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MPMBP down-regulates Toll-like receptor (TLR) 2 ligand-induced proinflammatory cytokine production by inhibiting NF-κB but not AP-1 activation

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ABSTRACT

MPMBP is a novel non-nitrogen-containing bisphosphonate (non-NBP) which possesses anti-bone resorptive activity and an antioxidant side chain. This study aimed to assess the effects of MPMBP on the production of proinflammatory cytokines and chemokines by the macrophage-like cell line, J774.1, in the presence of Toll-like receptor (TLR) agonists. J774.1 cells were pretreated with or without MPMBP for 5 min, and then incubated with or without Pam_3Cys-Ser-$(Lys)_4$ (Pam_3CSK_4, a TLR2 agonist) or lipid A (a TLR4 agonist) for 24 h. MPMBP down-regulated TLR2 ligand-induced production of IL-6, MCP-1, MIP-1α, and TNF-α, but not TLR4 ligand-induced proinflammatory cytokine production, and was not cytotoxic in J774.1 cells. Cu-CPT22, a TLR2 antagonist, down-regulated Pam_3CSK_4-induced production of IL-6, MCP-1, and MIP-1α, but not TNF-α. MPMBP inhibited the translocation of NF-κB p65, but not p50, RelB, or p52, and inhibited the activation of JNK, but not p38 MAPK or ERK, in J774.1 cells stimulated with Pam_3CSK_4. Moreover, MPMBP did not down-regulate AP-1 activation in J774.1 cells stimulated with Pam_3CSK_4 or lipid A. Our findings suggest that MPMBP inhibits proinflammatory cytokine production in J774.1 cells by suppressing NF-κB p65 activation in the TLR2, but not TLR4, pathway.

1. Introduction

MPMBP is a novel non-nitrogen-containing bisphosphonate (non-NBP) which possesses anti-bone resorptive activity and an antioxidant side chain [1]. Non-NBPs induce apoptosis of osteoclasts, but not other cell types such as murine macrophage-like J774.1 cells. These agents inhibit bone resorption to a lesser extent than NBPs, which have undesirable side effects such as gastric damage, inflammatory effects, and jaw osteonecrosis [2–5]. In contrast to NBPs, non-NBPs have been reported to have anti-inflammatory effects, although the underlying mechanism is unclear. In addition, etidronate, a non-NBP, was also found to reduce both pain and atherosclerotic plaques [6–9]. As proinflammatory cytokines induce pain and promote atherosclerotic plaque formation, etidronate might decrease pain and the formation of atherosclerotic plaques by inhibiting proinflammatory cytokine production [10–13]. Another non-NBP, clodronate, inhibited pathogenic bacteria-induced production of IL-1, a proinflammatory cytokine, and neuropathic pain [14–16]. IL-1, IL-6, and tumor necrosis factor-α (TNF-α) promote the differentiation of osteoclast precursors into mature osteoclasts, and monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) promote neutrophil migration [17]. The production of proinflammatory cytokines is induced not only by live bacteria but also bacterial components, such as lipopolysaccharide (LPS), in the cell wall of gram-negative bacteria. Similar to other non-NBPs, MPMBP inhibits LPS-induced IL-1β production by J774.1 cells and prevents mouse collagen-induced arthritis and rat adjuvant arthritis caused by IL-6 and TNF-α [18–20]. Moreover, MPMBP has been reported to dose-dependently suppress osteoclast cell fusion, pit formation, and calcium release from calvaria cultured in the presence of LPS, prostaglandin E_2, IL-1β, and parathyroid hormone (PTH), which are bone resorption factors [21].

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns [2]. LPS is a well-known TLR4 ligand, but many commercial LPS molecules include contaminant ligands that activate TLR2 [22–24]. In addition to recognizing whole bacteria, TLR2 recognizes many cell wall components, including peptidoglycan, lipoprotein, and lipoteichoic acid [25–27]. Pam_3Cys-Ser-$(Lys)_4$ (Pam_3CSK_4), a TLR2 agonist, is a synthetic lipopeptide that mimics the acylated amino terminus of bacterial lipoprotein [28]. Lipid A, a TLR4 agonist, is the lipophilic portion and bioactive moiety of LPS [29,30]. In addition to lipids, TLR4

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also recognizes proteins, such as heat shock proteins and fibrinogen [31,32]. Both Pam_3CSK_4 and lipid A can induce osteoarthritis via TLR2 and TLR4 pathways [33,34].

We previously reported that etidronate, a non-NBP, inhibited the activation of nuclear factor-κB (NF-κB), a transcription factor implicated in the production of IL-6, MCP-1, MIP-1α, and TNF-α [4,35]. NF-κB belongs to a family of transcription factors composed of five subunits (RelA/p65, RelB, p50, p52, and c-Rel). The complex formed by RelA/p65 and p50 is referred to as canonical NF-κB1 [36], whereas noncanonical NF-κB2 consists of RelB and p52. We also reported that etidronate inhibits proinflammatory cytokine production in J774.1 cells by suppressing NF-κB p65 activation in the TLR2, but not TLR4, pathway. Both TLR2 and TLR4 signaling can activate NF-κB, but the signals are transduced via different pathways [37].

AP-1 is another transcription factor which promotes the production of proinflammatory cytokines and is a component of TLR signaling [38,39]. AP-1 is a dimeric complex composed of members from the JUN, FOS, ATF, or MAF protein families, although c-Jun and c-Fos activation are typically used to assess AP-1 activity. Unlike NF-κB, etidronate does not inhibit the activation of p38 mitogen-activated protein kinase (MAPK), an important molecule that functions upstream of AP-1 [4]. The MAPK signaling pathway, which includes c-Jun N-terminal kinases (JNK), p38 MAPK, and ERK, plays an important role in the activation of both NF-κB and AP-1 [40,41].

This study aimed to assess the anti-inflammatory effects of MPMBP on TLR2 or TLR4 ligand-induced IL-6, MCP-1, MIP-1α, and TNF-α production by J774.1 cells, as well as whether MPMBP inhibits the activation of NF-κB, AP-1, and the MAPK pathway.

2. Materials and methods

2.1. Reagents and antibodies

MPMBP was synthesized as described previously [1] and dissolved in sterile PBS (Fig. 1). Pam_3CSK_4, a TLR2 agonist, was obtained from InvivoGen (San Diego, CA, USA). Lipid A (compound 506), a TLR4 agonist, was purchased from Peptide Institute (Osaka, Japan). Actin (I-19, #sc-1616) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit monoclonal anti-phospho-(Thr202/Tyr204)-ERK1/2 (#4370), anti-phospho-(Thr180/Tyr182)-p38 MAPK (#4511), anti-phospho-(Thr183/Tyr185)-JNK1/2 (#4668), anti-ERK1/2 (#4695), anti-p38 MAPK (#8690), and anti-JNK1/2 (#9252) antibodies, as well as horseradish peroxidase (HRP)-conjugated, affinity-purified goat anti-rabbit IgG antibody (#7074), were purchased from Cell Signaling Technology (Danvers, MA, USA). Cu-CPT22, a TLR2 antagonist, was purchased from Selleck (Houston, TX, USA).

2.2. Cell culture

Murine macrophage-like J774.1 cells were obtained from the RIKEN Bioresource Center (Ibaraki, Japan). Cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS: HyClone™, GE Healthcare, South Logan, UT, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) in an incubator at 37 °C and 5% CO_2. J774.1 cells were used as confluent monolayers at passages 10 through 17.

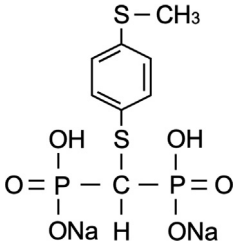

Fig. 1. Chemical structure of MPMBP.

2.3. Cytokine measurements

Confluent J774.1 cells (2×10^5 cells/well) were cultured in 96-well flat-bottomed plates (Falcon®, BD Biosciences, Franklin Lakes, NJ, USA) for 18 h, and then washed twice with serum-free medium and pretreated with or without MPMBP (1, 10, 100 μM) for 5 min. Cells were then incubated in the presence or absence of Pam_3CSK_4 (1–100 ng/ml) or lipid A (1–100 ng/ml) in RPMI-1640 containing 10% FBS for 24 h. Levels of secreted mouse IL-6, MCP-1, MIP-1α, and TNF-α in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA: mouse MCP-1, eBioscience, San Diego, CA, USA; mouse IL-6, MIP-1α, and TNF-α, R&D systems, Minneapolis, MN, USA). For the inhibition assay, cells were pretreated with or without MPMBP or Cu-CPT22 (2.5, 5, or 10 μM) at the indicated concentrations for 1 h, and then incubated with or without 10 ng/ml Pam_3CSK_4.

2.4. Cell viability

Cell viability was assessed by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)–2H–tetrazolium (MTS) to formazan by living cells. Briefly, MTS solution (Cell Titer 96® AQueous One Solution Assay; Promega Corp., Madison, WI, USA) was added directly to each well and incubated for 1 h at 37 °C. Absorbance was measured at 490 nm (reference: 655 nm), as described above. There was a linear response between cell number and absorbance at 490 nm.

LDH levels were also measured to assess cell death. LDH activity in supernatants (2% Triton X-100-treated cells corresponded to a total activity of 100%) was determined using the Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Basel, Switzerland). The amount of formazan formed was determined by measuring absorbance at 490 nm (reference: 655 nm) using an iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA).

2.5. Nuclear protein extraction and transactivation analysis

Confluent J774.1 cells (3×10^6 cells/well) were cultured in 60 mm dishes (Falcon®) for 18 h, washed twice with serum-free medium, and pretreated with or without MPMBP (1, 10, 100 μM) for 5 min. Cells were then incubated in the presence or absence of Pam_3CSK_4 (10 ng/ml) or lipid A (10 ng/ml) in RPMI-1640 containing 10% FBS for the indicated durations. Nuclear protein extracts were prepared with the TransAM™ nuclear extract kit according to the manufacturer's protocol (Active Motif Japan, Tokyo, Japan). Briefly, J774.1 cells were scraped into phosphate-buffered saline (PBS) in the presence of phosphatase and protease inhibitors, centrifuged, resuspended in 1 × hypotonic buffer, and kept on ice for 15 min. After addition of detergent, lysates were centrifuged at 14,000 × *g* for 30 s. Pellets were resuspended in complete lysis buffer (20 mM HEPES, pH 7.5, 350 mM NaCl, 20% glycerol, 1% Igepal CA630, 1 mM $MgCl_2$, 0.5 mM ethylene diaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol-*O,O′*-bis-[2-aminoethyl]-N,N,N′,N′,-tetraacetic acid (EGTA), 1 mM DTT, and phosphatase and protease inhibitors) and vortexed. After incubation on ice and centrifugation, supernatants were collected and protein concentrations were determined using the BCA™ protein assay kit (Pierce, Rockford, IL, USA).

NF-κB and AP-1 activation was determined by the TransAM ELISA kit (Active Motif Japan). This assay is based on a colorimetric reaction, and is an alternative to the electrophoretic mobility-shift assay. Oligonucleotides containing NF-κB or AP-1 consensus binding sites were immobilized in each well of a 96-well plate. Nuclear extracts (8 μg for p65 and p50; 4 μg for p52 and RelB; 10 μg for c-Jun and c-Fos) were

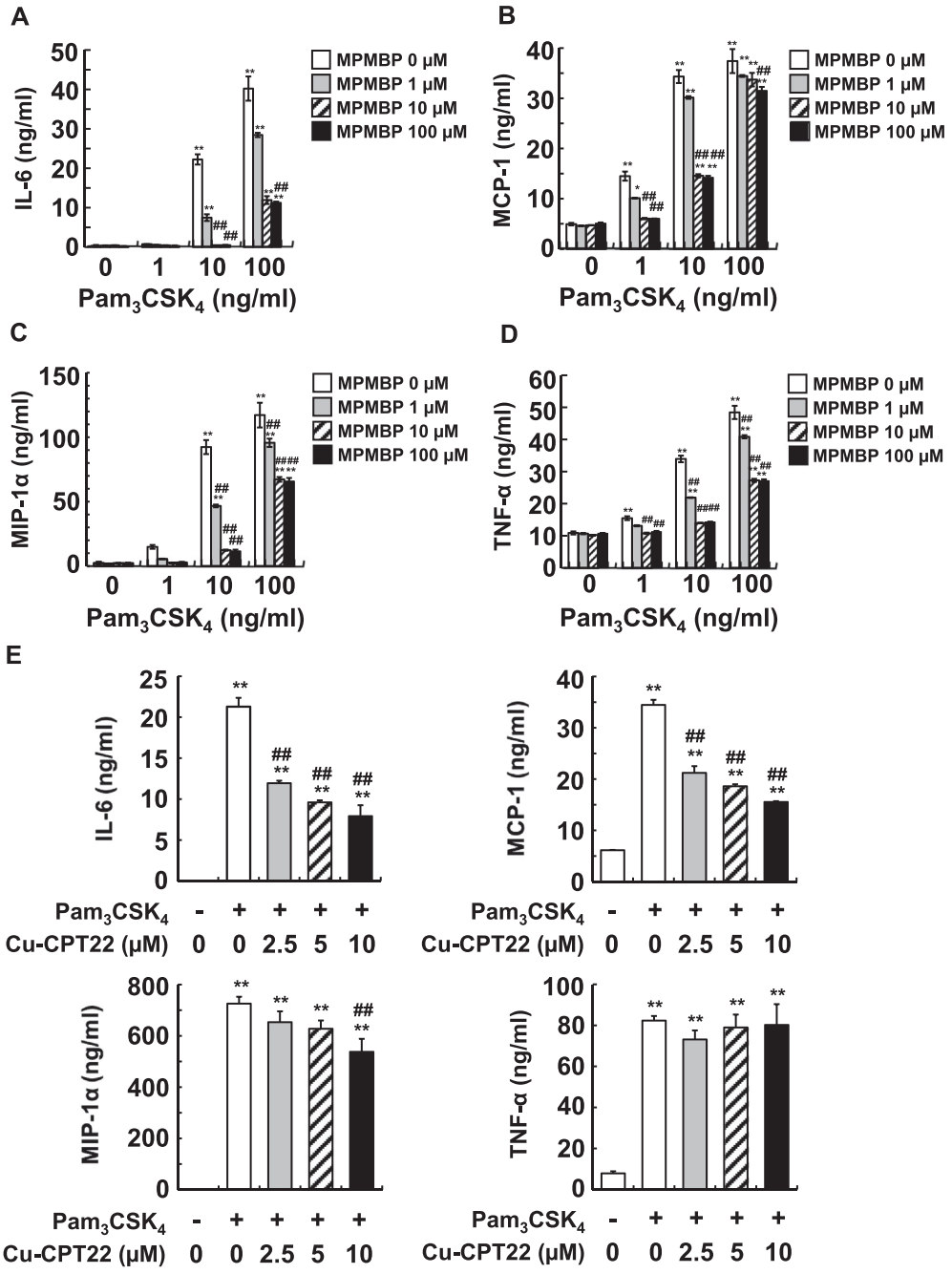

Fig. 2. Pretreatment of J774.1 cells with MPMBP down-regulates Pam_3CSK_4-induced proinflammatory cytokine production. J774.1 cells were preincubated without or with MPMBP (0, 1, 10, or 100 μM) for 5 min and then incubated without or with Pam_3CSK_4 (1, 10, or 100 ng/ml) for 24 h. Culture supernatants were collected and levels of IL-6 (A), MCP-1 (B), MIP-1α (C), and TNF-α (D) were measured by ELISA. (E) Pretreatment with Cu-CPT22. Results are presented as the mean (SE) of triplicate cultures from three independent experiments. $^{**}p < 0.01$ compared to vehicle. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to Pam_3CSK_4 alone.

added to each well, and the plate was incubated for 1 h with moderate agitation. Wells were then washed three times with washing buffer (100 mM phosphate buffer, pH7.5, 500 mM NaCl, and 1% Tween 20) and incubated with the relevant antibody (1:1000 in antibody binding buffer) for 1 h at room temperature. Subsequently, wells were incubated for 1 h with diluted HRP-conjugated antibody (1:1000 in antibody binding buffer) before addition of 100 μl developing solution (3,3′,5,5′ tetramethylbenzidine substrate solution diluted in 1% DMSO) and a 5-min incubation. The reaction was stopped by addition of 0.5 M H_2SO_4 solution. Absorbance was read on a spectrophotometer at 450 nm (reference: 655 nm).

2.6. Western blotting

Nuclear extracts were prepared with the TransAM™ nuclear extract kit according to the manufacturer's protocol. Nuclear extracts (15–30 μg) were fractionated on a 10–20% gradient SDS-PAGE gel (ATTO, Tokyo, Japan) and transferred to a PVDF membrane (GE Healthcare, Hercules, CA, USA) by electroblotting. The blot was blocked for 1 h with 0.5% (phosphos-JNK and JNK, p38, ERK1/2) or 2.5% (wt/vol) skim milk and 0.1% Tween 20 in TBS (TBS-T) and incubated overnight at 4 °C with primary antibody (1:1000). The blot was then washed four times with TBS-T, followed by incubation for 15 min with secondary antibody (1:10000) at room temperature. After washing, the blot was analyzed using EzWestLumi plus (ATTO) and

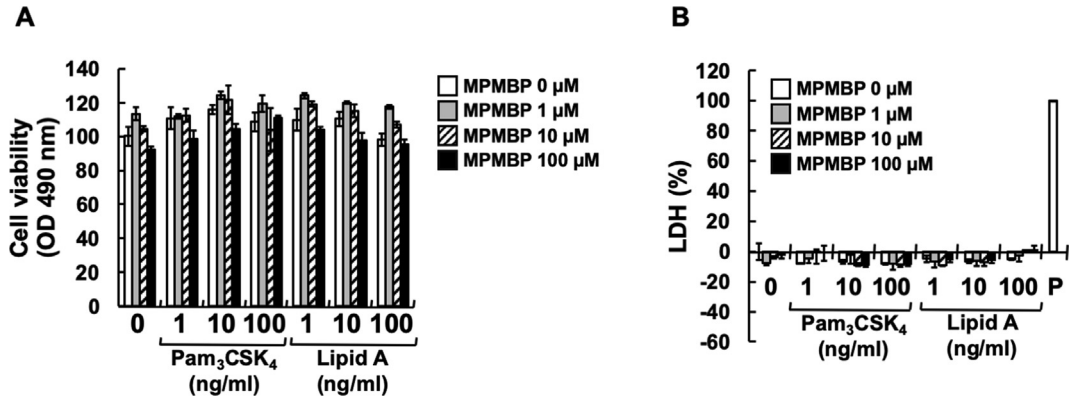

Fig. 3. Pretreatment of J774.1 cells with MPMBP does not induce cytotoxicity. J774.1 cells were preincubated without or with MPMBP (0, 1, 10, or 100 μM) for 5 min and then incubated without or with Pam_3CSK_4 (1, 10, or 100 ng/ml) or lipid A (1, 10, or 100 ng/ml) for 24 h. To assess cell viability (A), the optical density (OD) of cells incubated in medium alone without MPMBP pretreatment was set at 100%. LDH levels were also measured to assess cell death (B). Results are presented as the mean (SE) of triplicate cultures from three independent experiments.

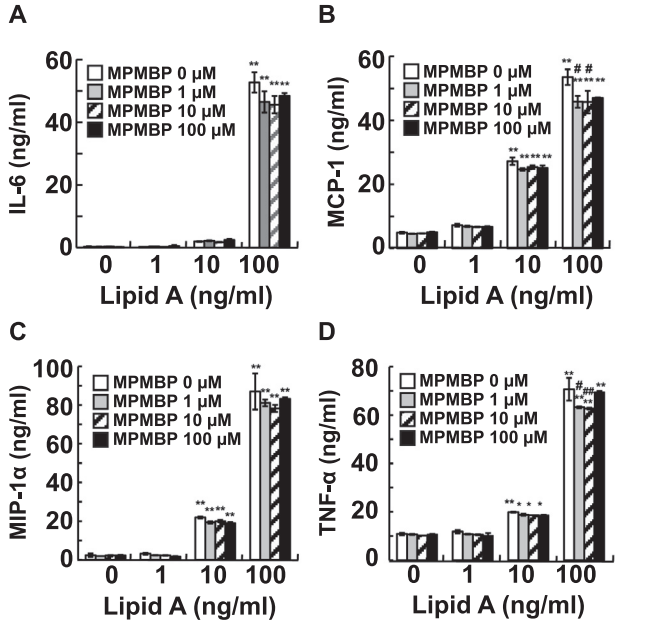

Fig. 4. MPMBP pretreatment does not inhibit lipid A-induced proinflammatory cytokine production. J774.1 cells were preincubated without or with MPMBP (0, 1, 10, or 100 μM) for 5 min and then incubated without or with lipid A (1, 10, or 100 ng/ml) for 24 h. Culture supernatants were collected and levels of IL-6 (A), MCP-1 (B), MIP-1α (C), and TNF-α (D) were measured by ELISA. Results are presented as the mean (SE) of triplicate cultures from three independent experiments. $^{**}p < 0.01$ compared to vehicle. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to lipid A alone.

Light Capture II (ATTO). The molecular mass of a given protein was estimated by comparison with the positions of standard proteins (Bio-Rad).

2.7. Data analysis

Data were analyzed using one-way analysis of variance (ANOVA) and the Bonferroni or Dunn method. Results are presented as the mean ± standard error (SE) of triplicate wells. $P < 0.05$ was considered significant. All experiments were performed at least three times independently.

3. Results

3.1. MPMBP down-regulates IL-6, MCP-1, MIP-1α, and TNF-α production by J774.1 cells incubated with Pam_3CSK_4, but is not cytotoxic

We first examined the effects of MPMBP pretreatment on Pam_3CSK_4-treated J774.1 cells. Treatment with MPMBP alone did not induce IL-6, MCP-1, MIP-1α, and TNF-α production at detectable levels (Fig. 2). However, pretreatment with 1 μM MPMBP for 5 min significantly down-regulated IL-6 production by J774.1 cells incubated with Pam_3CSK_4. In addition, MPMBP significantly down-regulated the production of MCP-1, MIP-1α, and TNF-α by J774.1 cells incubated with 1, 10, and 100 ng/ml Pam_3CSK_4. Pretreatment with Cu-CPT22, a TLR2 antagonist, down-regulated the production of IL-6, MCP-1, and MIP-1α, but not TNF-α, by J774.1 cells incubated with 10 ng/ml Pam_3CSK_4 for 24 h (Fig. 2E).

The possibility that MPMBP down-regulates proinflammatory cytokine production in J774.1 cells through cytotoxicity was also examined. As shown in Fig. 3, Pam_3CSK_4 did not reduce the viability of J774.1 cells, and pretreatment with MPMBP did not kill J774.1 cells incubated with Pam_3CSK_4. These results suggest that down-regulation of Pam_3CSK_4-induced cytokine production by MPMBP is not a result of cytotoxicity.

3.2. MPMBP does not down-regulate proinflammatory cytokine production by J774.1 cells incubated with lipid A

We next examined whether MPMBP down-regulates proinflammatory cytokine production induced by lipid A. As shown in Fig. 4, pretreatment with MPMBP did not down-regulate the lipid A-induced production of IL-6, MCP-1, MIP-1α, and TNF-α by J774.1 cells.

3.3. MPMBP down-regulates NF-κB p65, but not AP-1, activation by J774.1 cells incubated with Pam_3CSK_4

We further assessed whether MPMBP down-regulates Pam_3CSK_4-induced cytokine production by inhibiting NF-κB activation. The transcriptional activation domain exists on the C-terminal side of p65 and RelB and is required for the transcription of target genes. Treatment of J774.1 cells with 10 ng/ml Pam_3CSK_4 for 1 h induced the activation of NF-κB p65, but not RelB (Fig. 5A). Pretreatment with MPMBP inhibited NF-κB p65 translocation by up to about 50%, but not p52 and RelB translocation, in J774.1 cells incubated with 10 ng/ml Pam_3CSK_4 for 3 h (Fig. 5B). Moreover, MPMBP did not suppress lipid A-induced activation of NF-κB p65, p52, and RelB.

To determine whether MPMBP regulates AP-1 activation induced by

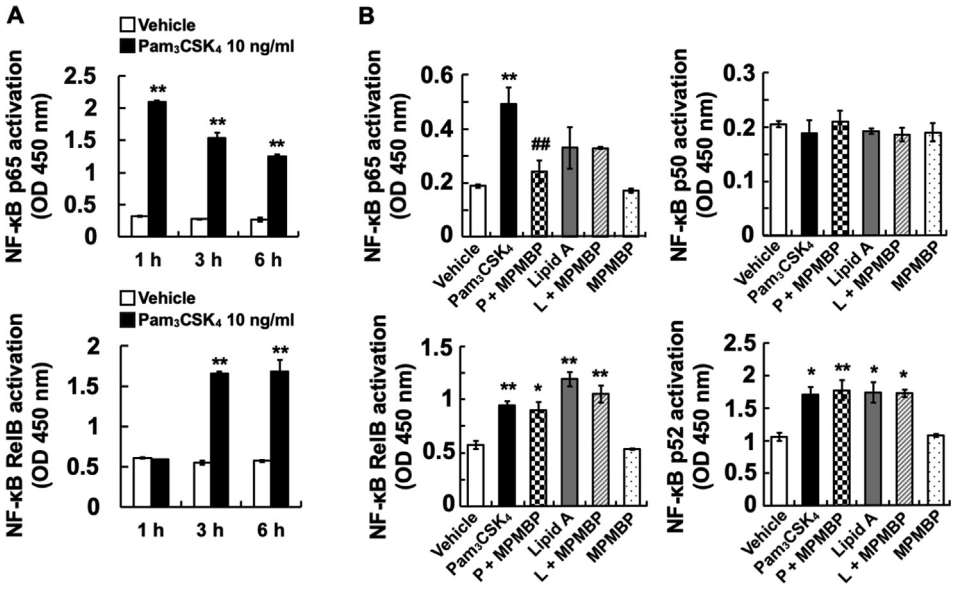

Fig. 5. MPMBP inhibits NF-κB p65 activation in J774.1 cells incubated with Pam_3CSK_4, but not lipid A. (A) Time-course of Pam_3CSK_4-induced NF-κB p65 (upper) and RelB (bottom) activation by J774.1 cells. (B) J774.1 cells were pre-incubated without or with 100 μM MPMBP for 5 min and then incubated without or with Pam_3CSK_4 (10 ng/ml) or lipid A (10 ng/ml) for 3 h. The DNA-binding activity of NF-κB p65 (upper, left), p50 (upper, right), RelB (bottom, left), and p52 (bottom, right) in nuclear extracts of J774.1 cells was determined using an ELISA-based transcription factor assay. Results are presented as the mean (SE) of triplicate cultures from three independent experiments. $^{**}p < 0.01$ compared to vehicle. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to Pam_3CSK_4 alone.

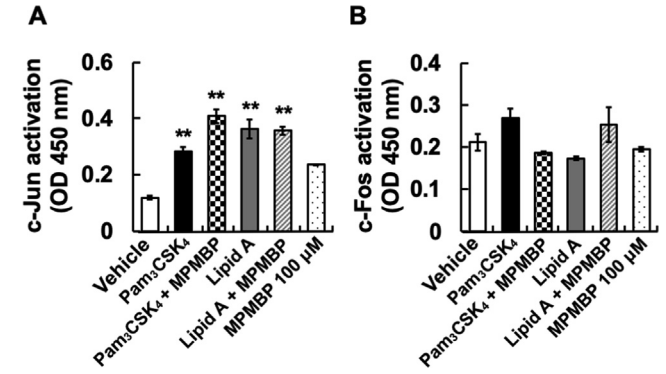

Fig. 6. MPMBP does not down-regulate AP-1 activation in J774.1 cells. J774.1 cells were preincubated without or with 100 μM MPMBP for 5 min and then incubated without or with Pam_3CSK_4 (10 ng/ml) or lipid A (10 ng/ml) for 3 h. (A) The DNA-binding activity of AP-1 c-Jun (A) and c-Fos (B) in nuclear extracts of J774.1 cells was determined using an ELISA-based transcription factor assay. Results are presented as the mean (SE) of triplicate cultures from three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to vehicle.

TLR ligands, c-Jun and c-Fos translocation was examined in J774.1 cells pretreated with MPMBP. As shown in Fig. 6, pretreatment with MPMBP did not inhibit Pam_3CSK_4-induced c-Jun and c-Fos translocation. These results suggest that MPMBP inhibits proinflammatory cytokine production in J774.1 cells upstream of NF-κB p65 activation in the TLR2 pathway.

3.4. MPMBP down-regulates JNK activation by J774.1 cells incubated with Pam_3CSK_4 but not lipid A

The effect of MPMBP on Pam_3CSK_4-induced activation of MAPK was also tested. As shown in Fig. 7, pretreatment with MPMBP inhibited Pam_3CSK_4-induced phosphorylation of JNK, but not lipid A-induced JNK activation. In addition, pretreatment with MPMBP did not inhibit the phosphorylation of p38 and ERK1/2 induced by TLR2 and TLR4 ligands. These results suggest that MPMBP inhibits proinflammatory cytokine production in J774.1 cells upstream of NF-κB activation via JNK in the TLR2 pathway.

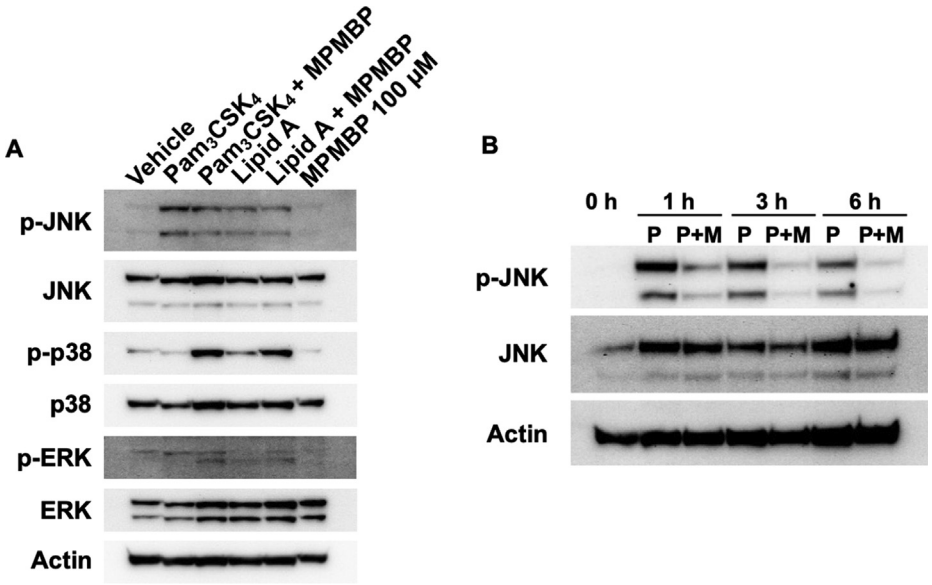

Fig. 7. MPMBP down-regulates Pam_3CSK_4-induced JNK activation, but not p38 MAPK and ERK activation, in J774.1 cells. (A) J774.1 cells were pre-incubated without or with 100 μM MPMBP for 5 min and then incubated without or with Pam_3CSK_4 (10 ng/ml) or lipid A (10 ng/ml) for 3 h. (B) Time-course of Pam_3CSK_4-induced JNK activation in J774.1 cells. Western blot analysis was performed for the indicated proteins from nuclear extracts. Representative blots of three independent experiments are shown.

4. Discussion

Non-NBPs, which include etidronate, clodronate, and tiludronate, are used in clinical settings despite their limited anti-bone resorptive effects. Unlike NBPs, non-NBPs do not inhibit the mevalonate pathway for cholesterol synthesis. However, both non-NBPs and NBPs are incorporated into the phosphate chain of ATP-containing compounds and have been suggested to improve cardiovascular disease [42–44]. Zoledronate, a NBP, has been reported to attenuate angiotensin II-induced abdominal aortic aneurysms by down-regulating the activation of JNK and NF-κB [45]. Moreover, etidronate has been shown to be effective in reducing the number of atherosclerotic aortic plaques [7]. However, an adverse effect associated with etidronate is osteomalacia, which results from etidronate's calcification inhibitory action [9,46,47].

Recent studies have found that infection by microorganisms can cause microvascular diseases by inducing the production of proinflammatory cytokines, including chemokines [48,49]. Chemokines facilitate atherosclerotic plaque development by promoting leukocyte extravasation. In this study, we found that MPMBP down-regulated IL-6, MCP-1, MIP-1α, and TNF-α production by J774.1 cells incubated with a TLR2 ligand by inhibiting NF-κB p65 activation. IκBα binds to the p65 subunit of the p50-p65 heterocomplex through ankyrin repeats and inhibits translocation of the p50-p65 complex, but not the p50 homodimer [50,51]. The activation of canonical NF-κB1 requires the degradation of IκBα by the ubiquitin-proteasome pathway. Thus, MPMBP may inhibit IκBα degradation by suppressing the ubiquitin-proteasome pathway through inhibition of ATP metabolism, which activates the ubiquitin-proteasome pathway, by functioning as an intracellular ATP analog [52,53].

IκB kinase (IKK) is an enzyme that phosphorylates IκBα and p100, which generates p52. The IKKα/IKKβ/IKKγ complex induces phosphorylation of IκBα, whereas the IKKα homodimer phosphorylates p100 and activates non-canonical NF-κB signaling [54]. Our data suggests that MPMBP does not down-regulate lipid A-induced NF-κB p65 activation. This is likely because, in addition to the MyD88/IKKγ-dependent pathway, TLR4 signaling also includes the MyD88-independent IKKε pathway, which is not involved in TLR2 signaling [55,56].

Signaling through TLR2 and TLR4 induces NF-κB activation by degrading IκBα via ubiquitination. Some E3 ubiquitin ligases promote the polyubiquitination and degradation of IκBα and TLR signaling components. However, other types of ubiquitin ligases are also involved. Casitas B-cell lymphoma-b (CBLB) degrades TLR4 and inhibits TLR-dependent production of proinflammatory cytokines [57–60]. Itch, an E3 ubiquitin ligase, has been shown to mediate osteoclast formation and bone loss, and this effect was prevented by zoledronic acid, an NBP [61]. Thus, MPMBP might inhibit the degradation of IκBα by acting on E3 ubiquitin ligases. Praja2, another E3 ubiquitin ligase, promotes TLR2 signaling and M1 macrophage polarization, which promotes inflammation, by activating JNK and p38 pathways through ubiquitination of malignant fibrous histiocytoma amplified sequence 1 (MFHAS1), a predicted oncoprotein [60,62,63]. MFHAS1 determines the difference between TLR2 and TLR4 signaling because MFHAS1 inhibits TLR4 signaling by inhibiting c-Jun [64]. This suggests the possibility that MPMBP up-regulates MFHAS1 expression and function and inhibits TLR2 signaling in J774.1 cells. In addition, inhibition of M1 phenotype has been suggested as an effective therapeutic strategy in ischemic stroke [65].

However, our results demonstrate that Cu-CPT22, a specific inhibitor of TLR2, down-regulated the production of IL-6, MCP-1, and MIP-1α, but not TNF-α, by J774.1 cells incubated with Pam_3CSK_4. MPMBP could inhibit TLR2 agonist-induced production of TNF-α, which plays a crucial role in activating osteoclasts [17]. It is important that MPMBP did not down-regulate TLR4 ligand-induced TNF-α production because the cytokine is also an important cytokine that protects against opportunistic pathogen, such as *Candida albicans*. According to one study, Cu-CPT22, unlike MPMBP, inhibits the activation of p38, but not JNK, in the kidneys of diabetic rats [66]. Cu-CPT22 has been also used as an inhibitor of Th1 differentiation, electroacupuncture analgesia, and inflammation induced by TLR2 agonists but not an anti-bone resorptive agent [67–69].

MPMBP was found to down-regulate the phosphorylation of JNK induced by a TLR2 ligand. BPs induce gastric damage by inhibiting ATP-sensitive potassium (K_{ATP}) channel signaling [3]. K_{ATP} channel block inhibits TLR2-mediated NF-κB activation by suppressing the activation of JNK [40]. Thus, MPMBP could potentially serve as an arteriosclerotic agent since up-regulated K_{ATP} channels increase inflammation and plaque vulnerability in macrophages [70]. The inhibition of TLR2/JNK/NF-κB signaling also reportedly attenuates the inflammatory response induced by amyloid β peptide [71]. Anisalcohol, a flavor compound contained in vanilla, inhibited neuro-inflammatory toxicity by suppressing the phosphorylation of JNK and NF-κB activation in BV2 microglia [72]. However, JNK deficiency up-regulates CD23 expression and antifungal response [73].

In conclusion, our results suggest that MPMBP can supplement the pharmacological effects on proinflammatory cytokine production in J774.1 cells by suppressing NF-κB p65 activation in the TLR2, but not TLR4, pathway.

CRediT authorship contribution statement

Riyoko Tamai: Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Keiko Suzuki:** Investigation, Writing - review & editing, Resources, Project administration, Funding acquisition. **Izumi Mashima:** Funding acquisition. **Yusuke Kiyoura:** Funding acquisition.

Declaration of Competing Interest

Tamai R, Mashima I, and Kiyoura Y declare no potential conflicts of interest with respect to the authorship and/or publication of this article. Tohoku University and Keiko Suzuki hold a patent on the anabolic effects of MPMBP (patent #:2010-527665).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.106085.

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