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Protein phosphatase magnesium-dependent 1A induces inflammation in rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a highly inflammatory autoimmune disease. Although proinflammatory cytokines, including tumor necrosis factor (TNF) and interleukin (IL)-6, play a key role in the pathogenesis of RA, the causes of chronic inflammation are not fully understood. Here, we report that protein phosphatase magnesium-dependent 1A (PPM1A) levels were increased in RA synovial fluid compared with osteoarthritis (OA) synovial fluid and positively correlated with TNF levels. In addition, PPM1A expression was increased in synovial tissue from

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Author Contributions

Conceptualization: BL, YSS, DHS, WHR; Acquisition of data: BL, CR, JSR, HJ, JP, MK; Analysis and interpretation of data: BL, YSS, DHS, TSG, HNL, SGL, SK, MK, SIL; Writing and editing of the manuscript: BL, YSS, SGL, DHS, WHR; Funding acquisition: YSS, DHS; Study supervision: DHS, WHR

Conflict of Interest

The authors declare no potential conflicts of interest.

RA patients and joint tissue from a mouse model of arthritis. Finally, extracellular PPM1A induced inflammation by stimulating macrophages to produce TNF through toll-like receptor 4 (TLR4) and myeloid differentiation primary response protein 88 (MyD88) signaling pathway. Our findings suggest that extracellular PPM1A may contribute to the pathogenesis of RA by functioning as a damage-associated molecular pattern (DAMP) to induce inflammation.

Keywords

PPM1A; Inflammation; DAMP; Rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammation, which leads to joint pain, bone destruction, and progressive disability. It is also associated with systemic complications by affecting extra-articular tissues such as the skin and lungs, and the cardiovascular system [1]. Proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-6, and IL-1β have a pathophysiological role in RA [1], but the underlying cause of inflammation in this disease is largely unknown.

Damage-associated molecular patterns (DAMPs) are endogenous molecules that are released after tissue injury or cell death, which induce inflammation by activating the innate immune system [2–4]. Several DAMPs have been identified so far, including high-mobility group box 1 (HMGB1), histones, S100 proteins, and heat-shock proteins (HSPs) [2]. They are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), thereby activating inflammatory signaling pathways that contribute to the pathogenesis of inflammatory diseases such as RA [2, 5, 6].

Protein phosphatase magnesium-dependent 1A (PPM1A) is a serine/threonine phosphatase that regulates bone morphogenetic protein (BMP) and transforming growth factor β (TGF-β) signaling pathways by dephosphorylating its substrates such as p38 mitogen-activated protein kinase (MAPK) and Smad1/2/3 [7–9]. Although PPM1A is an intracellular protein located in the nucleus and cytoplasm [10–12], the presence of extracellular PPM1A has also been reported. In the previous study, we have reported that the levels of PPM1A protein and anti-PPM1A autoantibodies were elevated in the sera from ankylosing spondylitis (AS) patients [13]. However, the function of extracellular PPM1A was unclear. In this study, we report that PPM1A levels are increased in both human RA and a murine model of arthritis. In addition, extracellular PPM1A induces TNF production in macrophages through the activation of TLR4. Our findings suggest that extracellular PPM1A contributes to the pathogenic inflammation observed in RA by functioning as a DAMP.

Materials and Methods

Patient samples

The demographic and clinical characteristics of the patients with osteoarthritis (OA) and RA included in this research are summarized in Table 1. Patients with RA were classified according to the 2010 ACR/EULAR rheumatoid arthritis classification criteria. All patients

provided informed consent. The synovial fluid and data used for this study were provided by the Gyeongsang National University Hospital, a member of the Korea Biobank Network. This study was approved by the Institutional Review Board (IRB) of the Hospital of Gyeongsang National University (Permit No: GNUH 2014–02-013–021). Synovial tissues from patients with RA or OA were collected in accordance with human subject protocols approved by the Stanford University IRB as previously described [14].

Enzyme-linked immunosorbent assay (ELISA)

PPM1A (Cloud-Clone Corp.) and TNF (R&D Systems) levels in RA and OA synovial fluid were determined by ELISA according to manufacturer's protocol.

Immunohistochemistry

Immunohistochemistry was performed as previously described [13]. Briefly, synovial tissue specimens embedded in the optimal cutting temperature (OCT) compound were cut on a cryostat and fixed in 4% paraformaldehyde. The tissue was permeabilized with 0.1% Triton X-100, and tissue sections were blocked with 5% normal goat serum. For detection of PPM1A, 3 μg/ml of anti-PPM1A antibody (Abcam) or control rabbit IgG (Cell Signaling Technology) was used, and the signal was developed with Vectastain Elite ABC kit and DAB substrate (Vector Laboratories) according to the manufacturer's instructions. To obtain joint tissues from arthritic mice, K/BxN (F1 mice of KRN TCR transgenic mice x NOD) serum transfer arthritis was induced by intraperitoneal injection of 100 μl of K/BxN on day 0 and 2, as previously described [14]. Sections of paraffin-embedded joint tissue from K/BxN serum transfer arthritic mice were deparaffinized, rehydrated, and subjected to antigen retrieval as described previously [14]. For detection of PPM1A, the same method was used as described above. Quantification of PPM1A on immunohistochemical images was performed with ImageJ software as previously described [15]. Optical density (OD) values of 3,3′-Diaminobenzidine (DAB) and hematoxylin staining were determined from three different areas of each image. Relative PPM1A expression was calculated with the ratio of DAB/hematoxylin.

Cells and reagents

RAW264.7 macrophages were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine (Thermo Fisher Scientific). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich. Palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4), high molecular weight polyinosinic-polycytidylic acid (poly(I:C) HMW), CpG (oligodeoxynucleotides (ODN) 2395), and LPS inhibitor polymyxin B were purchased from InvivoGen. Proteinase K was purchased from Thermo Fisher Scientific, and PPM1A was either purchased from Creative BioMart or purified by Professor Soohyun Kim.

Bone marrow-derived macrophages (BMMs)

We generated mouse BMMs from wild-type C57BL/6, TLR2 deficient, TLR4 deficient Tlr4$^{lps\text{-}del}$, TLR9 deficient, or myeloid differentiation primary response protein 88 (Myd88) deficient mice (The Jackson Laboratory) as previously described [16]. Briefly, bone-marrow

cells were collected by flushing the femur and tibia of mice with α-minimal essential medium (α-MEM; Thermo Fisher Scientific) using a 1-ml syringe. Red blood cells (RBCs) were removed by treating with ACK lysis buffer (Lonza) and filtering through a 70-μm cell strainer (Becton Dickinson). The filtered cells were cultured in α-MEM supplemented with 10% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine in 5% CO_2 at 37°C. After 16 to 24 hours, non-adherent cells were collected, plated on 100-mm dishes, and differentiated into BMMs for 7 days in the presence of 30 ng/ml of macrophage colony-stimulating factor (M-CSF; PeproTech).

Macrophage stimulation assays

Macrophage stimulation assays were performed as previously described [16]. Briefly, RAW264.7 macrophages or BMMs (8×10^4/well) in 96-well plates were incubated with Pam3CSK4, poly(I:C) HMW, LPS, CpG, or PPM1A for 24 hours. TNF levels in the supernatants of macrophage cultures were measured by ELISA according to manufacturer's instructions (PeproTech). To exclude the possibility of endotoxin contamination, macrophages were stimulated in the presence of 20 μg/ml polymyxin B in the stimulation assays of Fig 3B. In the stimulation assays of Fig 3C, PPM1A protein was pre-incubated with proteinase K (20 μg/ml) at 55°C for 4 hours in the presence of β-mercaptoethanol (0.3 mM) (Thermo Fisher Scientific) followed by boiling for 10 minutes to inactivate proteinase K.

Statistical analysis

Unpaired *t* test and Spearman's correlation test were used to analyze the data with GraphPad Prism software. *p* values less than 0.05 were considered to indicate significant differences.

Results

PPM1A is increased in RA and positively correlates with TNF

To investigate the function of extracellular PPM1A in RA, we first measured PPM1A and TNF levels in synovial fluid from patients with RA or OA using ELISA. As shown in Fig. 1A and B, PPM1A and TNF levels were increased in RA synovial fluid compared with OA synovial fluid. Interestingly, PPM1A levels were positively correlated with those of TNF, a key inflammatory cytokine involved in the pathogenesis of RA (Fig. 1C). We next examined whether PPM1A expression was also increased in the inflamed synovial tissues. Immunohistochemical images and quantification analysis revealed that PPM1A expression was increased in human RA synovial tissue compared with that in OA (Fig. 2A and B). In addition, PPM1A expression was increased in the joint tissue from K/BxN serum transfer model of arthritis compared with control mice (Fig. 2C and D). These results suggest that the increase in PPM1A may be correlated with the inflammation observed in human RA and mouse model of arthritis.

Extracellular PPM1A induces TNF production in macrophages.

Many DAMPs are intracellular proteins that are released into extracellular compartments, owing to cellular stress or tissue damage [2, 5]. PPM1A is also an intracellular protein involved in BMP and TGF-β signaling pathways [8, 9]. Although we have shown that

extracellular PPM1A levels were increased in the sera of AS patients compared to healthy controls [13], the role of extracellular PPM1A was unclear. Furthermore, the elevated PPM1A levels in RA synovial fluid and the correlation of PPM1A with TNF evoked the possibility that extracellular PPM1A induces inflammation. To investigate this possibility, we performed macrophage stimulation assays. When macrophages were treated with recombinant human PPM1A, TNF was induced in a dose-dependent manner (Fig. 3A). When macrophages were treated with PPM1A in the presence of the LPS inhibitor polymyxin B, PPM1A-induced TNF production was not abrogated whereas LPS-induced TNF production was completely inhibited (Fig. 3B). In addition, pre-treatment of PPM1A with proteinase K inhibited PPM1A-induced TNF production but not Pam3CSK4- or LPS-induced TNF production (Fig. 3C). These results suggest that extracellular PPM1A functions as a DAMP to induce inflammation by stimulating macrophages to produce TNF, and the immunostimulatory effect of PPM1A did not result from endotoxin contamination.

PPM1A induces TNF production via TLR4 and MyD88 pathway.

We next investigated the mechanism of PPM1A-induced TNF production. Because TLRs are the most well-characterized pattern recognition receptors (PRRs) for DAMPs [2, 17], we examined whether PPM1A also induces TNF production through TLRs. Bone marrow-derived macrophages (BMMs) were obtained from wild-type, TLR2, TLR4, or TLR9 deficient mice and stimulated with PPM1A. As shown in Fig. 3D, PPM1A-induced TNF production was significantly decreased in TLR4-deficient macrophages, but not in TLR2- or TLR9-deficient macrophages. We next investigated whether MyD88, an essential adaptor protein for TLR signaling except for TLR3 [18], is involved in PPM1A-induced TNF production. When MyD88-deficient macrophages were treated with PPM1A, TNF production was also significantly decreased (Fig. 3E). These results show that extracellular PPM1A induces TNF production via TLR4-MyD88 signaling pathway.

Discussion

Although inflammation plays a central role in the pathogenesis of RA, it is still incompletely understood what causes chronic inflammation in RA. Here, we suggest that extracellular PPM1A, which is increased in synovial fluid and tissues of RA patients compared with OA patients, induces inflammation through TLR4-MyD88 signaling pathway by functioning as a DAMP.

While RA is driven by autoimmunity and high inflammation responses, OA is considered a low-grade inflammatory disease caused by mechanical loading [19]. Although synovial fluid or tissues from healthy subjects would be ideal controls for our study, it was very difficult to obtain these samples from healthy subjects. Moreover, our study focused on DAMP function of PPM1A in RA. Therefore, synovial fluid and tissues from OA patients were used as controls for RA in our study. A further study comparing synovial fluid and tissues from healthy subjects with those from OA patients may be helpful to reveal the role of PPM1A in OA.

DAMPs are immunostimulatory molecules that are released at the site of tissue damage [2]. It is well established that DAMPs contribute to the pathogenesis of RA [2, 20]. For example,

HMGB1 is a nuclear protein that stabilizes nucleosome structure and regulates gene expression. However, HMGB1 can be released into the extracellular space, thereby exerting immunostimulatory function through PRRs such as TLR2, TLR4, and receptor for advanced glycation end products (RAGE) [2, 20]. In patients with RA, HMGB1 levels were increased in the serum and synovial fluid [21, 22]. In addition, administration of anti-HMGB1 antibodies reduced the clinical scores in animal models of arthritis [23, 24]. Likewise, intracellular PPM1A can be released into the extracellular space, thereby inducing inflammation by activating TLR4. Further research will be necessary to examine whether neutralization of extracellular PPM1A could ameliorate arthritis.

It is possible that under chronic inflammation, DAMPs are uncontrollably released from injured cells, which will in turn damage other tissues, leading to a vicious cycle that amplifies inflammation and DAMP production [2, 20]. Therefore, selective inhibition of DAMP signaling by blocking the interaction of DAMPs with their receptors or interfering with downstream signaling pathways will be promising therapeutic strategies to treat inflammatory diseases such as RA. In fact, TLR antagonists/inhibitors are currently being developed and their safety and efficacy are examined in clinical trials [25, 26].

In conclusion, our data suggest that extracellular PPM1A, which is increased in RA, can contribute to the pathogenesis of RA by inducing inflammation. Our results and future research on the role of extracellular PPM1A in other inflammatory diseases could suggest PPM1A as a therapeutic target for inflammatory diseases.

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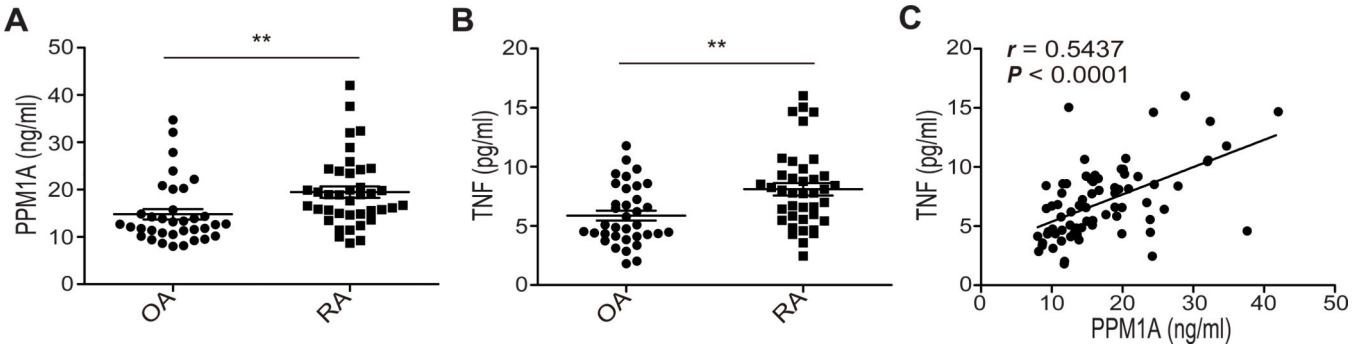

Fig. 1.

PPM1A levels are increased in RA and correlate with TNF levels. (A and B) PPM1A (A) and TNF (B) levels in RA (n=39) or OA (n=35) synovial fluid were measured by ELISA. Data are shown as the mean ± standard error of the mean (SEM). The *p* value was calculated by an unpaired *t* test. $^{**}p < 0.01$. (C) Spearman's correlation test was used to analyze the correlation between PPM1A and TNF.

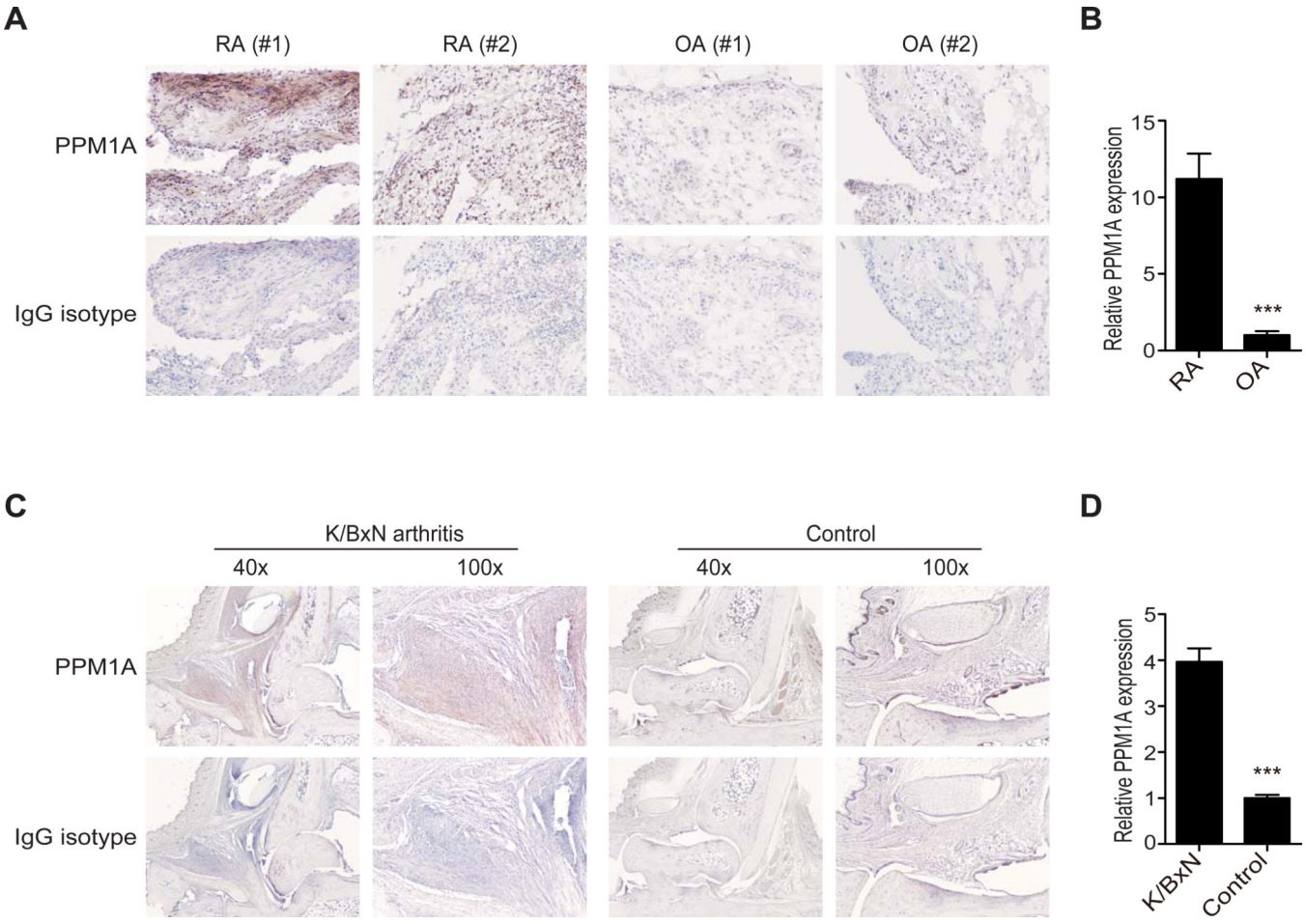

Fig. 2.

PPM1A expression is elevated in human RA synovial tissue and joint tissue from K/BxN serum transfer mouse model of arthritis. (A) Representative immunohistochemical images of synovial tissues from patients with RA or OA stained with anti-PPM1A antibody or IgG isotype control. Images are shown at ×100 magnification. (B) Three different areas of PPM1A-stained images from (A) were quantified and the relative PPM1A expression in RA was compared with that in OA. Data are shown as the mean ± SEM. The *p* values were calculated by an unpaired t test. ***$p < 0.001$. (C) Representative immunohistochemical images of ankle joint tissue from K/BxN serum transfer arthritic mice or control mice stained with anti-PPM1A antibody or IgG isotype control. Images are shown at ×40 and ×100 magnification. (D) Three different areas of PPM1A-stained images from (C) at ×100 magnification were quantified, and the relative PPM1A expression in K/BxN arthritis was compared with that in control mice. Data are shown as the mean ± SEM. The *p* values were calculated by an unpaired t test. ***$p < 0.001$.

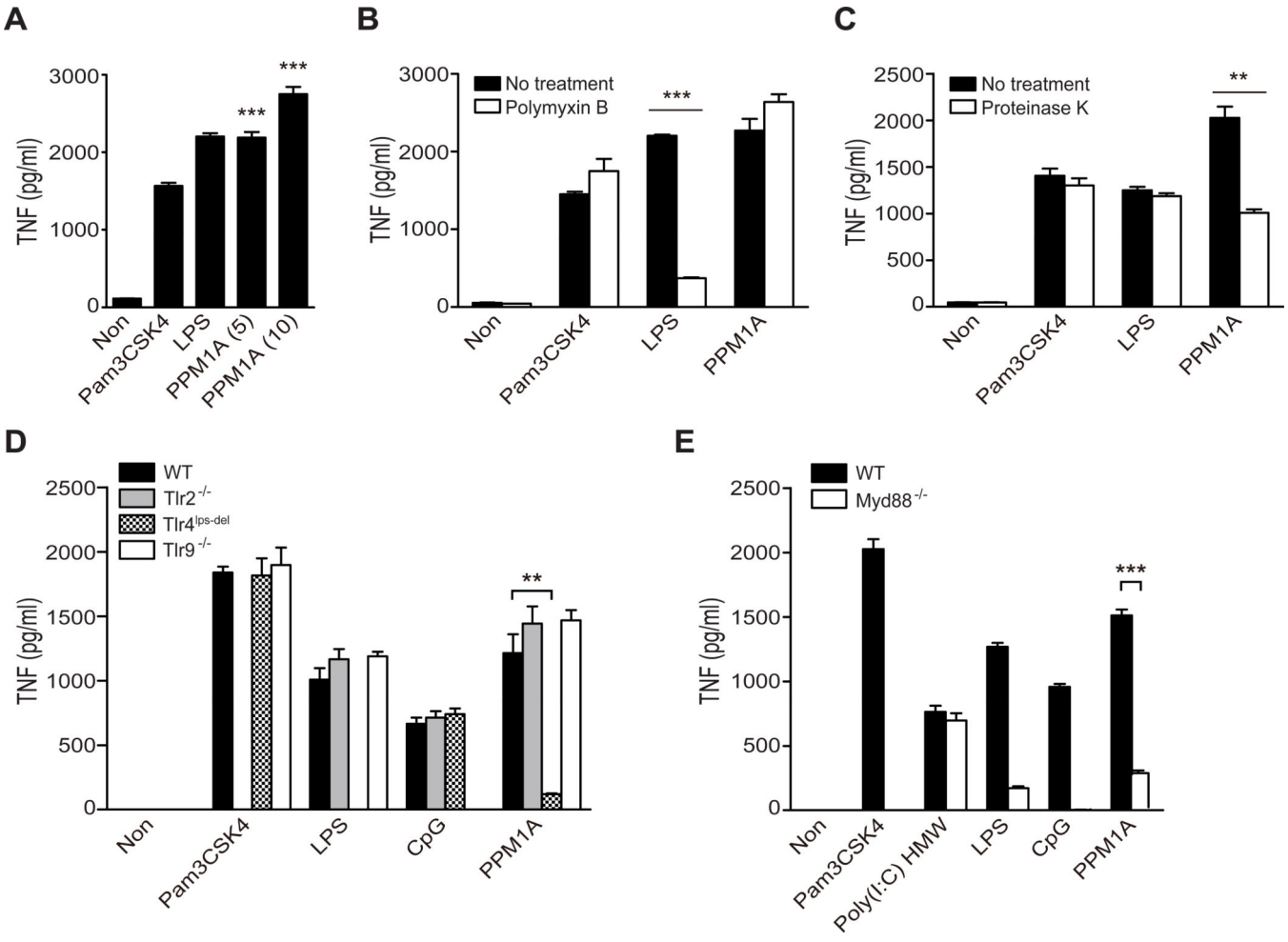

Fig. 3.

PPM1A induces TNF production in macrophages via TLR4-Myd88. (A) RAW264.7 macrophages were stimulated for 24 hours with the indicated concentrations (in μg/ml) of PPM1A and TNF levels in the supernatants were measured with ELISA. Palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4) (10 ng/ml) and lipopolysaccharide (LPS) (1 ng/ml) were used as positive controls. PPM1A-induced TNF levels were compared with levels in no treatment control (Non). (B) RAW264.7 macrophages were stimulated with PPM1A (10 μg/ml) in the presence or absence of polymyxin B (20 μg/ml), an inhibitor of LPS. LPS (1 ng/ml) was used as a positive control for confirming the efficacy of polymyxin B and Pam3CSK4 (10 ng/ml) was used as a negative control. (C) RAW264.7 macrophages were stimulated with PPM1A (10 μg/ml) after treatment with proteinase K. Pam3CSK4 (10 ng/ml) and LPS (1 ng/ml) were used as negative controls for the efficacy of proteinase K. (D) Bone-marrow-derived macrophages (BMMs) from wild-type (WT), TLR2-deficient ($Tlr2^{-/-}$), TLR4-deficient ($Tlr4^{lps\text{-}del}$), or TLR9-deficient ($Tlr9^{-/-}$) mice were stimulated for 24 hours with PPM1A (10 μg/ml) and TNF levels in the supernatants were measured with ELISA. TLR2 ligand Pam3CSK4 (10 ng/ml), TLR4 ligand LPS (1 ng/ml), and TLR9 ligand CpG (10 μg/ml) were used as positive controls for TLR2, TLR4, and TLR9 deficiency, respectively. (E) BMMs from wild-type or Myd88-deficient ($Myd88^{-/-}$) mice were

stimulated for 24 hours with PPM1A (10 μg/ml) and TNF levels in the supernatants were measured with ELISA. Pam3CSK4 (10 ng/ml), LPS (1 ng/ml), and CpG (10 μg/ml) were used as positive controls for Myd88 deficiency. TLR3 ligand poly(I:C) HMW (100 μg/ml), which does not require MyD88 for its signaling, was used as a negative control for Myd88 deficiency. Data are shown as the mean ± SEM of triplicates. The p values were calculated by an unpaired t test. $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 1.

Characteristics of patients

Characteristics	OA (n=35)	RA (n=39)
Age (years)	73.3 ± 1.4	65.2 ± 2.0
Sex	F = 24, M = 11	F = 29, M = 10
Disease duration (months)	52.5 ± 5.0	28.9 ± 2.3
ESR (mm/h)	26.0 ± 4.9	48.9 ± 7.6
CRP (mg/l)	15.5 ± 5.6	26.6 ± 4.4
RF (IU/ml)	9.9 ± 0.8	166.3 ± 31.9
Anti-CCP (U/ml)	1.6 ± 0.6	173.3 ± 26.4
Use of DMARDs (%)	N/A	100

Data are mean±SEM. Abbreviations: RA, rheumatoid arthritis; OA, osteoarthritis; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; Anti-CCP, anti-cyclic citrullinated peptide; DMARD, disease-modifying anti-rheumatic drug; N/A, not applicable.