Effect of IL-17 for monocyte chemotactic protein production by human temporomandibular joint synovial fibroblasts

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Abstract

IL-17, which is produced primarily by Th17 cells, has recently emerged as a key player in the pathogenesis of numerous autoimmune and inflammatory diseases. The effects of IL-17 in temporomandibular disorders (TMD) were investigated using gene expression profiling of synovial fibroblasts isolated from TMD patients. Microarray analysis indicated that CCL8 (also called monocyte chemotactic protein 2; MCP-2) was found to be the most highly upregulated gene by IL-17 among the genes with known functions. The MCP chemokines, MCP-1, MCP-2, and MCP-3, have considerable structural homology and are members of the cysteine–cysteine (C-C) chemokine family that mainly modulates monocyte/macrophage recruitment in multiple inflammatory diseases. Real-time PCR analysis showed that the gene expressions of MCP-1, MCP-2, and MCP-3 were significantly higher in IL-17-treated synovial fibroblasts than in non-treated controls. MCP-1 protein production was increased by IL-17 in a time-dependent manner. Additionally, the protein production of MCP-1 was increased by IL-17 treatment in synovial fibroblast samples isolated from three TMD patients. Furthermore, IL-17 signaling was mainly through the NF□B activation pathway for MCP-1 production in synovial fibroblasts. These results suggest that the MCP chemokine production by IL-17 is likely to contribute to promotion of the inflammatory condition in TMD.

Introduction

Synovial membrane lines the intra-articular surrounding capsule, except for the cartilage of the articular eminence and the glenoid fossa of the temporal bone, the mandibular condyle, and the articular disc of the temporomandibular joint (TMJ) (1). It has been suggested that an inflammatory synovial membrane frequently occurs with the intracapsular pathological conditions of TMJ, such as disc displacement (DD)/internal derangement (ID) and/or osteoarthritis (OA) (2, 3). The concentration of inflammatory factors have been increased in the synovial fluids from patients with intracapsular pathological conditions (4, 5). It has been reported that synovial fibroblasts produce many inflammatory mediators and tissue degradation enzymes (6-8) that contribute to both degradative and inflammatory processes associated with the pathological condition in the TMJ (6-8). However, little is known about the mechanisms of the inflammatory factors in the progression of the pathological condition in the TMJ.

Interleukin (IL)-17 is secreted by active Th17 cells, which have important roles in numerous autoimmune and inflammatory diseases (9, 10). IL-17 (also called IL-17A) is also thought to have an association with the pathological conditions of rheumatoid arthritis (RA) and OA, because IL-17 is detectable in serum and knee synovial fluid from patients with OA and RA (11, 12). It has also been suggested that the IL-17 concentration is positively associated with the severity and/or activity of arthritic diseases (12, 13). In addition, IL-17 was identified as a contributor to the promotion of angiogenesis, synovial hyperplasia, and cartilage degradation in *in vitro* experiments (14-17). IL-17 was also detected in the synovial fluid from pathological conditions of the TMJ (18), but its role in the TMJ is not fully understood.

The purpose of this study is to examined the roles of IL-17 in intracapsular pathological conditions of the TMJ. Human synovial fibroblasts were isolated from the synovial membrane of patients with DD/ID or OA in the TMJ, and then gene expression profiling was examined in synovial fibroblasts treated with IL-17.

Materials and Methods

Isolation and Culture of Synovial Fibroblasts

Human synovial tissue was obtained from three patients who underwent arthroscopy for ID or open TMJ surgery for OA. TMJ1 was obtained from a 23-year-old woman and used for the oligonucleotide microarray analysis, real-time PCR, and ELISA. TMJ2 was obtained from a 26-year-old woman and used for ELISA. TMJ3 was obtained from a 59-year-old man and used for ELISA. All patients provided informed consent for the surgery and for the use of their tissue specimens for research purposes. The isolation and primary culture of synovial fibroblasts were performed according to the guidelines established by the Institutional Review Board of Nihon University School of Dentistry at Matsudo (Ethics Committee Registration Number: EC10-037, EC15-039, and EC17-15-039).

The synovial fibroblasts isolated from the synovial tissues of patients with DD and/or OA of the TMJ were prepared using the outgrowth method previously reported (6). In brief, synovial tissue was washed by phosphate-buffered saline (PBS) and minced by a scalpel. The minced synovial tissue was placed in a 35-mm tissue culture dish and then covered with a sterilized glass coverslip. The culture medium used was Ham's F12 (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Technologies, Gravesano, Switzerland), 100 μg/ml penicillin G (Meiji, Tokyo, Japan), 100 μg/ml kanamycin sulfate (Meiji), and 250 ng/ml fungizone (Gibco, Grand Island, NY, USA). The medium was changed every 3 days. Confluent synovial fibroblasts were detached with 0.025% trypsin (Gibco) and 0.02% EDTA in PBS, and then subcultured in Ham's F12 supplemented with 10% FBS and antibiotics. For the experiments, synovial fibroblasts obtained from passages 6 to 8 were used.

Total RNA Extraction

Synovial fibroblasts were plated at 1 x 10^6 cells per 100-mm dish in Ham's F12 medium containing 10% FBS and antibiotics. Confluent-stage synovial fibroblasts were cultured in

medium containing 2% FBS for 24 h, and they were then treated with or without 10 ng/ml IL-17 (PeproTech Inc, Rocky Hill, NJ, USA) for various lengths of time. Total cellular RNA from synovial fibroblasts was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and then stored at -80°C until use.

DNA Microarray Analysis

Total RNA from synovial fibroblasts treated with IL-17 (10 ng/ml) for 4 h and from the cells of untreated controls were profiled on a SurePrint G3 Human Gene expression 8x60K v2 Microarray (Agilent Technologies Inc, Santa Clara, CA, USA). The array was scanned using an Agilent DNA Microarray scanner. The above procedure was performed according to Agilent protocols. Gene expression analysis of the DNA microarray was performed using Gene Spring GX software (Agilent). Data were normalized using raw data from each array as a reference. The IL-17-response genes were considered to show a greater than twofold difference in intensity between untreated controls and IL-17-treated synovial fibroblasts as determined by the GeneSpring GX software program. The dataset is deposited in the gene expression omnibus (GEO), series GSE74668.

Real-time PCR

Complementary DNA (cDNA) was synthesized from total RNA using a GeneAmp RNA PCR kit (Thermo Fisher Scientific Inc, Waltham, MA, USA). Real-time PCR was performed using a TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific Inc.). The PCR mixture (20 μl) contained 2 μl of cDNA. Amplification was performed using a Quantstudio 6 (Thermo Fisher Scientific Inc.) with pre-heating at 95°C for 20 s, followed by 40 cycles of: 95°C for 1 s, and 60°C for 20 s. Gene transcription was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ΔC_T). The relative expression ratios were calculated using the $2^{-\Delta\Delta C_T}$ method (19). All analyses were performed in three replicates, and the results were confirmed by three independent experiments.

ELISA

Synovial fibroblasts were seeded at a density of 5 x 10^4 cells per well onto 24-well plates with Ham's F12 medium containing 10% FBS. Confluent cells were cultured for 24 h in the same medium containing 2% FBS. After incubation with IL-17 for the appropriate amount of time, culture supernatants were collected and stored at -80°C until use. The MCP-1 levels in the conditioned medium were measured using an ELISA kit (R&D Systems, McKinley, MN, USA), according to the manufacturer's protocol. The ELISA experiments were independently performed six times.

2.7. Inhibition of IRAK 1/4, PI3K, TAK1, and IKKβ.

Synovial fibroblasts were plated at a density of 5 x 10^4 cells per well in 24-well plates with Ham's F12 medium containing 10% FBS. Confluent cells were cultured for 24 h in medium containing 2% FBS. The inhibition experiments were performed using the following inhibitors: Interleukin-1 Receptor-Associated-Kinase-1/4 (IRAK-1/4) inhibitor (20 μM) (Merck KGaA, Darmstadt, Germany), the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (20 μM) (Merck KGaA), the transforming growth factor-β-activated kinase 1 (TAK1) inhibitor 5z-7-oxozeaenol (1 μM) (Merck KGaA), and the inhibitor of the NFκB kinase β subunit (IKKβ) PS-1145 (10 μM) (Cayman Chemical, Ann Arbor, MI, USA). The cells were pre-treated with the inhibitor reagents for 30 min, followed by incubation with IL-17 (10 ng/ml). After 8 h, the culture supernatants were collected and stored at -80˚C until use. The MCP-1 levels in the conditioned medium were measured using an ELISA kit (R&D systems).

Statistical Analysis.

The data are expressed as means ± standard deviations (SD) and analyzed by one-way analysis of variance (ANOVA). Post hoc analyses were carried out using the Student-Newman-Keuls (SNK) multiple comparison test. $P<0.05$ were considered to indicate significance.

Results

Microarray Analysis

The expressions of 50,739 genes on the DNA microarray in untreated control cells and IL-17-treated cells were compared. A total of 1,710 genes showed greater than 2-fold changes in expression with IL-17 treatment; the expressions of 389 genes were up-regulated, and the expressions of 1,321 genes were down-regulated (data not shown). Table 1 lists the 10 most upregulated genes by IL-17. Four chemokine genes are listed among the top 10 up-regulated genes in synovial fibroblasts treated with IL-17 (Table 1). CCL8 (monocyte chemotactic protein 2; MCP-2), which is one of the chemokine superfamily members, was found to be the most highly upregulated gene by IL-17 among the genes with known functions. MCP proteins are specifically chemotactic for monocytes and lymphocytes (20). The MCP chemokines, MCP-1, MCP-2, and MCP-3, have considerable structural homology and are redundant in biological properties (21-23). All MCP chemokines were included in the genes upregulated by IL-17 in synovial fibroblasts (Table 2). The gene expression of MCP-1 (CCL2) was the highest among the IL-17-response genes.

Time Course of IL-17-induced Gene Expression

MCP chemokines were upregulated in synovial fibroblasts by IL-17 treatment for 4 h using the microarray analysis. Therefore, the time-course of IL-17 induction of the expressions of these genes was measured in synovial fibroblasts. Their expressions were measured using real-time PCR in synovial fibroblasts with or without IL-17 for 2, 4, 8, 12, or 24 h. The gene expression of MCP-1 was significantly higher in synovial fibroblasts treated with IL-17 for 2 h to 24 h compared to untreated controls, and it was most upregulated with 4-h treatment (Fig. 1A). The gene expressions of MCP-2 and MCP-3 were significantly upregulated in synovial fibroblasts by IL-17 treatment for 4 to 24 h (Fig. 1B, C). The peak of upregulation in MCP-2 expression was also at 4 h (Fig. 2B). In contrast, the peak of MCP-3

was at 12 h (Fig. 1C).

Effect of IL-17 on MCP-1 Protein Production

MCP-1 is a well-known chemokine that is expressed in synovial cells, osteoblasts, and chondrocytes, and has a role in bone metabolism and OA. MCP-1 was the most abundantly expressed among MCPs in synovial fibroblasts in this microarray analysis. Therefore, MCP-1 was selected to examine the effects of IL-17 on protein production in MCP chemokines. The time-course of MCP-1 protein production was examined in synovial fibroblasts incubated with or without 10 ng/ml IL-17 for 4, 8, 12, or 24 h. The MCP-1 concentration in the conditioned media was increased by IL-17 in a time-dependent manner over the entire 24-h period (Fig. 2).

In the next experiment, the effect of IL-17 on MCP-1 protein production was examined in three synovial fibroblast samples isolated from three different patients. Although the increase of concentration varied among the conditioned media from the three synovial fibroblast samples from the three individual patients, MCP-1 protein concentrations were significantly increased in synovial fibroblasts treated with 10 ng/ml IL-17 for 24 h compared to the untreated control cells in all three samples (Fig. 3).

Effects of signaling inhibitors on IL-17-induced MCP-1 production

Several previous studies suggested that the expression and production of cytokines induced by IL-17 are mediated by NFκB activation. Therefore, whether inhibitors of the signaling pathway for NFκB activation affect IL-17-induced MCP-1 production in synovial fibroblasts was investigated. The induction of MCP-1 by IL-17 was decreased in synovial fibroblasts by pre-treatment with LY294002 (a PI3K inhibitor), (5z)-7-oxozeaenol (a TAK1 inhibitor), and PS-1145 (an IKKβ inhibitor), although MCP-1 production was not affected by pre-treatment with an IRAK-1/4 inhibitor (Fig. 4).

Discussion

In this study, synovial fibroblasts were isolated from patients with DD/ID and/or OA in the TMJ, and the IL-17-regulated genes in synovial fibroblasts were analyzed using a high-throughput DNA microarray. The gene expression of CCL8 (also called MCP-2) was the most upregulated by IL-17 among the already known genes using a high-throughput DNA microarray.

MCPs, which are members of the cysteine–cysteine (C-C) chemokine family, mainly modulate monocyte and/or macrophage recruitment in multiple inflammatory diseases (22, 23). High levels of MCPs have been detected in synovial tissues and fluids of individuals suffering from RA, osteoarthritis, and reactive arthritis (24, 25). The MCPs, which are expressed in synovial cells, osteoblasts, and chondrocytes (26-28), are ligands of C-C-chemokine-receptor-2 (CCR2) (29, 30). The CCR2-positive (CCR2+) macrophages are also abundant in human synovial tissue with RA and OA (31, 32). It has been suggested that the CCL2(MCP-1)/CCR2 signaling axis preferentially mediates monocyte accumulation and promotes tissue damage and inflammation in RA and OA. In several mouse models of chronic inflammation, deficiencies in MCP-1 or CCR2 protect against inflammation and tissue damage (33). In addition, it has been demonstrated that inhibition of MCP-1 synthesis or MCP-1 binding to CCR2 defend against development of mouse OA in part by attenuating macrophage accumulation in the synovial joints (34). In the TMJ region, CD68-positive cells, which are generally known to be of macrophage lineage, are more abundant in the synovial tissue of patients with painful joint clicking or OA (35). Our previous study showed that the level of MCP-1 was increased in synovial fluids from painful TMD patients compared to no-pain TMD patients (28). A recent study showed that the CCR2 selective antagonist RS504393 significantly abrogated migration of murine monocyte/macrophage cell line RAW264.7 cells promoted by a chemotactic factor produced from mouse TMJ synoviocyte-like cells, indicating that MCP-1 secreted from TMJ synoviocyte-like cells promotes migratory activity of RAW264.7 cells (36). Therefore, the expression of MCPs was examined in synovial fibroblasts because CCR2-expressing inflammatory monocytes/macrophages recruited to tissues drive local inflammation and tissue damage.

Next, the kinetics of the expression of MCP chemokines were examined using real-time PCR, and all gene expressions of MCPs were upregulated in synovial fibroblasts by IL-17-treatment until 24 h, although the time of the peak upregulated varied by MCP. In addition, the gene expressions of MCP-2 and MCP-3 were not significantly upregulated in synovial fibroblasts by IL-17 treatment for 2 h although the gene expressions of MCP-1 were significantly upregulated. The transcription is influenced by many enhancers and by multiple factors bound to the promoter sequences. We suggested that the differentiation for expression pattern may be influenced an individual DNA sequences for promoter region of each MCPs. MCP-1 is the most well-known among MCPs, and MCP-1 showed the most abundant gene expression among the MCPs in synovial fibroblasts in this microarray analysis. Therefore, the protein production of MCP-1 was examined, and it was found to be increased in an IL-17 time- and dose-dependent manner. IL-17 also stimulated MCP-1 protein production in all three synovial fibroblast samples isolated from the three patients. It was suggested that IL-17 stimulates the gene expression and protein production of MCP-1 in synovial fibroblasts from the human TMJ.

Signaling pathway analysis indicated that MCP-1production was stimulated by NFκB. IL-17 activates NFκB through a heterodimeric receptor subunit of IL-17 receptors A and C (10) in several cell types (37, 38). We already confirmed the expression of IL-17 receptors A and C in synovial fibroblasts from the human TMJ (8). It has been reported that IL-17 signaling shares the downstream transcription factors of IL-1β and/or TNF-α signaling (38). We supposed that the majority of signaling molecules for NFκB activation by IL-17 may be similar to those by IL-1β and TNF-α. To investigate IL-17-mediated NFκB activation, the effects of kinase inhibitors of NFκB activation signaling on MCP-1 production were examined in synovial fibroblasts stimulated with IL-17. The increase of MCP-1 production by IL-17 was decreased by treatment with LY294002 (a PI3K inhibitor), (5z)-7-oxozeaenol (a TAK1 inhibitor), and PS-1145 (an IKKβ inhibitor), but it was not affected by treatment with an IRAK-1/4 inhibitor. IL-17 signal transduction may share TAK1 and its downstream signals leading to NFκB activation with IL-1β signal transduction. PI3K/Akt signaling, one of the

TNF-α-dependent NFκB activation pathways (39), was also implicated in IL-17-induced MCP-1 production. These data suggest that IL-17 signaling mainly involved the NF□B activation pathway for MCP-1 production in synovial fibroblasts. On the other hand, the protein level of MCP-1 was low in the synovial fibroblasts treated with LY294002 compared to untreated control sample. TAK1 is associated with MAPK signaling. The MCP-1 production may be taken the MAPK signaling pathway in untreated synovial fibroblasts.

The present study demonstrated that IL-17 upregulated the expression of MCP chemokines through the NFκB activation pathway, which might be important in promoting monocyte/macrophage attraction to synovial tissue and fluid of TMD. Abundant monocytes/macrophages and the interaction of monocytes/macrophages and synovial fibroblasts possibly exacerbate the symptoms of synovitis or promote its transition to chronic inflammation in the TMJ. We suggest that the MCP chemokine production stimulated by IL-17 is likely to contribute to the promotion and to increase the inflammatory condition in TMD.

Conclusions

IL-17 induces the mRNA expression of MCP chemokines, as well as the protein production of MCP-1 in synovial fibroblasts. IL-17 appears to transduce signals for MCP-1 production via NFκB activation. These data provide insights into the cellular mechanisms by which IL-17 participates in the activation of synovial fibroblasts in the inflamed TMJ.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure legends

Table 1 Ranking of up regulated genes by treatment with IL-17

Rank	GeneSymbol	GenBank	Fold change	Description
1			65.3	unknown
2	XLOC_010851		54.6	BROAD Institute lincRNA
3	CCL8	NM_005623	51.3	chemokine (C-C motif) ligand 8 (CCL8)
4	CXCL1	NM_001511	49.8	chemokine (C-X-C motif) ligand 1 (CXCL1)
5	CXCL1	NM_001511	48.7	chemokine (C-X-C motif) ligand 1 (CXCL1)
6	CXCL2	NM_002089	38.8	chemokine (C-X-C motif) ligand 2 (CXCL2)
7	CXCL3	NM_002090	35.9	chemokine (C-X-C motif) ligand 3 (CXCL3)
8	XLOC_004023		35.7	BROAD Institute lincRNA
9	OLIG2	NM_005806	34.5	oligodendrocyte lineage transcription factor 2 (OLIG2)
10	CXCL2	NM_002089	33.8	chemokine (C-X-C motif) ligand 2 (CXCL2)

Rank: ranking of upregulated gene by IL-17.
Fold: intensity of IL-17 treatment/intensity of control.

Table 2 DNA microarray data of MCPs

Gene	GenBank	Fold change	Intensity (Raw data)	
			Control	IL-17
MCP1 (CCL2)	NM_002982	2.9	35563.2	105781.0
MCP2 (CCL8)	NM_005623	51.3	6.3	325.6
MCP3 (CCL7)	NM_006273	11.1	363.0	4067.9

Rank: ranking of upregulated gene by IL-17.
Fold: intensity of IL-17 treatment/intensity of control.

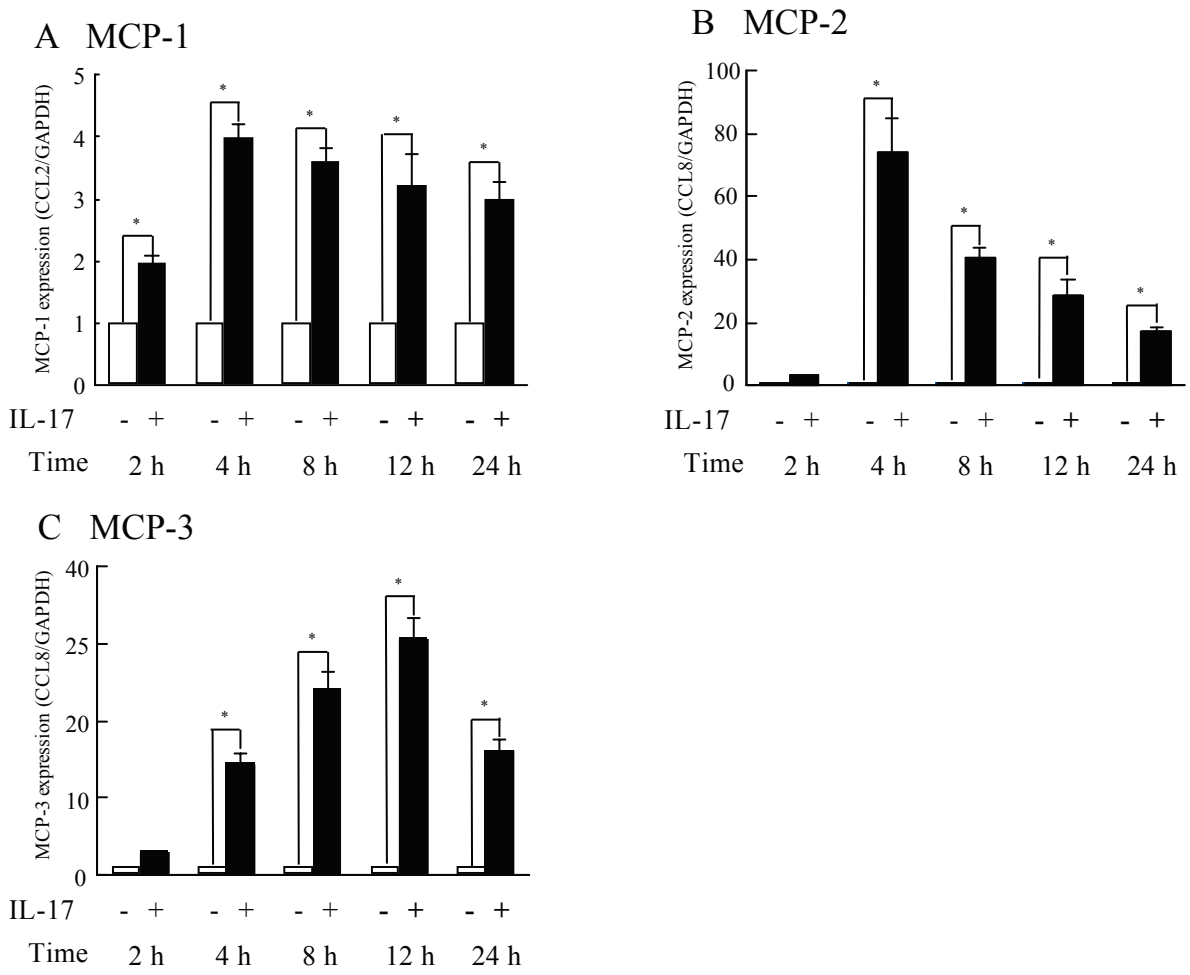

Fig. 1: Time course of the gene expression of MCPs. The gene expression in synovial fibroblasts was analyzed using real-time PCR following culture of the cells with or without IL-17 (10 ng/ml) for 4, 8, 12 or 24 h. A, MCP-1; B, MCP-2; C, MCP-3. Data are shown as means ± SD (n = 3), *P<0.01.

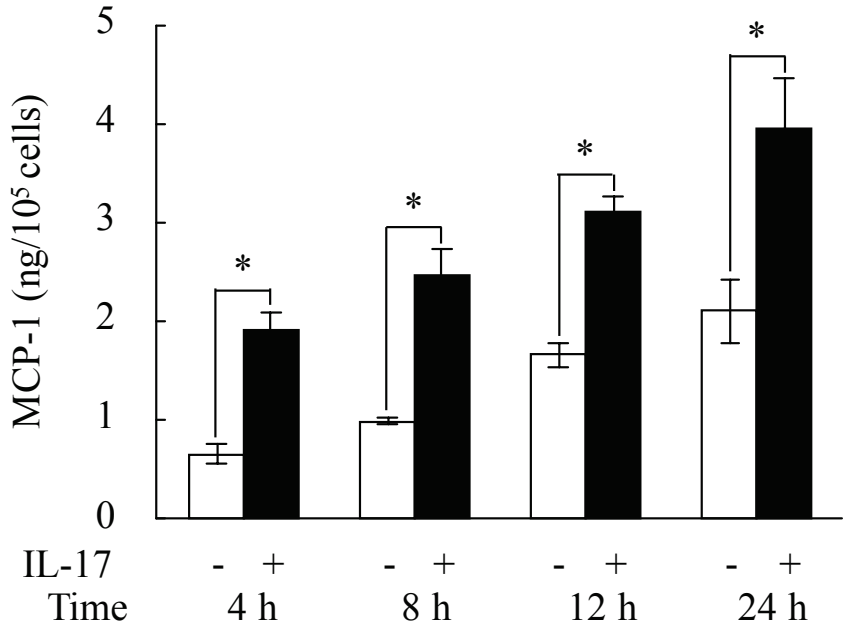

Fig. 2: Time course of IL-17-induced MCP-1 protein production. Synovial fibroblasts were treated with 10 ng/ml IL-17 for 4, 8, 12, or 24 h. The MCP-1 protein levels in the conditioned medium were then assayed using ELISA. Data are shown as means ± SD (n = 6), *P<0.01.

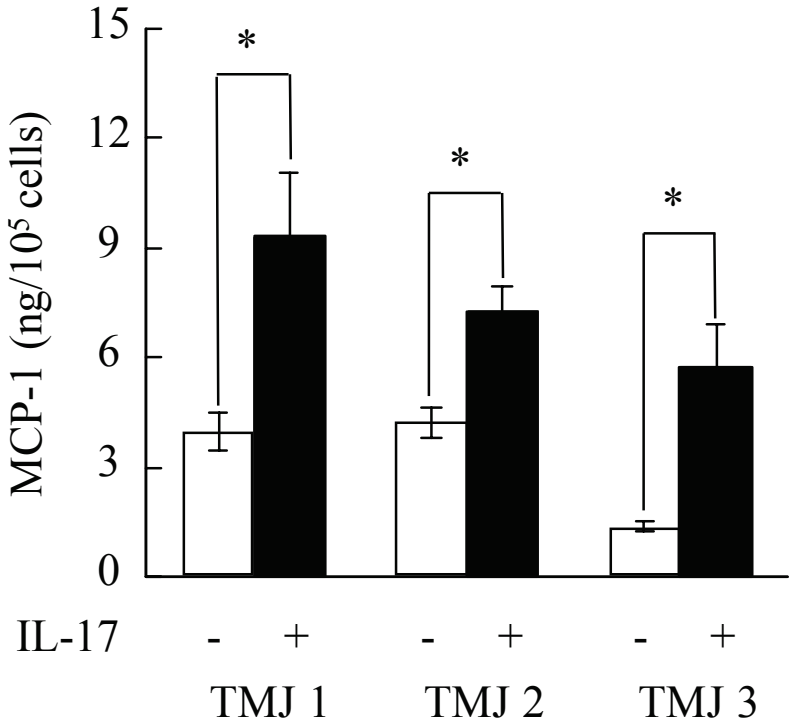

Fig. 3: Effects of IL-17 on MCP-1 protein production by three human synovial fibroblast samples. Synovial fibroblast samples were isolated from three patients with TMD (TMJ1-3). The cells were treated with 10 ng/ml IL-17 for 24 h, following which the MCP-1 protein levels in the conditioned medium were then assayed using ELISA. Data are shown as means ± SD (n = 6). *P < 0.01.

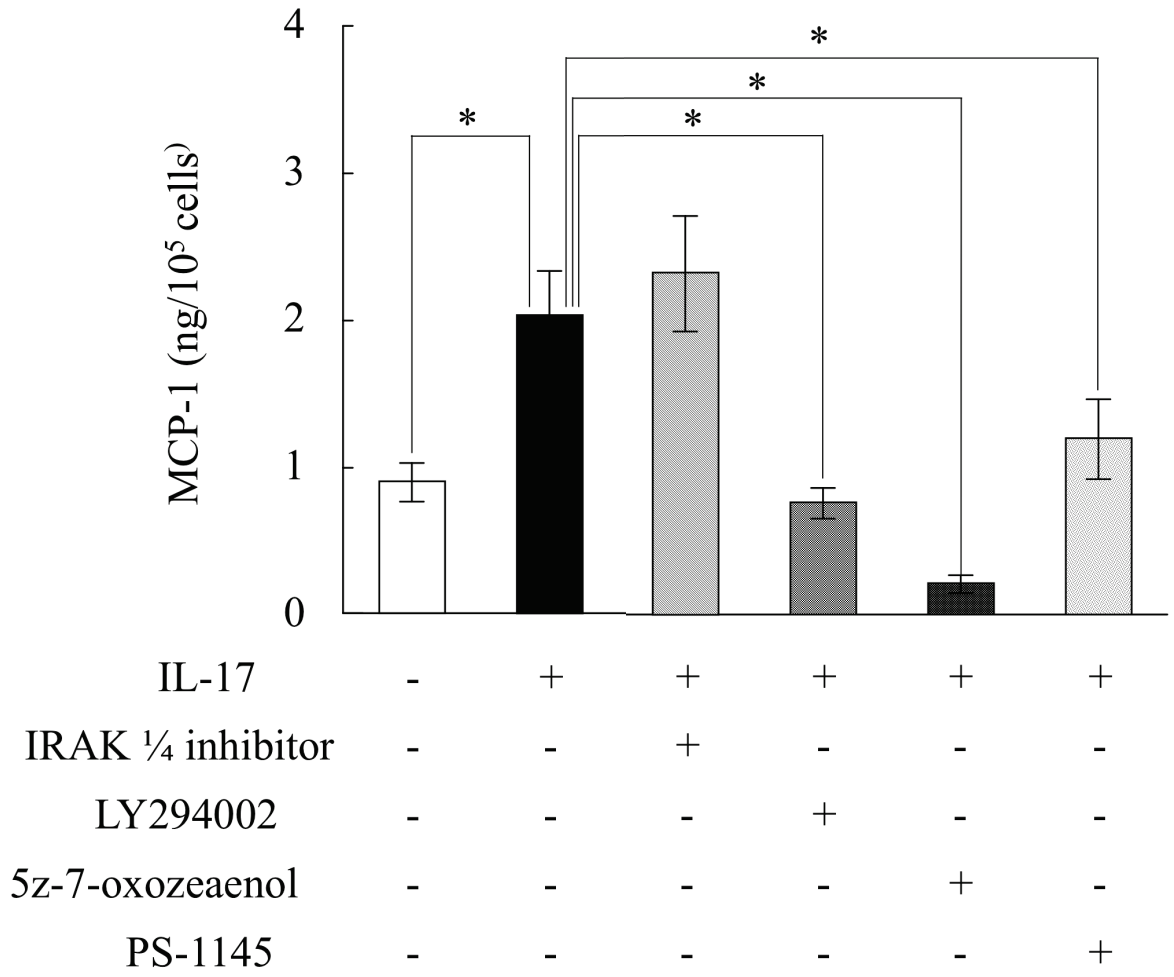

Fig. 4: Effect of inhibitors of the signaling pathway for NFκB activation on IL-17-induced MCP-1 production. Synovial fibroblasts were pre-treated with the IRAK 1/4 inhibitor (20 μM), 20 μM LY294002, 1 μM 5z-7- oxozeaenol, or 10 μM PS-1145 for 30 min, and they were then treated with 10 ng/ml IL-17 for 8 h, following which the MCP-1 protein levels in the conditioned medium were assayed using ELISA. Results are expressed as means ± SD (n = 4), *$P < 0.01$.