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Specialized Pro-Resolving Mediators Do Not Inhibit the Synthesis of Inflammatory Mediators Induced by Tumor Necrosis Factor-α in Synovial Fibroblasts

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ABSTRACT

Background Tumor necrosis factor (TNF)-α, a proinflammatory cytokine, is involved in the pathogenesis of rheumatoid arthritis (RA). The omega-3 unsaturated fatty acid-derived metabolites resolvin (Rv) D1, RvE1, and maresin-1 (MaR1) have been reported as anti-inflammatory lipid mediators and are known as specialized pro-resolving mediators (SPMs). In this study, we aimed to investigate the anti-inflammatory effects of SPMs on TNF-α-induced responses in synovial fibroblasts.

Methods We investigated the effects of SPMs on gene expression and/or production of cyclooxygenase-2 (COX-2), microsomal prostaglandin E synthase-1 (mPGES-1), interleukin (IL)-6, and matrix metalloproteinase (MMP)-3, which are involved in TNF-α-induced synovitis in RA or OA synovial fibroblasts, by quantitative real-time PCR. We also investigated the effects of SPMs on the mitogen-activated protein kinase (MAPK) signaling pathway by western blotting. Anti-inflammatory effects of SPMs were evaluated by applying SPMs to cultured synovial fibroblasts, followed by TNF-α stimulation.

Results The induction of COX-2, mPGES-1, IL-6, and MMP-3 by TNF-α in synovial fibroblasts was not suppressed by omega 3-derived SPMs regardless of their origin such as RA or OA. SPMs had no effect on lipid mediator receptor gene expression induce by TNF-α and did not inhibit the TNF-α-activated MAPK signaling pathway. The production of COX-2 and IL-6 protein was significantly decreased by p38 inhibitor.

Conclusion Despite reports on the anti-inflammatory effect of omega 3-derived SPMs, its anti-inflammatory effect on TNF-α-induced responses was not observed in synovial fibroblasts. The reason may be that SPMs have no suppressive effect on p38 activation, which plays an important role in the production of inflammatory cytokines in synovial fibroblasts.

Key words fatty acids, unsaturated; resolvin D1; rheumatoid arthritis; synoviocytes; tumor necrosis factor-α

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized primarily by synovitis of the joint synovium. In addition, subsequent progressive bone and cartilage destruction greatly reduces a patient's quality of life. The major pathological condition is inflammation caused by tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 derived from macrophages and synovial cells in synovial tissues. Prostaglandins (PGs), the expression of which is enhanced by the stimulation of these cytokines, play an important role in RA pathogenesis.[1–5] PGE_2 strongly promotes pathology, including processes related to synovial cell proliferation, angiogenesis, and osteoclast activation, in RA.[6] Synovial fibroblasts are key players in the progression of RA, inducing inflammation via the secretion of various cytokines and chemokines, such as IL-8 or regulated on activation, normal T cell expressed and secreted (RANTES), which act as chemoattractants, promoting the invasion of immune cells, such as macrophages or neutrophils. Furthermore, synovial fibroblasts contribute to cartilage degradation, as well as inducing angiogenesis, pannus hyperplasia, and bone erosion.[1, 7] The

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Abbreviations: BLT1, leukotriene B4 receptor 1; CMKLR1, chemerin chemokine-like receptor 1; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FPR2, N- formyl peptide receptor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IgG, immunoglobulin G; IL, interleukin; JNK, c-Jun N-terminal kinase; LXA4, lipoxin A4; MAPK, mitogen-activated protein kinase; MaR1, maresin-1; MEK, mitogen activated protein kinase; MMP, matrix metalloproteinase; mPGES-1, microsomal prostaglandin E synthase-1; NF-κB, nuclear factor-kappa B; NSAIDs, non-steroidal anti-inflammatory drugs; OA, osteoarthritis; p-, phospho-; PG, prostaglandin; PUFA, polyunsaturated fatty acid; PVDF, poly vinylidene di-fluoride; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor-κB ligand; Rv, resolvin; SAPK, stress-activated protein kinase; SPM, specialized pro-resolving mediator; TBS-T, tris-buffered saline-Tween; TNF, tumor necrosis factor; ω, omega

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production of proinflammatory cytokines and receptor activator of nuclear factor-κB ligand (RANKL) by synovial fibroblasts directly promotes osteoclastogenesis.

PGs are produced via the arachidonic acid cascade. The fatty acid arachidonic acid is converted to PGH_2 by cyclooxygenase (COX). Then, PGH_2 is transformed by specific synthase into PGs (PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$) and thromboxane (TX) A_2.[8] PGE_2 is converted from PGH_2 by microsomal prostaglandin E synthase-1 (mPGES-1). Based on these factors, COX-2 and mPGES-1 have a strong influence on the onset of synovitis.[9]

Polyunsaturated fatty acids (PUFAs) consist of omega (ω)-3 and -6 types, depending on the position of the double bond in the molecule. ω-6 PUFA-derived lipid metabolites, such as PGs and leukotrienes, play a central role in the early stages of the inflammatory response. ω-3 PUFAs, which are contained in many fish oils, represented by ω-3 eicosapentaenoic acid and docosahexaenoic acid, have anti-inflammatory and cardiovascular protective effects.[10–13] In addition, ω-3 PUFA-derived metabolites are transiently produced via the activation of leukocytes at the site of inflammation, and exert a local anti-inflammatory effect.[14] Resolvin (Rv) E1, RvD1, and maresin-1 (MaR1) are biosynthesized from ω-3 essential fatty acids, respectively, and collectively termed specialized pro-resolving mediators (SPMs) based on their potent pro-resolving actions. These SPMs act as potent regulators of neutrophil infiltration, cytokine and chemokine production, and the clearance of apoptotic neutrophils by macrophages, which promote a return to tissue homeostasis via their specific receptors.[15] These SPMs produced at the site of inflammation resolve inflammation.[13, 16–21] In addition, it has been reported that RvE1 suppresses osteoclast differentiation in joint regions.[22–24] We found that RvE1 suppresses the IL-17-induced receptor activator of nuclear factor kappa-B ligand (RANKL) expression in osteoblasts, and further suppresses RANKL-induced osteoclast and cell differentiation to inhibit osteoclast formation and bone resorption.[25] In an adjuvant-induced arthritis model rat, the intraperitoneal administration of 17 (R)-hydroxydocosahexaenoic acid (HDoHE), which is a precursor of RvD1, exerted a pain-relieving effect and a decrease in TNF-α and IL-1β locally in the joint.[26] RvD1 levels decreased while connective tissue growth factor (CTGF) levels, which promotes synovial fibroblasts, pannus formation, and the damage of cartilage as well as bone, increased in the serum of patients with RA, and RvD1 suppresses pannus formation via decreasing CTGF by upregulation of miRNA-146a-5p in collagen-induced arthritis.[27] In another study, patients with RA showed lower lipoxin A4 (LXA4), RvD1, and RvE1 levels compared to healthy individuals; however, the levels of these SPMs are not related RA activites.[28] Randomized controlled trials in patients with early RA have indicated that groups administered fish oil had longer remissions times and fewer transitions to second-line treatment than the untreated groups.[29, 30] On the other hand, the intake of dietary long-chain ω-3 PUFAs was found to decrease the risk of developing RA.[31] Proudman et al.[29] reported that a high intake of fish oil increased the rate of American College of Rheumatology remission compared to a low intake of fish oils. However, no difference was observed in the Disease Activity Score-28 for RA with erythrocyte sedimentation rate (ESR) scores between the control and fish oil groups. In a meta-analysis, Goldberg et al.[32] reported on the effects of ω-3 PUFAs on RA or joint pain secondary to inflammatory bowel disease and dysmenorrhea. Dietary supplementation with PUFAs reduced patient-reported joint pain intensity, morning stiffness duration, painful and/or tender joints, and the use of non-steroidal anti-inflammatory drugs (NSAIDs), whereas physician-assessed pain did not change. The results of another meta-analysis indicated a reduction in NSAID consumption with ω-3 PUFA use.[33] However, the tender and swollen joint count, morning stiffness, and physical function did not improve in a statistically significant manner.[33]

Negative large-scale test results have been reported regarding the anti-inflammatory effects of SPMs derived from ω-3 fatty acids in recent years. The intake of ω-3 fatty acids derived from marine organisms was not found to have a significant protective effect on the incidence of cardiovascular disease and cancer.[13, 34] Another study found that ω-3 fatty acids did not significantly change the incidence of cardiovascular disease compared to placebo.[35]

Thus, although SPMs are expected to promote the resolution of inflammation and may help to prevent the progression of an acute inflammatory response to chronic inflammation in patients with arthritis, their anti-inflammatory effects remain controversial. Furthermore, the effects of SPMs on synovial fibroblasts, key players in RA patients, remain unclear. In this study, we investigated the effects of ω-3 lipid mediators on TNF-α-induced synthesis of inflammatory mediators in synovial fibroblasts.

MATERIALS AND METHODS

Cell culture and reagents

MH7A (Riken Bio Resource Research Center, Tsukuba, Japan) is a cell line isolated from intra-articular soft

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Table 1. Primers for real-time PCR

Genes	Assay ID[a]	RefSeq	Exon boundary	Product length (bp)
COX-2	Hs00153133	NM_000963	5–6	75
mPGES-1	Hs01115610	NM_004878.4	2–3	136
IL-6	Hs00985639	NM_000600.4	2–3	66
MMP-3	Hs00968305	NM_002422.4	6–7	126
CMKLR1	Hs01081979	NM_001142343.1	4	73
FRP2	Hs02759175	NM_001005738.1	2	98
GAPDH	Hs02758991	NM_001256799.2	6–7	93

CMKLR1, Chemokine like receptor 1; COX-2, Cyclooxygenase-2; FRP2, N-formyl peptide receptor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, Interleukin-6; MMP-3, matrix metalloproteinase-3; mPGES-1, microsomal prostaglandin E synthetase-1. a: TaqMan Gene Expression Assay (Applied Biosystems).

tissues of the knee joints of patients with RA and established by transfection with the SV40 T antigen.[36] HT91989516 (National Institute of Biomedical Innovation, Ibaraki, Japan) was extracted during artificial knee joint replacement surgery in patients with knee osteoarthritis (OA). MH7A cells were cultured in RPMI-1640 (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) with 10% fetal bovine serum (FBS), 100 µg/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere containing 5% carbon dioxide. OA fibroblasts were cultured in D-MEM (Fujifilm Wako Pure Chemical Corporation) with 10% FBS, 100 µg/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified 5% carbon dioxide atmosphere. RvD1 and MaR1 were purchased from Cayman Chemical Company (Ann Arbor, MI), and RvE1 was purchased from Toronto Research Chemicals (Toronto, Canada). These were pre-added 1 h before recombinant human TNF-α (R&D systems, Minneapolis, MN) stimulation, according to previous reports.[16, 37–39] MEK inhibitor (U0126) was purchased from Cell Signaling Technology (Danvers, MA). JNK inhibitor (SP600125) was purchased from Selleck Chemicals (Houston, TX), and a p38 inhibitor (SB202190) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). These were pre-added 1 h before TNF-α stimulation.

Quantitative real-time PCR

MH7A cells or OA fibroblasts were plated in 6-well plates at a density of 1.0×10^5 cells/mL. Total RNA was isolated from cultured cells using the RNeasy Plus mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. The mRNA was reverse-transcribed into cDNA using the Super Script VILO Master Mix (Invitrogen, Carlsbad, CA). The resultant cDNA was subjected to real-time PCR using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA). Specific primers (Table 1) were purchased from Thermo Fisher Scientific. PCR was conducted using the TaKaRa PCR Thermal Cycler Dice system (Takara Bio, Kusatsu, Japan) under the following conditions: initial holding at 25°C for 10 min, 42°C for 60 min, and 85°C for 5 min. Real-time PCR was performed on a ViiA7 Real-Time PCR system (Thermo Fisher Scientific) for 40 cycles at 95°C for 1 s and 60°C for 20 s. The expression levels of COX-2, mPGES-1, IL-6, and MMP-3 were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All real-time PCR experiments were conducted in triplicate and analyzed using the comparative $2^{-\Delta\Delta Ct}$ relative quantification cation method.

Enzyme-linked immunosorbent assay

The amounts of PGE_2 and IL-6 in the culture medium were determined using a commercially available enzyme-linked immunosorbent assay kit (Enzo Life Sciences, Farmingdale, NY) (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The data were converted to pg/mL. Finally, duplicate assays were conducted on each sample and the absorbance was recorded at 405 nm.

Western blotting

MH7A cells or OA fibroblasts were cultured in 60-mm dishes at a density of 1.0×10^6 cells/mL. TNF-α was added after culturing to 70–80% confluency. After washing with cold phosphate buffered saline, radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl, pH 7.4, 1% Triton-X100, 0.25% sodium deoxycholate, 150 mmol/L sodium chloride, 1 mmol/L ethyleneglycol-bis (β-aminoethyleter)-N, N, N, N-tetraacetic acid, 0.1% SDS, 0.5 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and protease inhibitor cocktail (Merck Kgaa, Darmstadt, Germany)] and collected using a cell

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lifter. The collected cells were subjected to rolling at 4°C for 10 min and then centrifuged at 13,000 rpm and 4°C for 5 min. The protein concentration in the supernatant was measured using a standard assay (Bio-Rad Laboratories, Irvine, CA). The samples were adjusted in concentration with radioimmunoprecipitation assay buffer (Fujifilm Wako Pure Chemical Corporation) and 4× sample buffer (Bio-Rad Laboratories) before separating by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Thereafter, the protein was transferred to a polyvinylidene difluoride (PVDF) blotting membrane (GE Healthcare Life Sciences, Marlborough, MA) at 100 V for 75 min. To prevent the nonspecific reaction of the transferred PVDF membrane, blocking was conducted for 1 h with 5% skim milk. The primary antibody, diluted with 5% skim milk, was incubated overnight at 4°C. The following primary antibodies were used: COX-2 antibody (1:200; Cayman Chemical), mPGES-1 antibody (1:200; Cayman Chemical), IL-6 antibody (1:1000; Cell Signaling Technology, Danvers, MA), MMP-3 antibody (1:200; Santa Cruz Biotechnology, Dallas, TX), β-actin antibody (1 μg/mL) (Medical and Biological Laboratories, Nagoya, Japan), chemokine-like receptor 1 (CMCKLR1) antibody (1:100; Cayman Chemical), leukotriene B_4 receptor 1 (BLT1) antibody (1:200; Cayman Chemical), and formyl peptide receptor 2 (FPR2) antibody (1:200; Santa Cruz). To study signal transduction, the primary antibodies used were phospho-p42/p44 [p-extracellular signal-regulated kinase (ERK)1/2] antibody (1:2000; Cell Signaling Technology), p42/p44 (ERK1/2) antibody (1:1000; Cell Signaling Technology), p-p38 antibody (1:1000, Cell Signaling Technology), p38 antibody (1:1000; Cell Signaling Technology), p- stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) antibody (1:1000; Cell Signaling Technology), SAPK/JNK antibody (1:1000; Cell Signaling Technology), p-Akt antibody (1:1000; Cell Signaling Technology), Akt antibody (1:1000; Cell Signaling Technology), p-NF-κB p65 antibody (1:1000; Cell Signaling Technology), and NF-κB p65 antibody (1:1000; Cell Signaling Technology). After washing the PVDF membrane three times with Tris-buffered saline-Tween (TBS-T), a secondary antibody diluted with 5% skim milk was reacted for 1 h at room temperature. The secondary antibodies used were: goat anti-mouse immunoglobulin G (IgG) antibodies (1:2000; Santa Cruz Biotechnology) and anti-rabbit IgG antibodies (1:2000, Cell Signaling Technology). After washing the PVDF membrane three times with TBS-T, enhanced chemiluminescence was conducted using Amersham ECL Prime kit (GE Healthcare Life Science) and detected with Image Quant LAS4000 (GE Healthcare Life Science).

Statistical analysis

Data were analyzed using GraphPad Prism 7 (GraphPad Software, San Diego, CA). Multiple groups were compared using one-way analysis of variance (ANOVA) and Dunn's multiple comparison test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

TNF-α induced COX-2, mPGES-1, IL-6, and MMP-3 mRNA expressions as well as PGE_2 and IL-6 release in MH7A cells

Since TNF-α is an important cytokine that induces PGE_2 release via increasing COX-2 and mPGES-1 expression in synovial fibroblasts,[40] we examined the effects of TNF-α on the expression of COX-2, mPGES-1, IL-6, and MMP-3 mRNA using real-time reverse transcription PCR (RT-PCR), and PGE_2 and IL-6 protein release using enzyme-linked immunosorbent assay in MH7A cells. Treatment with TNF-α (10 ng/mL) enhanced the expression of COX-2, mPGES-1, and IL-6 mRNA in MH7A cells (Figs. 1A–C). The maximal TNF-α-enhancing effects were observed after 12 h of culture. TNF-α increased the MMP-3 mRNA expression in a time-dependent manner (Fig. 1D). When the cells were treated with TNF-α (10 ng/mL) for 36 h, the PGE_2 and IL-6 concentrations in the culture medium increased in a time-dependent manner (Figs. 1E and F).

When the MH7A cells were stimulated with TNF-α at various concentrations (0–100 ng/mL) for 12 h, the COX-2, mPGES-1, IL-6, and MMP-3 mRNA levels were enhanced in a concentration-dependent manner (Figs. 2A–D). COX-2, mPGES-1, and IL-6 proteins in the MH7A cells were enhanced by treatment with TNF-α for 24 h in a concentration-dependent manner, according to the results of western blot analysis (Figs. 2E, F, and G). By contrast, MMP-3 protein was not enhanced (Fig. 2H).

Effects of ω-3 PUFA-derived lipid mediators on COX-2, mPGES-1, IL-6, and MMP-3 mRNA expressions in MH7A cells

SPMs have been reported to exert pro-resolving and cartilage-protective actions in response to inflammatory arthritis, as well as a reduction of pro-inflammatory cytokine production *in vivo*.[17, 19, 26, 41] Therefore, we evaluated the effects of SPMs on TNF-α stimulation in synovial fibroblasts. To this end, MH7A cells were cultured with or without 10 nM and 100 nM RvE1, RvD1, and MaR1 in the presence of 10 ng/mL TNF-α for 12 h. The mRNA expression levels of COX-2,

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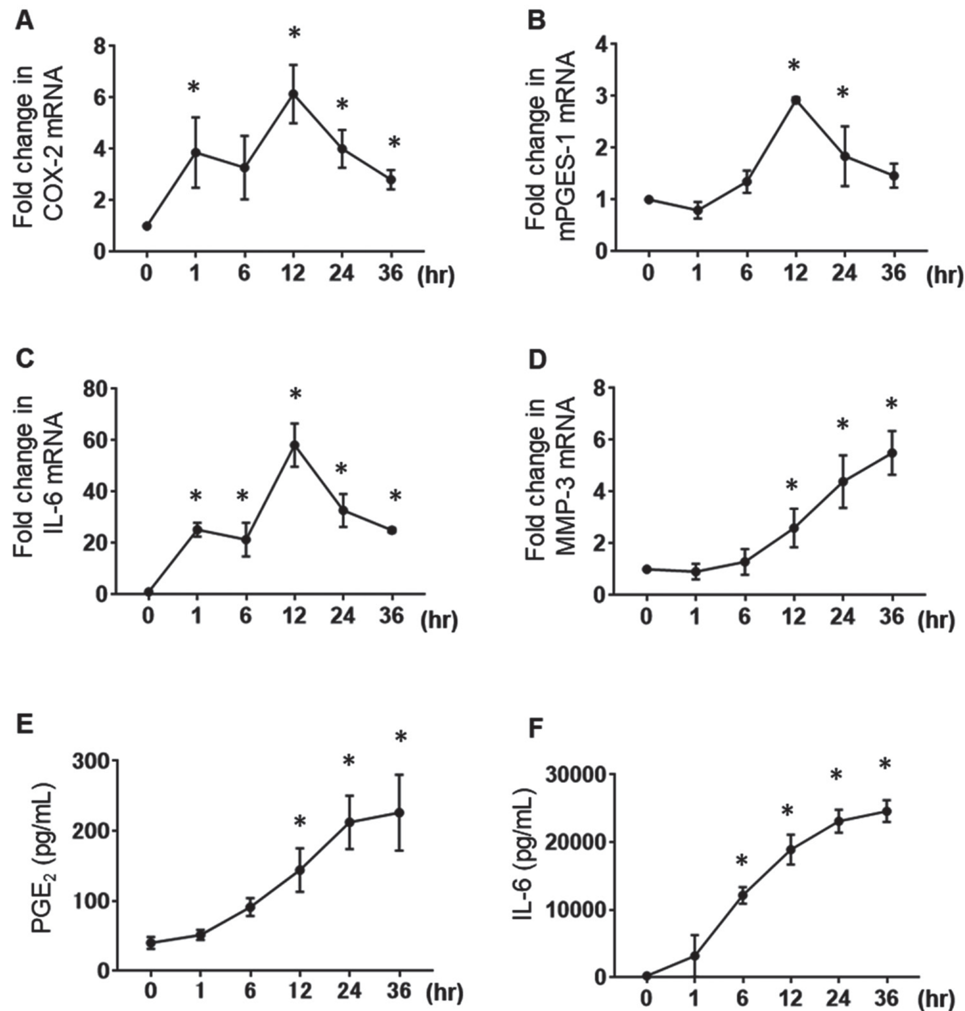

Fig. 1. Effects of TNF-α on MH7A cells. MH7A cells were stimulated with 10 ng/mL TNF-α for 1, 6, 12, 24, or 36 h. The mRNA expression levels of COX-2 (**A**), mPGES-1 (**B**), IL-6 (**C**), and MMP-3 (**D**) were determined by real-time PCR. Data are presented as the mean ± SEM of three independent experiments (*$P < 0.05$ vs. 0 h). The PGE_2 (**E**) and IL-6 (**F**) production levels in MH7A cells stimulated with 10 ng/mL TNF-α for 1, 6, 12, 24, or 36 h were determined using enzyme-linked immunosorbent assay. Data are expressed as the mean ± SD ($n = 3$; *$P < 0.05$ vs. 0 h).

mPGES-1, IL-6, and MMP-3 were determined by real-time PCR. The mRNA expression levels of COX-2 (Fig. 3A), mPGES-1 (Fig. 3B), IL-6 (Fig. 3C), MMP-3 (Fig. 3D) were increased by TNF-α; however, RvE1 failed to decrease the expression of these inflammatory markers (Figs. 3A–D). Similar results were observed for RvD1 (Figs. 3E–H) and MaR1 (Figs. 3I–L). These mRNA expression levels by SPMs without TNF-α were not increased in MH7A cells (Supplementary Fig. S1).

Effects of ω-3 PUFA-derived lipid mediators on COX-2, mPGES-1, IL-6, and MMP-3 mRNA expressions in synovial fibroblasts derived from OA

Since MH7A cells were derived from RA patients, we examined the effects of SPMs on synovial fibroblasts from patients with OA. The mRNA expression levels of COX-2 (Fig. 4A), mPGES-1 (Fig. 4B), IL-6 (Fig. 4C), and MMP-3 (Fig. 4D) were increased by TNF-α stimulation. However, these inflammatory markers were not inhibited by RvE1 (Figs. 4A–D). Similar results were observed for RvD1 (Figs. 4E–H) and MaR1 (Figs. 4I–L). Similar to MH7A cells, the anti-inflammatory effect of ω-3 SPMs was not observed in OA synovial fibroblasts. These mRNA expression levels by SPMs without TNF-α were not increased in OA fibroblasts (Supplementary Fig. S2). Thus, this result suggests that SPMs do not have an anti-inflammatory effect in synovial fibroblasts regardless of their origin such as RA or OA.

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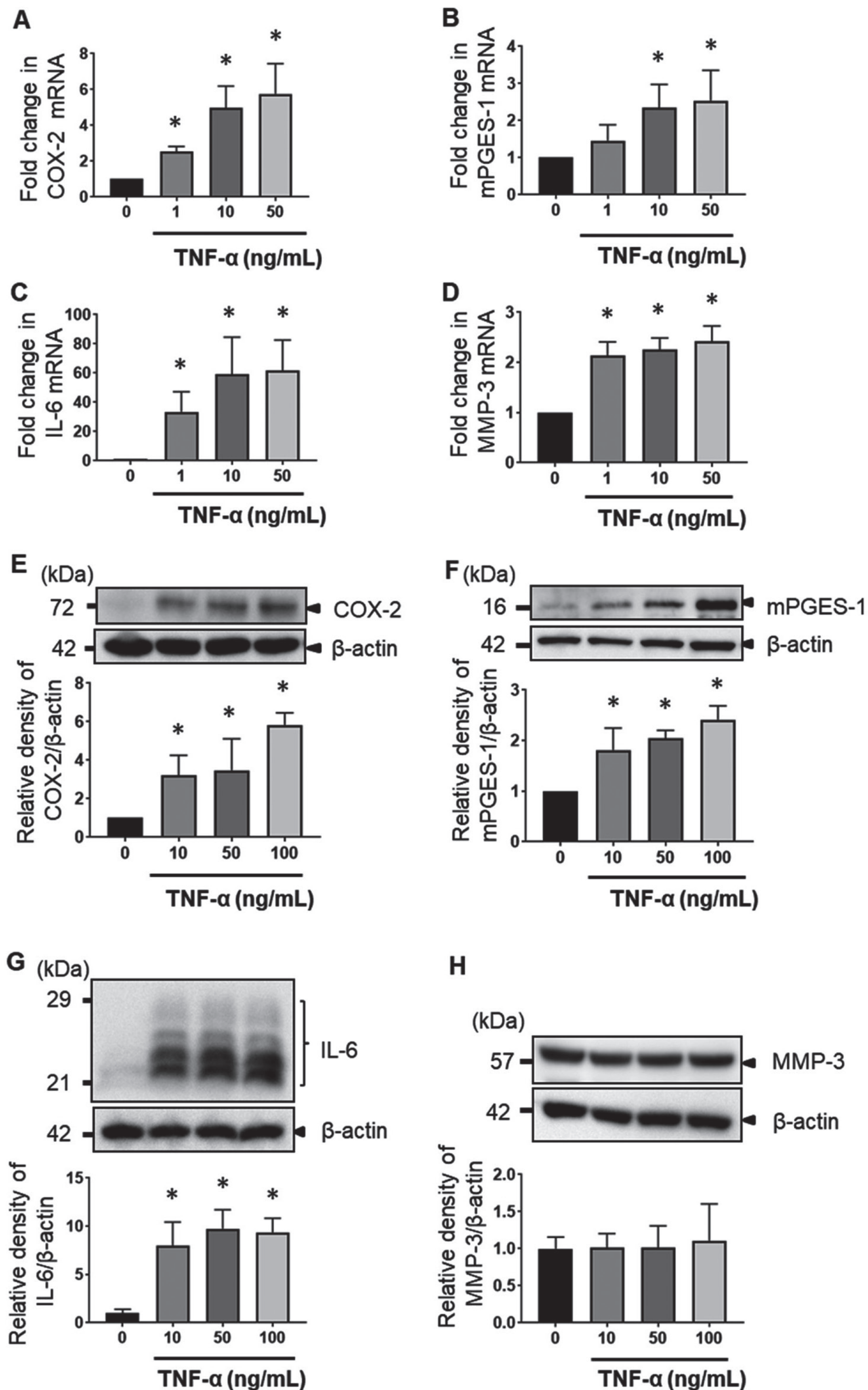

Fig. 2. MH7A cell response with increasing dose of TNF-α. MH7A cells were cultured in the presence of 1, 10, 50, and 100 ng/mL TNF-α. The mRNA expression levels of COX-2 (**A**), mPGES-1 (**B**), IL-6 (**C**), and MMP-3 (**D**) were determined by real-time PCR. The expression of COX-2 (**E**), mPGES-1 (**F**), IL-6 (**G**), and MMP-3 (**H**) in cell lysates was determined by western blot analysis. The data are expressed as the relative protein expression of targets/β-actin. Data are presented as the mean ± SEM of three independent experiments (*P < 0.05 vs. untreated).

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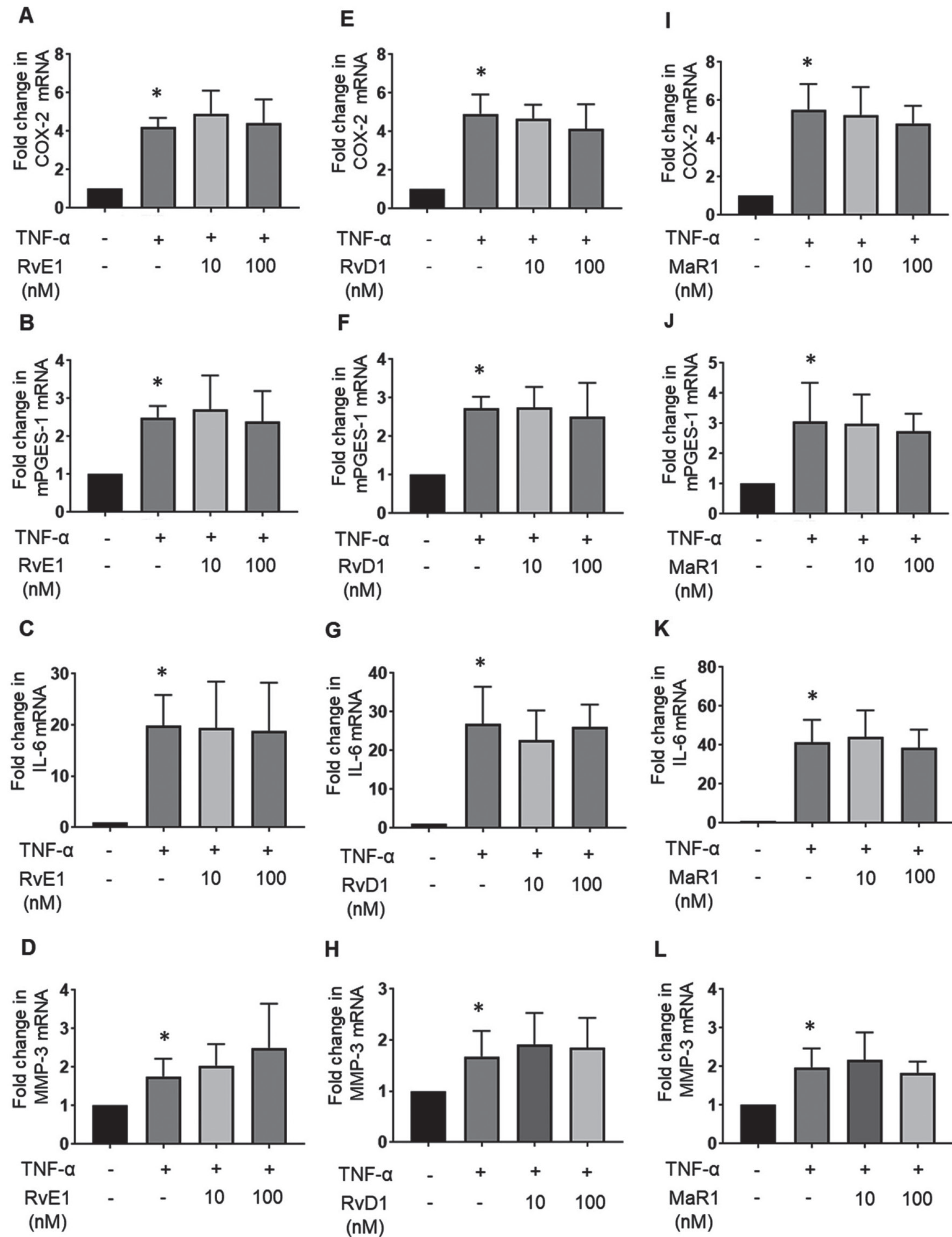

Fig. 3. Effects of SPMs on TNF-α-treated MH7A cells. MH7A cells were cultured with or without 10 nM and 100 nM RvE1, RvD1, and maresin-1 (MaR1) in the presence of 10 ng/mL TNF-α for 12 h. The effects of RvE1 on the following substances were examined using real-time PCR: the mRNA expression levels of COX-2 (**A**), mPGES-1 (**B**), IL-6 (**C**), and MMP-3 (**D**) or RvD1 on COX-2 (**E**), mPGES-1 (**F**), IL-6 (**G**), and MMP-3 (**H**) or MaR1 on COX-2 (**I**), mPGES-1 (**J**) and IL-6 (**K**), and MMP-3 (**L**). The plots represent three independent experiments. Data are presented as the mean ± SEM of three independent experiments (*$P < 0.05$ vs. untreated).

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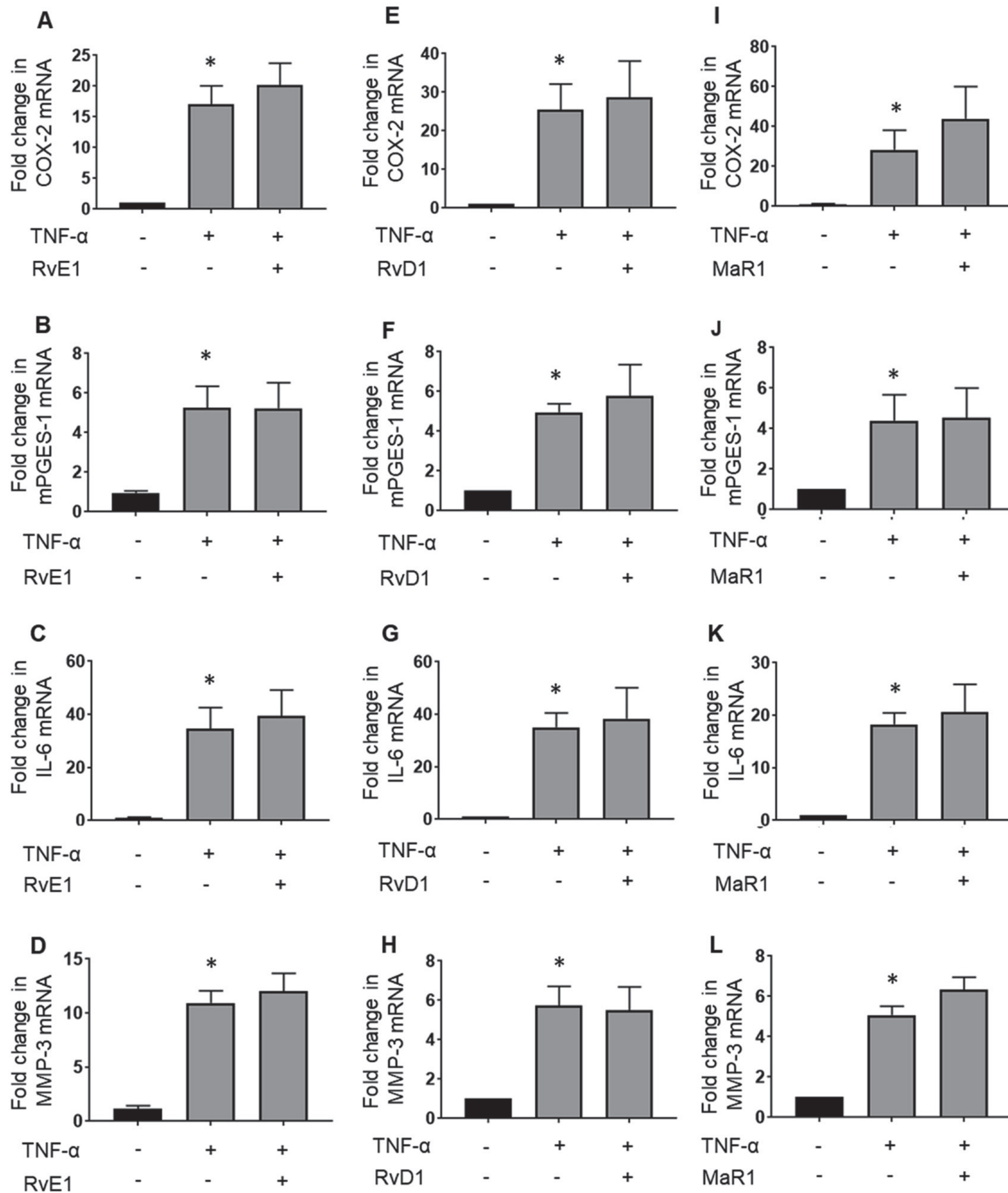

Fig. 4. Effects of SPMs on osteoarthritis-derived synovial fibroblasts treated with TNF-α. Osteoarthritis-derived synovial fibroblasts were cultured with or without 100 nM RvE1, RvD1, and MaR1 in the presence of 10 ng/mL TNF-α for 12 h. The effects of RvE1 on the following substances were examined using real-time PCR: the mRNA expression levels of COX-2 (**A**), mPGES-1 (**B**), IL-6 (**C**), MMP-3 (**D**), or RvDl on COX-2 (**E**), mPGES-1 (**F**), IL-6 (**G**), and MMP-3 (**H**) or MaR1 on COX-2 (**I**), mPGES-1 (**J**), IL-6 (**K**), and MMP-3 (**L**). The plots represent three independent experiments. Data are presented as the mean ± SEM of three independent experiments (*$P < 0.05$ vs. untreated).

Effects of TNF-α on expression of SPM receptors in MH7A cells

SPMs bind to G-protein-coupled receptors (GPCRs) and induce specialized biological actions.[42, 43] To elucidate the mechanism underlying the anti-inflammatory effect of SPMs, the expression of chemerin chemokine-like receptor 1 (CMKLR1), an RvE1 receptor, was evaluated using real-time PCR and western blotting. MH7A cells were stimulated with 10 ng/mL TNF-α for 1, 6, 12, 24, 36, or 48 h. The expression of CMKLR1 mRNA was increased after stimulation with TNF-α for 1 h, according to the results of real-time PCR. However, no difference

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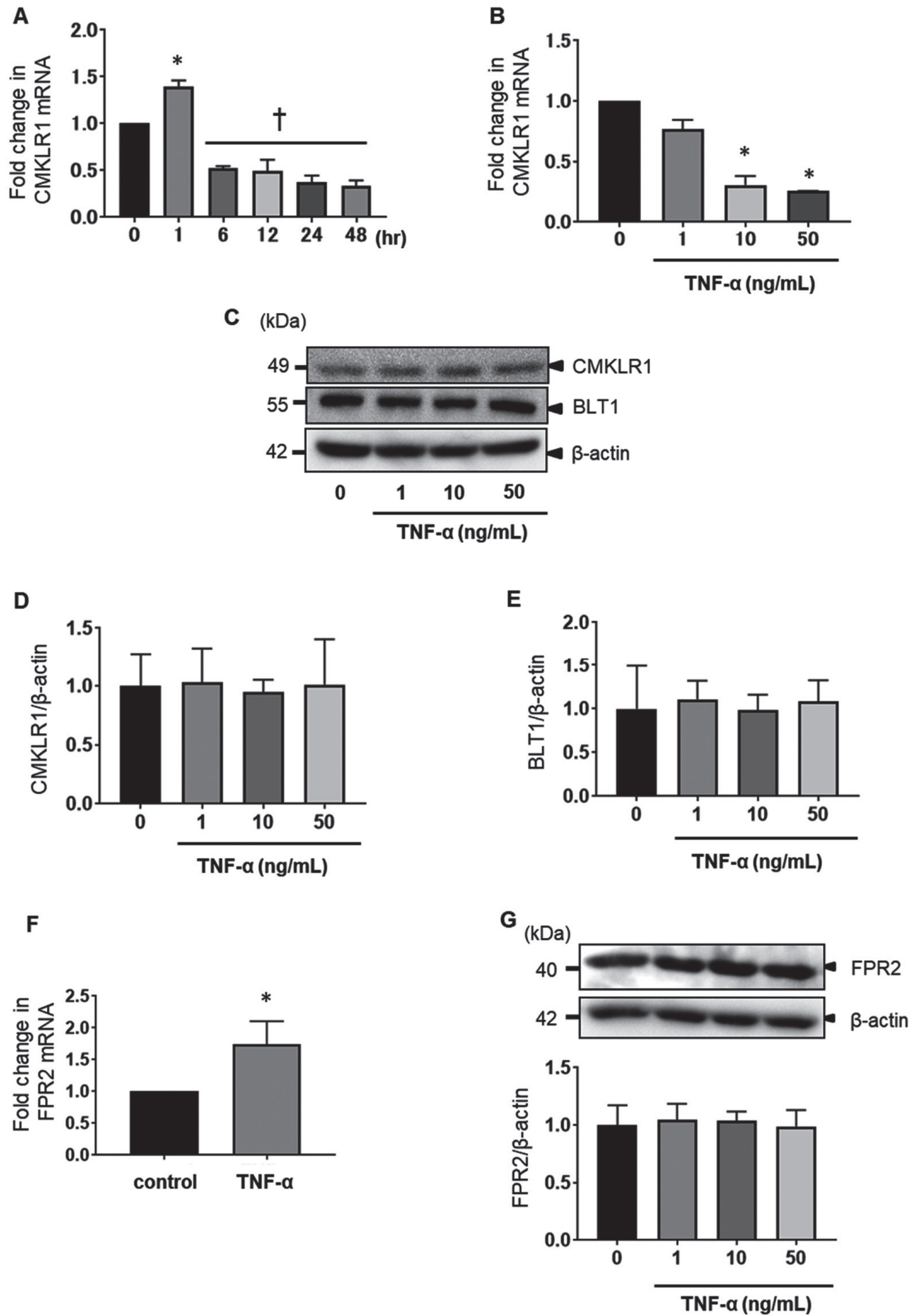

Fig. 5. Effects of TNF-α on the expression of chemerin chemokine-like receptor 1 (CMKLR1), leukotriene B_4 receptor 1 (BLT1), and FPR2 in MH7A cells. MH7A cells were stimulated with 10 ng/mL TNF-α for 1, 6, 12, 24, or 48 h (**A**) and in the presence of 1, 10, and 50 ng/mL TNF-α for 12 h (**B**). The mRNA levels of CMKLR1 were determined using real-time PCR. Data are presented as the mean ± SEM of three independent experiments (*P < 0.05, **P < 0.01 vs. 0 h). MH7A cells were incubated for 24 h with 0–50 ng/mL TNF-α. The expression of CMKL1 and BLT1 was determined by western blot analysis (**C**, **D**, and **E**). The mRNA level of FPR2 was determined in the presence of 10 ng/ml TNF-α for 12 h using real-time PCR (**F**). The expression of FPR2 in the presence of 10 ng/ml TNF-α for 24 h was determined by western blot analysis (**G**). Data are presented as the mean ± SEM of three independent experiments (*P < 0.05 vs. control. †P < 0.05 vs. TNF-α).

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was observed at 6–48 h (Fig. 5A). CMKLR1 mRNA expression was further reduced by stimulation with 10–50 ng/mL TNF-α, according to real-time PCR (Fig. 4B). The expression of CMKLR1 and another RvE1 receptor, BLT1, did not change after 24 h of stimulation with TNF-α (Figs. 5C–E). The mRNA expression of N-formyl peptide receptor 2 (FPR2), an RvD1 receptor, was increased after stimulation with TNF-α, according to real-time PCR (Fig. 5F); however, the expression of FPR2 did not change after 24 h of stimulation with TNF-α, according to western blotting (Fig. 5G). The mRNA expression level of CMKLR1 was decreased after stimulation with TNF-α for 6-48 h; however, the protein levels of CMKLR1, BLT1, and FPR2 in MH7A cells were not affected by TNF-α. Therefore, these results suggest that SPMs can act via SPMs receptors.

Effect of ω-3 PUFA-derived lipid mediators on TNF-α-induced MAPK signal activation in MH7A cells

In synovial fibroblasts, MAPK signaling plays an important role in TNF-α-induced inflammation responses.[42] Three MAPK signaling pathways have been characterized: MEK/ERK1/2, JNK, and p38 MAPK signaling pathways. We examined the contribution of MAPK signaling pathways to TNF-α-induced COX-2, mPGES-1, and IL-6 expression in MH7A cells using MAPK inhibitors. TNF-α-induced COX-2 expression was inhibited in the presence of the MEK inhibitor U0126 and the p38 inhibitor SB202190 (Fig. 6A). By contrast, mPGES-1 expression was not inhibited (Fig. 6B). TNF-α-induced IL-6 expression was inhibited in the presence of the p38 inhibitor SB202190 and enhanced in the presence of U0126 (Fig. 6C). Next, we evaluated the bioactivity of SPMs in the MH7A cells. SPMs are known to activate the PI3K/AKT and ERK signaling pathways via receptors.[16, 44] To evaluate the bioactivity of SPMs in MH7A cells, we investigated the signal activity of SPMs in the absence of TNF-α. As a result, RvE1, RvD1, and MaR1 were found to enhance ERK activation (Fig. 6D), while RvD1 and MaR1 enhanced Akt activation (Supplementary Fig. S3).

Next, we examined the effects of SPMs on the MAPK signaling pathway in MH7A cells. The expression of p-ERK, p-p38, p-JNK, and p-NF-κB was enhanced by 15–30 min after TNF-α stimulation; however, the expression of p-ERK, p-p38, p-JNK, and p-NF-κB was not inhibited by RvE1 (Figs. 7A–D). Similar results were observed for RvD1 (Figs. 7E–H) and MaR1 (Figs. 7I–L).

No significant effect of SPMs (RvE1, RvD1, and MaR1) on these signaling systems by TNF-α stimulation was observed. These results indicate that SPMs have no inhibitory effect on TNF-α-stimulated MAPK signaling and NF-κB phosphorylation in synovial fibroblasts.

DISCUSSION

In this study, we investigated the effects of SPMs on TNF-α-induced inflammatory responses in synovial fibroblasts. We found that SPMs exhibited a non-inhibitory effect on the TNF-α-induced inflammatory response in synovial fibroblasts. We also found that the expression of RvE1 receptors was not affected by TNF-α; however, the signaling pathways activated by TNF-α were not affected. The activation of MAPKs is involved in TNF-α-induced synovitis. In synovial fibroblasts, TNF-α enhances the expression of COX-2, mPGES-1, IL-6, and MMP-3 by the activation of MAPKs and NF-κB.[21, 40, 45–51] In our study, SPMs did not inhibit the activation of MAPKs and NF-κB, which may explain why SPMs are incapable of inhibiting the synthesis of COX-2, mPGES-1, IL-6, and MMP-3 induced by TNF-α. It has been reported that prophylactic and therapeutic RvE1 regimens did not ameliorate the incidence or severity of collagen-induced arthritis in mice, including the histopathological scores of synovial inflammation, chondrocyte death, cartilage erosion, bone erosion, proteoglycan depletion, and proinflammatory cytokine production.[52] These results suggest that SPM has a limited anti-inflammatory response in patients with RA.

Furthermore, it has been reported that p38 plays an important role in the production of inflammatory cytokines.[53–55] Our study suggests that p38 phosphorylation plays an important role in the expression of COX-2 and IL-6, but that SPMs could not suppress p38 phosphorylation, thus failing to suppress inflammatory cytokine production.

As a receptor, RvE1 uses CMKLR1 and BLT1, which are expressed on the cell surface of inflammatory cells and synovial fibroblasts.[56, 57] RvD1 and LXA4, an ω-6 derived lipid mediator, use FPR2 as a receptor.[58, 59] MaR1 has been reported to be mediated by the receptors for retinoic acid-related orphan receptor alpha (RORα) and leucine-rich repeat-containing G-protein coupled receptor 6 (LGR6); however, the mechanisms by which this regulation occurs remain poorly understood.[60] In our study, the protein levels of CMKLR1, BLT1 and FPR2 in synovial fibroblasts were not affected by TNF-α while the expression level of FPR2 mRNA was increased after stimulation with TNF-α. Ubiquitination is one of the mechanism for degradation of proteins. Connor et al. reported that TNF-α is not only capable of inducting expression of E3 ubiquitin ligases involved in the ubiquitination pathway but may also stimulate the

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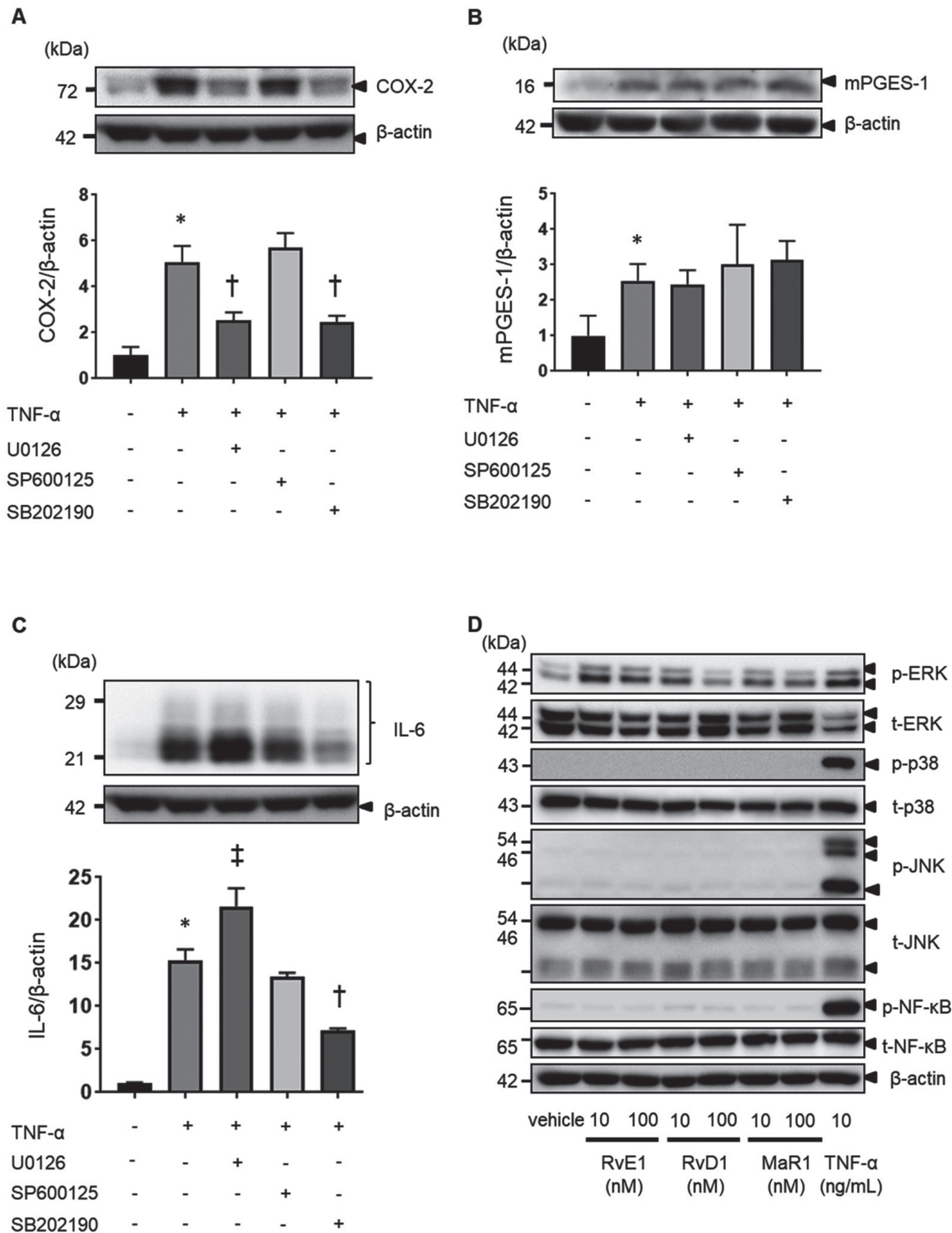

Fig. 6. Effects of ERK1/2, JNK, and p38 inhibitors on TNF-α-induced COX-2, mPGES-1, and IL-6 expression in MH7A cells. When MH7A cells were pretreated with the MEK inhibitor U0126 (10 μM), the JNK inhibitor SP600125 (10 μM), and the p38 inhibitor SB202190 (1 μM) for 1 h, the expression of TNF-α-induced COX2 (**A**), mPGES-1 (**B**), and IL-6 (**C**) in cell lysates was determined using western blot analysis. The data are expressed as the relative protein expression of targets/β-actin. Data are presented as the mean ± SEM of three independent experiments (*$P < 0.05$ vs. untreated. †$P < 0.05$ vs. TNF-α, ‡$P < 0.05$ vs. TNF-α). Effects of SPMs on the MAPK signaling pathway without TNF-α in MH7A cells (**D**). MH7A cells were cultured with 10 nM and 100 nM RvE1, RvD1, and MaR1 without TNF-α for 15 min. The expression of ERK, p38, JNK, and NF-κB in cell lysates was determined by western blot analysis.

proteasome itself in RA synovial fibroblasts.[61] Y. Zhang et al. reported that the inhibitory receptor, leucocyte-associated immunoglobulin (Ig)-like receptor-1 (LAIR-1), on cell surface could be shed from RA synovial fibroblasts following TNF-α stimulation.[62] The further study of leading to strengthened ubiquitination of SPMs receptors and shedding from the cell surface in response to TNF-α should be conducted.

Many types of cells are involved in the pathogenesis of RA, and the effects of SPMs have been

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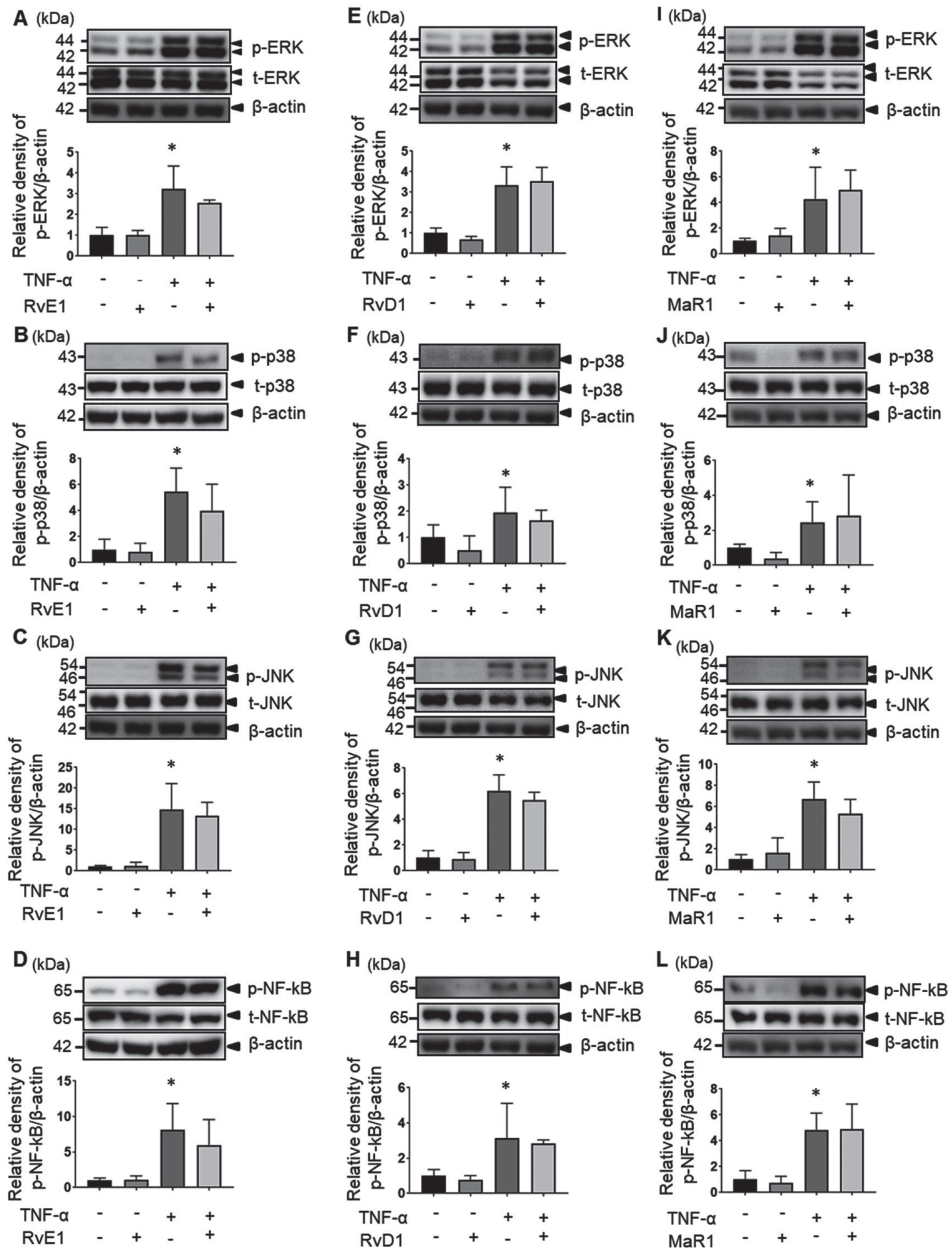

Fig. 7. Effects of SPMs on signaling pathways after stimulation of MH7A cells with TNF-α. MH7A cells were pretreated with SPMs for 1 h and then stimulated with 10 ng/mL TNF-α for 15 min. The effects of RvE1 on the following substances were examined: extracellular signal-regulated kinase (ERK) (**A**), p38 (**B**), c-Jun N-terminal kinase (JNK) (**C**), and transcription factor nuclear factor-κB (NF-κB) (**D**) or RvD1 on ERK (E), p38 (F), JNK (G), NF-kB (H), and maresin-1 (MaR1) on ERK (I), p38 (J), JNK (K), and transcription factor nuclear factor-κB (NF-κB) (L). The plots represent three independent experiments (*$P < 0.05$ vs. untreated).

investigated in several inflammatory cells. SPMs are able to modulate the inflammatory response of macrophages and neutrophils.[63] Furthermore, we previously reported on the inhibitory effect of RvE1 on osteoclasts.[25] We found that synovial fibroblasts originating from RA express CMKLR, BLT1, and FPR2; however,

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SPMs on TNF-α-induced inflammatory responses was not elucidated in MH7A cells and synovial fibroblasts originating from OA. Given the positive effects of SPMs on other inflammatory cells, our results may be due to the cell specificity of synovial fibroblasts to SPMs.

In conclusion, our study shows that the anti-inflammatory effect of ω-3-derived SPMs on TNF-α-induced response was not observed in synovial fibroblasts. This may come from non-inhibitory effects of SPMs on p38 activation induced by TNF-α. Further studies will be needed to elucidate the precise effects of SPMs in the synovial tissue of patients with RA, where many types of cells, including neutrophils, macrophages, lymphocytes, osteoclasts, chondrocytes, and synovial fibroblasts, exist and cause inflammation. It will also be necessary to investigate the effects of SPMs on other inflammatory cytokines such as RANTES, IL-8, and IL-17.

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