 6 (TRAF6), an intracellular signaling molecule. When NFATc1, an important transcriptional factor for osteoclastogenesis, is activated, it undergoes nuclear potential and activates TRAP, OSCAR, Cathepsin K, and NFATc1 itself (88). The osteoclasts are then organized into one large ring to distinguish the extracellular space from bone-absorbing spaces and they attach to bones (89). In addition, actin rings are dynamics, and are formed during bone reabsorption (90).

In this study, KMU-11342 inhibited multinucleated cell differentiation representing osteoclast differentiation, and inhibited F-actin rings formation. It also inhibited Cathepsin K, TRAP, NFATc-1, and OSCAR, which are specific indicators of osteoclast differentiation, and mRNA levels of MMP-9. In addition, c-Fos is a member of the Fos family that forms the activator protein-1 (AP)-1 family of heterogeneous dimer transcription factors with Jun protein (91). It serves as an essential factor for the differentiation of osteoblasts and macrophages (92). In this study, KMU-11342 inhibited c-Fos expression. MAPKs (ERK, JNK, and p38) signaling pathways are important for normal bone cell differentiation and activation. Stimulation of TLR and pro-inflammatory cytokiness in MAPK pathway regulate bone cell adhesion, migration, fusion, survival, and bone-like bone resorption (93).

TAK1 deficient monocytes did not form mature bone cells in response to RANKL, indicating that TAK1, in particular, is indispensable for RANKL-induced bone cell formation (94). NF-κB is a transcription factor with diversity that regulates the osteoclast formation, function, and survival (95). Studies have shown that the deletion of NF-κB p50 and p52 subunits causes osteoporosis due to the absence of osteoclasts (96,97), indicating that NF-κB is essential for differentiation into TRAP-positive osteoclasts in response to RANKL and other osteoclast-mediated cytokines (98). As shown in Figure 18 and 19, KMU-11342 inhibited NFATc-1 upregulation, and phosphorylation of ERK, JNK, and p38, and the phosphorylation and nuclear translocation of NF-κB.

In conclusion, I identified the anti-inflammatory effects of KMU-11342 via inhibition of TLR4/NF-κB/NLRP3 inflammasome signaling in LPS-treated RA-FLS and THP-1 cells. In addition, it was confirmed it also suppresses osteoclast differentiation. Therefore, KMU-11342 has the potential to be developed as a therapeutic drug for RA as it inhibits inflammation and osteoclastogenesis.

5. Summary

Pim kinase is a serine-threonine protein kinase that regulates an important role in cell proliferation, survival, and motor regulation. Pim-1 suggest that it can be as a therapeutic target in inflammatory regulation. However, the mechanisms underlying Pim-1 function, including Pim kinase in inflammation, remain insufficiently studied. In the present study, I investigated whether Pim-1 kinase plays an important role in the LPS-mediated inflammatory signaling pathway. In addition, I demonstrated the anti-inflammatory effects and mechanism of a novel Pim kinase inhibitor, KMU-11342 in THP-1, RA FLS, and osteoclastogenesis. Potential was identified as an inflammatory target of Pim phosphorylate using PIM447 and AZD1208, which are Pim kinase inhibitors. Pim kinase inhibitors inhibited LPS-induced upregulation of pro-inflammatory cytokine, TNF-α and IL-6 and phosphorylation of NF-κB p65 in THP-1 cells. Decomposition of Pim-1 into siRNA reduced mRNA expression and protein expression in both LPS-induced iNOS and COX-2. Pim-1 siRNA inhibited p-IKKα/β and p-NF-κB p65. In addition, Pim-1 siRNA inhibits the transfer of NF-κB p65 from the cytoplasm to the nucleus in LPS-treated THP-1 cells. Pim-1 siRNA inhibits phosphorylation of ERK, JNK, and p38 in the MAPKs pathway. Consequently, Pim-1 regulates LPS-mediated inflammation upstream of the NF-κB p65 and MAPKs pathways. Pim-1 siRNA inhibited NLRP3, pro-IL-1β, caspase-1 activation, and caspase-1 activation-dependent secretion of IL-1β. Pim-1 siRNA inhibited NLRP3, pro-IL-1β, Caspase-1 activation, and Caspase-1 activation-dependent secretion of IL-1β. Meaningfully, Pim-1 siRNA suppressed phosphorylation of TAK1 and

TAK1 inhibitor, (5Z)-7-Oxozeaenol inhibited expression level of pim-1 in LPS-mediated inflammatory signal pathway. In addition, kinase domain of Pim-1 play an important role in interaction with TAK1. Pim-1 siRNA inhibited LPS-induced phosphorylation of JAK1 and STAT3 in THP-1 cells. These result suggested that Pim-1 is closely related to the JAK/STAT pathway to regulate its expression and function. Next, using KMU-11342, which has a novel Pim kinase inhibitor effect, its potential as an RA therapeutic drug and its anti-inflammatory mechanism were confirmed. Taken together, I identified anti-inflammatory effect of KMU-11342 via inhibiting of TLR4/NF-κB/NLRP3 inflammasome signaling in LPS-treated RA-FLS and THP-1 cells. In addition, it was confirmed that the differentiation of osteoclasts was also suppressed by KMU-11342. In this study, KMU-11342 inhibited multinucleated cell differentiation representing osteoclast differentiation and showed an effect of inhibiting the formation of F-actin rings. Additionally, it was shown that KMU-11342 inhibited both Cathepsin K, TRAP, NFATc-1, c-Fos, OSCAR, which are specific indicators of osteoclast. KMU-11342, inhibition of RANKL induced TAK1/MAPKs/NF-κB signal pathway in osteoclast differentiation.

As a result of this study, KMU-11342 has the potential to be developed as a therapeutic drug for RA through inhibition of inflammation and osteoclastogenesis.

Reference

1. Mondello P, Cuzzocrea S, Mian M: Pim kinases in hematological malignancies: where are we now and where are we going? J Hematol Oncol 2014; 7: 95.

2. Mikkers H, Nawijn M, Allen J, Brouwers C, Verhoeven E, Jonkers J, et al.:. Mice deficient for all PIM kinases display reduced body size and impaired responses to hematopoietic growth factors. Mol Cell Biol 2004; 24: 6104-15.

3. Muraski JA, Rota M, Misao Y, Fransioli J, Cottage C, Gude N, et al.:. Pim-1 regulates cardiomyocyte survival downstream of Akt. Nat Med 2007; 13: 1467-75.

4. Katakami N, Kaneto H, Hao H, Umayahara Y, Fujitani Y, Sakamoto K, et al.: Role of pim-1 in smooth muscle cell proliferation. J Biol Chem 2004; 279: 54742-9.

5. Brault L, Gasser C, Bracher F, Huber K, Knapp S, Schwaller J: PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers. Haematologica 2010; 95: 1004-15.

6. Eichmann A, Yuan L, Bréant C, Alitalo K, Koskinen PJ: Developmental expression of pim kinases suggests functions also outside of the hematopoietic system. Oncogene 2000; 19: 1215-24.

7. Bachmann M, Möröy T: The serine/threonine kinase Pim-1. Int J Biochem Cell Biol 2005; 37: 726-30.

8. Narlik-Grassow M, Blanco-Aparicio C, Carnero A: The PIM family of serine/threonine kinases in cancer. Med Res Rev 2014; 34: 136-59.

9. Keane NA, Reidy M, Natoni A, Raab MS, O'Dwyer M: Targeting the Pim kinases in multiple myeloma. Blood Cancer J 2015; 5: e325-e.

10. Shen YM, Zhao Y, Zeng Y, Yan L, Chen BL, Leng AM, et al.: Inhibition of Pim-1 kinase ameliorates dextran sodium sulfate-induced colitis in mice. Dig Dis Sci 2012; 57: 1822-31.

11. Baek HS, Min HJ, Hong VS, Kwon TK, Park JW, Lee J, et al.: Anti-Inflammatory Effects of the Novel PIM Kinase Inhibitor KMU-470 in RAW 264.7 Cells through the TLR4-NF-κB-NLRP3 Pathway. Int J Mol Sci 2020; 2: 5138.

12. De Vries M, Heijink IH, Gras R, den Boef LE, Reinders-Luinge M, Pouwels SD, et al.: Pim1 kinase protects airway epithelial cells from cigarette smoke-induced damage and airway inflammation. Am J Physiol Lung Cell Mol Physiol 2014; 307: L240-51.

13. Shin YS, Takeda K, Shiraishi Y, Jia Y, Wang M, Jackson L, et al.: Inhibition of Pim1 kinase activation attenuates allergen-induced airway hyperresponsiveness and inflammation. Am J Respir Cell Mol Biol 2012; 46: 488-97.

14. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al.:. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget 2018; 9: 7204-18.

15. Akira S, Uematsu S, Takeuchi O: Pathogen recognition and innate immunity Cell 2006; 124: 783-801.

16. Santos-Sierra S: Targeting Toll-like Receptor (TLR) Pathways in Inflammatory Arthritis: Two Better Than One? Biomolecules 2021; 11.

17. Joosten LA, Abdollahi-Roodsaz S, Dinarello CA, O'Neill L, Netea MG: Toll-like receptors and chronic inflammation in rheumatic diseases: new developments. Nat Rev Rheumatol 2016; 12: 344-57.

18. Sandra M. Sacre, Evangelos Andreakos, Serafim Kiriakidis, Parisa Amjadi, Anna Lundberg, Grey Giddins, et al.: The Toll-Like Receptor Adaptor Proteins MyD88 and Mal/TIRAP Contribute to the Inflammatory and Destructive Processes in a Human Model of Rheumatoid Arthritis. Am J Pathol 2007; 170: 518 - 525.

19. Li Yu,, a Liantang Wang, and Shangwu Chen: Endogenous toll-like receptor ligands and their biological significance. J Cell Mol Med 2010; 14: 2592 - 2603.

20. Kawasaki T, Kawai T: Toll-like receptor signaling pathways. Front Immunol 2014; 5: 461.

21. Sabio G, Davis RJ: TNF and MAP kinase signalling pathways. Semin Immunol 2014; 26: 237-45.

22. O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A: The JAK-STAT pathway: impact on human disease and therapeutic intervention. Annu Rev Med 2015; 66: 311-28.

23. Czaja AJ: Hepatic inflammation and progressive liver fibrosis in chronic liver disease. World J Gastroenterol 2014; 20: 2515-32.

24. Liu Z, Wang Y, Wang Y, Ning Q, Zhang Y, Gong C, et al.: Dexmedetomidine attenuates inflammatory reaction in the lung tissues of septic mice by activating cholinergic anti-inflammatory pathway. Int Immunopharmacol 2016; 35: 210-6.

25. Murakami A, Ohigashi H: Targeting NOX, INOS and COX-2 in inflammatory cells: chemoprevention using food phytochemicals. Int J Cancer 2007; 121: 2357-63.

26. Aho TL, Sandholm J, Peltola KJ, Mankonen HP, Lilly M, Koskinen PJ: Pim-1 kinase promotes inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser112 gatekeeper site. FEBS Lett 2004; 571: 43-9.

27. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, et al.: Non-canonical inflammasome activation targets caspase-11. Nature 2011; 479: 117-21.

28. Kayagaki N, Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-Takamura S, et al.:. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science 2013; 341: 1246-9.

29. Tursynbay Y, Zhang J, Li Z, Tokay T, Zhumadilov Z, Wu D, et al.: Pim-1 kinase as cancer drug target: An update. Biomed Rep 2016; 4: 140-6.

30. Saris CJ, Domen J, Berns A: The pim-1 oncogene encodes two related protein-serine/threonine kinases by alternative initiation at AUG and CUG. Embo J 1991; 10: 655-64.

31. Leverson JD, Koskinen PJ, Orrico FC, Rainio EM, Jalkanen KJ, Dash AB, et al.: Pim-1 kinase and p100 cooperate to enhance c-Myb activity. Mol Cell 1998; 2: 417-25.

32. Beharry Z, Mahajan S, Zemskova M, Lin YW, Tholanikunnel BG, Xia Z, et al.: The Pim protein kinases regulate energy metabolism and cell growth. Proc Natl Acad Sci USA 2011; 108: 528-33.

33. Koike N, Maita H, Taira T, Ariga H, Iguchi-Ariga SM: Identification of heterochromatin protein 1 (HP1) as a phosphorylation target by Pim-1 kinase and the effect of phosphorylation on the transcriptional repression function of HP1(1). FEBS Lett 2000; 467: 17-21.

34. Wang J, Cao Y, Liu Y, Zhang X, Ji F, Li J, et al.: PIM1 inhibitor SMI-4a attenuated lipopolysaccharide-induced acute lung injury

through suppressing macrophage inflammatory responses via modulating p65 phosphorylation. Int Immunopharmacol 2019; 73: 568-74.

35. Rainio EM, Sandholm J, Koskinen PJ. Cutting edge: Transcriptional activity of NFATc1 is enhanced by the Pim-1 kinase. J Immunol 2002; 168: 1524-7.

36. Kim K, Kim JH, Youn BU, Jin HM, Kim N: Pim-1 regulates RANKL-induced osteoclastogenesis via NF-κB activation and NFATc1 induction. J Immunol 2010; 185: 7460-6.

37. Nihira K, Ando Y, Yamaguchi T, Kagami Y, Miki Y, Yoshida K: Pim-1 controls NF-kappaB signalling by stabilizing RelA/p65. Cell Death Differ 2010; 17: 689-98.

38. Rahman I, MacNee W: Role of transcription factors in inflammatory lung diseases. Thorax 1998; 53: 601-12.

39. Liu T, Zhang L, Joo D, Sun SC: NF-κB signaling in inflammation. Signal Transduct Target Ther 2017; 2: 17023-.

40. Ha YJ, Choi YS, Han DW, Kang EH, Yoo IS, Kim JH, et al.: PIM-1 kinase is a novel regulator of proinflammatory cytokine-mediated responses in rheumatoid arthritis fibroblast-likesynoviocytes. Rheumatology (Oxford) 2019; 58: 154-64.

41. Turner MD, Nedjai B, Hurst T, Pennington DJ: Cytokines and

chemokines: At the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta 2014; 1843: 2563-82.

42. Takeda K, Akira S: TLR signaling pathways. Semin Immunol 2004; 16: 3-9.

43. Zhang JM, An J: Cytokines, inflammation, and pain. Int Anesthesiol Clin 2007; 45: 27-37.

44. Needleman P, Manning PT: Interactions between the inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) pathways: implications for therapeutic intervention in osteoarthritis. Osteoarthritis Cartilage 1999; 7: 367-70.

45. Bonizzi G, Karin M: The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol 2004; 25: 280-8.

46. Ghosh S, Karin M: Missing pieces in the NF-kappaB puzzle. Cell 2002; 109: S81-96.

47. Karin M, Greten FR: NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 2005; 5: 749-59.

48. Manzoor Z, Koh Y-S: Mitogen-activated Protein Kinases in Inflammation. Jbv 2012; 42: 189-95.

49. Huang P, Han J, Hui L: MAPK signaling in inflammation-associated

cancer development. Protein Cell 2010; 1: 218-26.

50. Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W: IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. J Biol Chem 1999; 274: 30353-6.

51. He Y, Hara H, Núñez G: Mechanism and Regulation of NLRP3 Inflammasome Activation. Trends Biochem Sci 2016; 41: 1012-21.

52. Martinon F, Burns K, Tschopp J: The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 2002; 10: 417-26.

53. Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA: Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science 2013; 341: 1250-3.

54. Lu YC, Yeh WC, Ohashi PS: LPS/TLR4 signal transduction pathway. Cytokine 2008; 42: 145-51.

55. Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, et al: Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol 2005; 6: 1087-95.

56. Sakurai H: Targeting of TAK1 in inflammatory disorders and cancer. Trends Pharmacol Sci 2012; 33: 522-30.

57. Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z,

Matsumoto K: The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature 1999; 398: 252-6.

58. Warfel NA, Kraft AS: PIM kinase (and Akt) biology and signaling in tumors. Pharmacol Ther 2015; 151: 41-9.

59. Hofmann AD, Takahashi T, Duess J, Gosemann JH, Puri P: Increased expression of activated pSTAT3 and PIM-1 in the pulmonary vasculature of experimental congenital diaphragmatic hernia. J Pediatr Surg 2015; 50: 908-11.

60. Szydłowski M, Dębek S, Prochorec-Sobieszek M, Szołkowska M, Tomirotti AM, Juszczyński P, et al.: PIM Kinases Promote Survival and Immune Escape in Primary Mediastinal Large B-Cell Lymphoma through Modulation of JAK-STAT and NF-κB Activity. Am J Pathol 2021; 191: 567-74.

61. McInnes IB, Schett G: The pathogenesis of rheumatoid arthritis. N Engl J Med 2011; 365: 2205-19.

62. Bartok B, Firestein GS: Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev 2010; 233: 233-55.

63. Huang QQ, Pope RM: The role of toll-like receptors in rheumatoid arthritis. Curr Rheumatol Rep 2009; 11: 357-64.

64. Elemam NM, Hannawi S, Maghazachi AA: Role of Chemokines and

Chemokine Receptors in Rheumatoid Arthritis. Immunotargets Ther 2020; 9: 43-56.

65. Sucur A, Jajic Z, Artukovic M, Matijasevic MI, Anic B, Flegar D, et al.: Chemokine signals are crucial for enhanced homing and differentiation of circulating osteoclast progenitor cells. Arthritis Res Ther 2017; 19: 142.

66. McInnes IB, Schett G: Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 2007; 7: 429-42.

67. Sano H, Hla T, Maier JA, Crofford LJ, Case JP, Maciag T, et al.: In vivo cyclooxygenase expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis. J Clin Invest 1992; 89: 97-108.

68. Kang RY, Freire-Moar J, Sigal E, Chu CQ: Expression of cyclooxygenase-2 in human and an animal model of rheumatoid arthritis. Br J Rheumatol 1996; 35: 711-8.

69. Beiche F, Brune K, Geisslinger G, Goppelt-Struebe M: Expression of cyclooxygenase isoforms in the rat spinal cord and their regulation during adjuvant-induced arthritis. Inflamm Res 1998; 47: 482-7.

70. Abd-El-Aleem SA, Ferguson MW, Appleton I, Bhowmick A, McCollum CN, Ireland GW: Expression of cyclooxygenase isoforms in normal human skin and chronic venous ulcers. J Pathol 2001; 195: 616-23.

71. Bruch-Gerharz D, Stahl W, Gerharz CD, Megahed M, Wingerath T, Sies H, et al.: Accumulation of the xanthophyll lutein in skin amyloid deposits of systemic amyloidosis (al type). J Invest Dermatol 2001; 116: 196-7.

72. Bruch-Gerharz D, Fehsel K, Suschek C, Michel G, Ruzicka T, Kolb-Bachofen V: A proinflammatory activity of interleukin 8 in human skin: expression of the inducible nitric oxide synthase in psoriatic lesions and cultured keratinocytes. J Exp Med 1996; 184: 2007-12.

73. Lawrence T: The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol 2009; 1: a001651.

74. Nejatbakhsh Samimi L, Farhadi E, Tahmasebi MN, Jamshidi A, Sharafat Vaziri A, Mahmoudi M: NF-κB signaling in rheumatoid arthritis with focus on fibroblast-like synoviocytes. Autoimmunity Highlights 2020; 11: 11.

75. Karami J, Aslani S, Tahmasebi MN, Mousavi MJ, Sharafat Vaziri A, Jamshidi A, et al: Epigenetics in rheumatoid arthritis; fibroblast-like synoviocytes as an emerging paradigm in the pathogenesis of the disease. Immunol Cell Biol 2020; 98: 171-86.

76. Schett G, Tohidast-Akrad M, Smolen JS, Schmid BJ, Steiner CW, Bitzan P, et al: Activation, differential localization, and regulation of the stress-activated protein kinases, extracellular signal-regulated kinase, c-JUN N-terminal kinase, and p38 mitogen-activated protein

kinase, in synovial tissue and cells in rheumatoid arthritis. Arthritis Rheum 2000; 43: 2501-12.

77. Ajibade AA, Wang HY, Wang RF: Cell type-specific function of TAK1 in innate immune signaling. Trends Immunol 2013; 34: 307-16.

78. Liu X, Zhang Z, Ruan J, Pan Y, Magupalli VG, Wu H, et al.: Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. Nature 2016; 535: 153-8.

79. Pope RM, Tschopp J: The role of interleukin-1 and the inflammasome in gout: implications for therapy. Arthritis Rheum 2007; 56: 3183-8.

80. Kolly L, Busso N, Palmer G, Talabot-Ayer D, Chobaz V, So A: Expression and function of the NALP3 inflammasome in rheumatoid synovium. Immunology 2010; 129: 178-85.

81. Yeung YT, Aziz F, Guerrero-Castilla A, Arguelles S: Signaling Pathways in Inflammation and Anti-inflammatory Therapies. Curr Pharm Des. 2018; 24: 1449-84.

82. MacFarlane LA, Todd DJ: Kinase inhibitors: the next generation of therapies in the treatment of rheumatoid arthritis. Int J Rheum Dis 2014; 17: 359-68.

83. Flanagan ME, Blumenkopf TA: Brissette WH, Brown MF, Casavant

JM, Shang-Poa C, et al. Discovery of CP-690,550: a potent and selective Janus kinase (JAK) inhibitor for the treatment of autoimmune diseases and organ transplant rejection. J Med Chem 2010; 53: 8468-84.

84. Feldmann M, Brennan FM, Maini RN: Rheumatoid arthritis. Cell 1996; 85: 307-10.

85. Teitelbaum SL: Bone resorption by osteoclasts. Science 2000; 289: 1504-8.

86. Ammann P, Rizzoli R, Bonjour JP, Bourrin S, Meyer JM, Vassalli P, et al.: Transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency. J Clin Invest 1997; 99: 1699-703.

87. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al.: Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998; 93: 165-76.

88. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al.: Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cel 2002; 3: 889-901.

89. Nakamura I, Takahashi N, Jimi E, Udagawa N, Suda T: Regulation of osteoclast function. Mod Rheumatol 2012; 22: 167-77.

90. Wilson SR, Peters C, Saftig P, Brömme D: Cathepsin K activity-dependent regulation of osteoclast actin ring formation and bone resorption. J Biol Chem 2009; 284: 2584-92.

91. Shaulian E, Karin M: AP-1 as a regulator of cell life and death. Nat Cell Biol 2002; 4: E131-6.

92. Wang ZQ, Ovitt C, Grigoriadis AE, Möhle-Steinlein U, Rüther U, Wagner EF: Bone and haematopoietic defects in mice lacking c-fos. Nature 1992; 360: 741-5.

93. Lee K, Seo I, Choi MH, Jeong D: Roles of Mitogen-Activated Protein Kinases in Osteoclast Biology. Int J Mol Sci 2018; 19: 3004.

94. Lamothe B, Lai Y, Xie M, Schneider MD, Darnay BG: TAK1 is essential for osteoclast differentiation and is an important modulator of cell death by apoptosis and necroptosis. Mol Cell Biol 2013; 33: 582-95.

95. Soysa NS, Alles N: NF-kappaB functions in osteoclasts. Biochem Biophys Res Commun 2009; 378: 1-5.

96. Iotsova V, Caamaño J, Loy J, Yang Y, Lewin A, Bravo R: Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. Nat Med 1997; 3: 1285-9.

97. Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, et al.: Requirement for NF-kappaB in osteoclast and B-cell

development. Genes Dev 1997; 11: 3482-96.

98. Boyce BF, Xing L, Franzoso G, Siebenlist U: Required and nonessential functions of nuclear factor-kappa B in bone cells. Bone 1999; 25: 137-9.

Pim-1 Regulates NLRP3 Inflammasome-mediated Inflammatory Signals

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(Abstract)

Pim-1 is a proto-oncogene that encodes for the serine-threonine protein kinase family. Recent studies have suggested that Pim-1 kinase plays an important role in inflammatory signaling. However, the underlying regulatory mechanism remains unclear. In this study, I investigated the regulatory mechanism of Pim-1 on inflammation, and the anti-inflammatory effect of a novel protein kinase inhibitor, KMU-11342. PIM-1 knockdown suppressed lipopolysaccharide (LPS)-induced up-regulation of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), phospho-Janus kinase, phospho-signal transducer and activator of transcription 3, phospho-extracellular signal-regulated kinase, phospho-c-Jun N-terminal kinase, phospho-p38, phospho-nuclear factor

kappa B p65 (p-NF-κB p65), nuclear translocation of NF-κB p65, and phospho-inhibitor of NF-κB kinase α/β (p-IKKα/β). PIM-1 knockdown inhibited up-regulation of Nod-like receptor family pyrin domain containing 3 (NLRP3) and cleavage of caspase-1 induced by combination of LPS and adenosine triphosphate (ATP). Additionally, phospho-transforming growth factor-β-activated kinase 1 (TAK1) was found to be associated with Pim-1 binding. KMU-11342 inhibited LPS-induced up-regulation of chemokines in human rheumatoid arthritis fibroblast-like synoviocyte (RA-FLS). KMU-11342 suppressed LPS-induced up-regulation of pro-inflammatory cytokines, iNOS, COX-2, p-IKKα/β, p-NF-κB p65, and nuclear translocation of NF-κB p65 in both RA-FLS and THP-1 cells. It suppressed up-regulation of NLRP3 and co-treatment with LPS and ATP-induced caspase-1 cleavage. KMU-11342 also inhibited receptor activator of NF-κB-induced osteoblast differentiation and up-regulation of nuclear factor of activated T-cells, cytoplasmic 1 and c-Fos in RAW264.7 cells. Taken together, these findings indicated that Pim-1 plays an important role in inflammatory signaling and KMU-11342 is a

promising anti-inflammatory agent.

Pim-1의 NLRP3 Inflammasome 매개 염증 신호 조절

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(초록)

Pim-1은 세린-트레오닌 단백질 키나아제 family 계열의 암호화된 proto-oncogene이다. 최근 연구에 따르면 Pim-1 키나아제가 염증 신호에 중요한 역할을 한다. 그러나, 조절 메커니즘은 아직 명확하게 설명되지 않았다. 본 연구에서는, Pim-1의 염증에서의 조절기전과 새로운 단백질 키나아제 억제제인, KMU-11342의 항염증 효과를 조사했다. Pim-1의 억제는 LPS로 유도된 염증성 사이토카인, 유도성 일산화질소 합성효소(iNOS), 사이클로옥시게나제-2 (COX-2), p-JAK, p-STAT, p-ERK, p-JNK, p-p38, NF-κB p65 인산화 및 핵전사의 상향 조절을 억제하였다.

Pim-1의 억제는 LPS와 ATP의 결합에 의해 유도된 NLRP3의 상향조절과 Caspase-1의 활성을 억제했다. 게다가, TAK1은 Pim-1의 결합과 관련이 있는 것으로 밝혀졌다. KMU-11342는 류마티스 관절염 환자의 섬유아세포 유사 활막세포(RA-FLS)에서 LPS로 유도된 케모카인의 상향 조절을 억제

하였다. KMU-11342는 RA-FLS 와 THP-1 세포 모두에서 LPS 유도 염증성 사이토카인, iNOS, COX-2, p-IKKα/β, p-NF-κB p65, NF-κB P65의 핵전이를 억제하였다. 또한 LPS와 ATP 유도 NLRP3 inflammasome을 상향 조절과 Caspase-1의 활성을 억제했다. KMU-11342는 RAW264.7 세포에서 RANKL 유도 파골세포 분화와 NFATc-1 및 c-Fos의 상향 조절을 억제하였다. 결과적으로, 이러한 연구 결과는 Pim-1이 염증 신호 전달에 중요한 역할을 하며, KMU-11342는 잠재적인 항염증제임을 시사한다.

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□ 논문 및 저서

「KMU-1170, a Novel Multi-Protein Kinase Inhibitor, Suppresses Inflammatory Signal Transduction in THP-1 Cells and Human Osteoarthritic Fibroblast-Like Synoviocytes by Suppressing Activation of NF-κB and NLRP3 Inflammasome Signaling Pathway」 Int. J. Mol Sci 2021. 1

「Anti-Inflammatory Effects of the Novel PIM Kinase Inhibitor KMU-470 in RAW 264.7 Cells through the TLR4-NF-κB-NLRP3 Pathway」 Int. J. Mol. Sci 2020. 6

「Effect of Methanol Extract from Cassia mimosoides var. nomame on Ischemia/Reperfusion-induced Renal Injury in Rats.」 Kor. J. Herbology. 2013. 11

「Hot Water Extract of Triticum aestivum L.(Common Wheat) Ameliorates Renal Injury by Inhibiting Apoptosis in a Rat Model of Ischemia/Reperfusio」 kor. J. Herbology 2013. 5

「Synthesis and Biological Evaluation of a Novel ^{177}Lu-DOTA-[Gly^{3}-Cyclized (Dap^{4}, D-Phe^{7}, Asp^{10})-Arg^{11}] a-MSH$_{3-13}$ Analogue for Melanocortin-1 Receptor-Positive Tumor Targeting」 Cancer Biother Radiopharm 2012. 8

「Methanol Extract of Goat's-beard (Aruncus dioicus) Reduces Renal Injury by Inhibiting Apoptosis in a Rat Model of Ischemia-Reperfusion」

Pre Nutr Food Sci 2012. 6

「소맥추출물과 그 구성성분을 이용한 허혈성 급성신부전 치료」 석사학위 논문 2011. 2.