

# Differential regulation by IFN- $\gamma$ on TNF- $\alpha$ -induced chemokine expression in synovial fibroblasts from temporomandibular joint

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**Abstract.** Tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$ , are inflammatory cytokines in the synovial fluid of patients with temporomandibular joint disorder (TMD). However, it remains unknown whether they participate in the regulation of various chemokine expression levels associated with TMD. The effects of TNF- $\alpha$  and IFN- $\gamma$  on the expression of several different inflammatory chemokines, including interleukin (IL)-8, C-X-C motif chemokine ligand (CXCL)1, C-C motif chemokine ligand (CCL)20, CXCL9, CXCL10, and CXCL11 in synovial fibroblasts obtained from the temporomandibular joint (TMJ) were examined. The results demonstrated that TNF- $\alpha$  increased the mRNA levels of all examined chemokines in synovial fibroblasts obtained from the TMJ. IFN- $\gamma$  treatment alone increased the mRNA expression levels of CXCR3 chemokines, including CXCL10, while they were significantly enhanced when administered in combination with TNF- $\alpha$  compared with either treatment alone. However, the combination of IFN- $\gamma$  and TNF- $\alpha$  resulted in lower mRNA expression levels of IL-8 and CXCL1 as compared with those induced by TNF- $\alpha$  alone. The nuclear factor- $\kappa$ B inhibitor, Bay 11-7082, decreased the TNF- $\alpha$ -mediated expression of IL-8 and CXCL10 in the absence, and presence of IFN- $\gamma$ . In addition, the JAK2 inhibitor, AG490, decreased CXCL10 expression when administered with TNF- $\alpha$  and IFN- $\gamma$ . Finally, the decrease in TNF- $\alpha$ -induced IL-8 caused by IFN- $\gamma$  was recovered by AG490. The results of the present study suggest that TNF- $\alpha$  and IFN- $\gamma$  function in a cooperative manner to regulate inflammatory chemokine expression in synovial

fibroblasts, which may contribute to the pathological condition of the TMJ.

## Introduction

The temporomandibular joint (TMJ), a synovial joint, similar to other articulating joints in the human body, provides diarthrodial articulation between the mandibular condyle and temporal bone. Its synovial membrane covers all of TMJ intra-articular structures, except for the articular cartilage of the eminence, fossa and mandibular condyle, and the articular disc (1). Pathological conditions of the TMJ, such as internal derangement and/or osteoarthritis, in patients with temporomandibular joint disorder (TMD) have been reported to be accompanied by inflammation of the synovial membrane (2,3). Synovitis, is defined as inflammation of the synovial membrane and characterized by chronic inflammatory changes, such as hyperplasia of the synovial lining as well as increases in new capillaries and small vessels along with immune cell infiltration (1), while various inflammatory mediators, such as cytokines have been detected in synovial fluid and tissue samples obtained from affected patients (4,5). Inflammatory mediators that modulate the functions of cells that compose the synovial membrane, such as synovial fibroblasts are considered to promote and shape the pathological condition of the TMJ.

TNF- $\alpha$  is produced by monocytes and macrophages, and known to be a pro-inflammatory cytokine with roles in inflammatory mediation and immune response (6). Furthermore, reported evidence has shown that this cytokine likely mediates acute and chronic inflammation associated with connective tissue degeneration in TMD. Indeed, TNF- $\alpha$  has been detected in the synovial fluid of patients with TMD (4,5), while transgenic mice with over-expression of TNF- $\alpha$  were found to develop remarkable arthritic changes in the TMJ (7). In other studies, TNF- $\alpha$  was reported to increase inflammatory chemokines, small peptides that induce leucocyte activation and migration, such as IL-8 and CCL20, in synovial fibroblasts obtained from patients with TMJ (8,9). On the other hand, IFN- $\gamma$  is also known as a Type II interferon produced by T-lymphocytes and natural killer cells (10). Although IFN- $\gamma$  as well as TNF- $\alpha$  have been found in synovial fluid samples from TMD patients, in contrast to those from healthy

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individuals (5), it is unknown whether either participates in induction of inflammatory chemokines, such as IL-8 in synovial fibroblasts from the TMJ.

CXCR3-agonistic chemokines including CXCL10 bind to the chemokine receptor CXCR3 expressed by activated T cells, and play important roles in inflammation via their T cell chemotactic and adhesion-promoting activities (11). In a previous study, CXCL10 was detected in the majority of synovial fluid samples from patients with internal derangement of the TMJ (12). Furthermore, TNF- $\alpha$  and IFN- $\gamma$  have been reported to be main inducers of CXCL10 in monocytes, skin fibroblasts, and endothelial cells (13). Together, these findings suggest regulation of CXCR3 chemokines such as CXCL10 in synovial fibroblasts by TNF- $\alpha$  and IFN- $\gamma$ , and also implicate their involvement in the development of pathological processes in the TMJ.

In the present study, we examined whether TNF- $\alpha$  and IFN- $\gamma$  participate in regulation of expression of various chemokines in pathological processes in the TMJ. We first investigated their effects on the expression of several different chemokines including CXCL10 in synovial fibroblasts, then examined the effects of IFN- $\gamma$  on regulation of expression of those chemokines and transcription factors affected by TNF- $\alpha$ .

## Materials and methods

**Cultures of synovial fibroblast from TMJs.** After obtaining informed consent for acquisition according to a protocol approved by the Ethical Committee of Hiroshima University (no. 930), human synovial tissue samples were obtained from a patient with condyle hypertrophy. Synovial fibroblasts were then isolated from the synovial membrane using an outgrowth method, as previously reported (9,14). Briefly, the samples were washed with PBS, then minced, placed in tissue culture flasks, and grown in Dulbecco's modified Eagle's medium (Sigma Chemical Co, St. Louis, MO, USA) containing 10% fetal calf serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> in air. Confluent cells were detached with 0.025% trypsin (Gibco, Grand Island, NY, USA) and 0.02% EDTA in PBS, then sub-cultured in medium. For the experiments, we used synovial fibroblasts obtained from 4 to 8 passages.

**RNA preparation.** Cells were exposed to either recombinant human IFN- $\gamma$  (10 ng/ml) or TNF- $\alpha$  (10 ng/ml) (both from R & D Systems, Minneapolis, MI, USA), or those in combination at various concentrations for 12 h. RNA from each culture was extracted using an RNAeasy Mini kit (Qiagen, Hilden, Germany).

**RNA extraction, RT-PCR, and real-time PCR.** We used gene-specific oligonucleotide primers for PCR analysis, as follows (Table I). Total RNA was prepared from the cell using an RNeasy Total RNA Isolation kit (Qiagen). One-step RT-PCR was performed using an RT-PCR High Plus System (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer's instructions. Single-stranded cDNA for RT-PCR and a quantitative real-time PCR template were synthesized using a First Strand cDNA Synthesis kit (Amersham Biosciences, Uppsala, Sweden). The RT-PCR conditions for assays of

the chemokines were 1x (95°C, 15 min), 35x (95°C, 2 min; 60°C, 30 sec; 72°C, 1 min) and 1x (72°C, 7 min), while those for  $\beta$ -actin were 1x (95°C, 15 min), 25x (95°C, 2 min; 60°C, 30 sec; 72°C, 1 min), and 1x (72°C, 7 min). Quantitative real-time PCR was performed using SYBR-Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) for 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Quantitative PCR analysis was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Values for quantification of chemokine mRNA levels are presented as the fold increases in chemokine mRNA expression in cells treated with TNF- $\alpha$  and IFN- $\gamma$  were calculated in comparison with mRNA expression in non-treated cells after normalization to that of  $\beta$ -actin, and shown as the mean  $\pm$  standard deviation from 3 independent experiments.

**Chemokine determination.** Cells were pre-cultured for 1 h in the presence or absence of Bay 11-7082, an NF- $\kappa$ B inhibitor (Invivogen, San Diego, CA, USA) and AG490, a JAK/STAT inhibitor (Cayman Chemical Co., Ann Arbor, MI, USA), then incubated with TNF- $\alpha$  or IFN- $\gamma$  for 24 h. Supernatants from the cultures were collected, and the concentrations of IL-8 and CXCL10 were measured using a DuoSet ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA).

**Western blot analysis.** Cells were harvested using a Mammalian Cell Lysis kit (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and proteins from each sample were separated on 10% SDS-polyacrylamide gels, then transferred to polyvinylidene fluoride membranes (Amersham Biosciences). After incubation with the specific antibody, immunoblots were labeled with an HRP-conjugated secondary antibody and developed using an ECL Advance Western Blotting Detection kit (GE Healthcare Life Sciences, Tokyo, Japan). Image data were analyzed using an LAS 4000 mini imaging system (Fuji Film, Tokyo, Japan). Phosphorylation of the proteins was evaluated by comparing the integrated density of the phosphorylated-bands revealed by western blotting. Densitometric scanning was performed using Kodak Digital Science 1D Software (Eastman Kodak, Rochester, USA) and the levels of phosphorylated proteins were compared with total protein values.

**NF- $\kappa$ B activity.** Nuclear extracts were prepared using a nuclear extraction kit (Cayman Chemical Co.), according to the manufacturer's instructions. Nuclear extract concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Inc.). The DNA-binding activity of NF- $\kappa$ B p65 was detected by use of an NF- $\kappa$ B (p65) Transcription Factor Assay kit (Cayman Chemical Co.). Nuclear extracts (each containing 10  $\mu$ g of protein) were added to 96-well plates coated with a specific double stranded DNA sequence containing the NF- $\kappa$ B response element. NF- $\kappa$ B was then detected by addition of a specific primary antibody directed against NF- $\kappa$ B, followed by an HRP-conjugated secondary antibody to provide a colorimetric readout obtained using a microplate reader (Bio-Rad Laboratories, Inc.). Relative NF- $\kappa$ B p65 DNA-binding activity

Table I. Effects of TNF- $\alpha$  and IFN- $\gamma$  on chemokine mRNA expression levels.

Chemokines	mRNA expression levels <sup>a</sup>		Primer sequences
	TNF- $\alpha$	IFN- $\gamma$	
CXCL9	29.9 $\pm$ 3.4 <sup>b</sup>	5.6 $\pm$ 1.5 <sup>b</sup>	5'-CATGCTGGTGAGCCAAGCAGTTTGAA-3' 5'-CACTTCTGTGGGGTGTGGGGACAAG-3'
CXCL10	212.9 $\pm$ 27.6 <sup>b</sup>	10.3 $\pm$ 0.82 <sup>b</sup>	5'-TGCAAGCCAATTTGTCCACGTGTTG-3' 5'-GCAGCTGATTTGGTGACCATCATTGG-3'
CXCL11	6.1 $\pm$ 1.1 <sup>b</sup>	6.8 $\pm$ 1.9 <sup>b</sup>	5'-AGAGGACGCTGTCTTTGCAT-3' 5'-GTCCTTTCACCCACCTTTCA-3'
CCL20	10,268.4 $\pm$ 1,425.6 <sup>b</sup>	1.3 $\pm$ 0.2	5'-TACTCCACCTCTGCGGCGAATCAGAA-3' 5'-GTGAAACCTCCAACCCAGCAAGGTT-3'
IL-8	592.1 $\pm$ 78.7 <sup>b</sup>	0.9 $\pm$ 0.2	5'-ATGACTTCCAAGCTGGCCGTGGCT-3' 5'-TCTCAGCCCTCTTCAAAAATTCTC-3'
CXCL1	82.2 $\pm$ 5.4 <sup>b</sup>	1.2 $\pm$ 0.3	5'-TACTCCACCTCTGCGGCGAATCAGAA-3' 5'-AACTATGGGGGATGCAGGA-3'

<sup>a</sup>Cells were exposed to 20 ng/ml of TNF- $\alpha$  and IFN- $\gamma$  for 12 h, then mRNA expressions of the indicated chemokines were examined. Results are shown as relative to  $\beta$ -actin, the internal control. Values are presented as the mean  $\pm$  standard deviation of 3 independent experiments.

<sup>b</sup>Significantly different from non-treated cells ( $P < 0.05$ ). TNF, tumor necrosis factor; IFN, interferon; CXCL, C-X-C motif chemokine ligand; IL, interleukin.

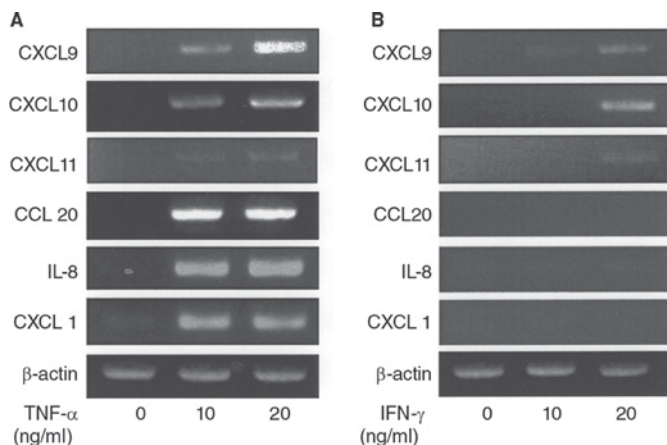


Figure 1. Effects of TNF- $\alpha$  and IFN- $\gamma$  on induction of mRNA expression of several chemokines in synovial fibroblasts from temporomandibular joint. (A) Cells were exposed to TNF- $\alpha$  (10, 20 ng/ml) for 12 h, then total RNA was isolated from the cells, and chemokine expression was examined using RT-PCR assays. Results are shown as relative to  $\beta$ -actin, the internal control. (B) Cells were exposed to IFN- $\gamma$  (10, 20 ng/ml) for 12 h, then total RNA was isolated from the cells, and expressions of chemokines were examined using RT-PCR. Results are shown as relative to  $\beta$ -actin, the internal control. TNF, tumor necrosis factor; IFN, interferon; CXCL, C-X-C motif chemokine ligand; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction.

in the nuclear extracts was normalized to that of the control cells.

**Statistical analysis.** Data were analyzed using Student's t-test or one-way analysis of variance (ANOVA), and the results are presented as the mean  $\pm$  standard deviation.

## Results

**Effects of TNF- $\alpha$  and IFN- $\gamma$  on various chemokine mRNA expressions in synovial fibroblasts.** We initially examined the effects of TNF- $\alpha$  on the mRNA expressions of various chemokines in synovial fibroblasts obtained from the TMJ. The mRNA levels of CXCL9, 10, 11, 20, IL-8, and CXCL1 were increased by TNF- $\alpha$  (Table I, Fig. 1A), whereas, addition of IFN- $\gamma$  to the culture resulted in only slight increase in CXCL9-11 expression (Table I, Fig. 1B), while no increase in expression of CCL20, IL-8, or CXCL1 expression observed (Table I, Fig. 1B).

**Effects of TNF- $\alpha$  and IFN- $\gamma$  on CXCL10 and IL-8 protein expressions in synovial fibroblasts.** To confirm the variations of mRNA expressions when stimulated with TNF- $\alpha$  or IFN- $\gamma$ , we examined CXCL10 and IL-8 protein expressions that were especially affected by TNF- $\alpha$  in CXCR3 and CXCR2 agonists, respectively. Those results showed that TNF- $\alpha$  increased CXCL10 and IL-8 expressions in a manner, similar to the increase in mRNA expression (Fig. 2). In contrast, addition of IFN- $\gamma$  resulted in an increase in CXCL10, but not IL-8 (Fig. 2).

**Effects of combined TNF- $\alpha$  and IFN- $\gamma$  on mRNA expressions of various cytokines in synovial fibroblasts.** We also examined the effects of the combination of IFN- $\gamma$  and TNF- $\alpha$  on the mRNA expression of various cytokines in synovial fibroblasts from TMJ. That combination enhanced CXCL9, CXCL10, CXCL11, and CCL20 mRNA expressions in comparison to stimulation with either alone (Fig. 3). Notably, the combinations of IFN- $\gamma$  and TNF- $\alpha$  dramatically increased the mRNA levels of CXCL9, CXCL10 and CXCL11 (CX3CR1 agonists) in comparison to TNF- $\alpha$  alone (Fig. 3). On the other hand, exposure to that in combination resulted in decreased mRNA

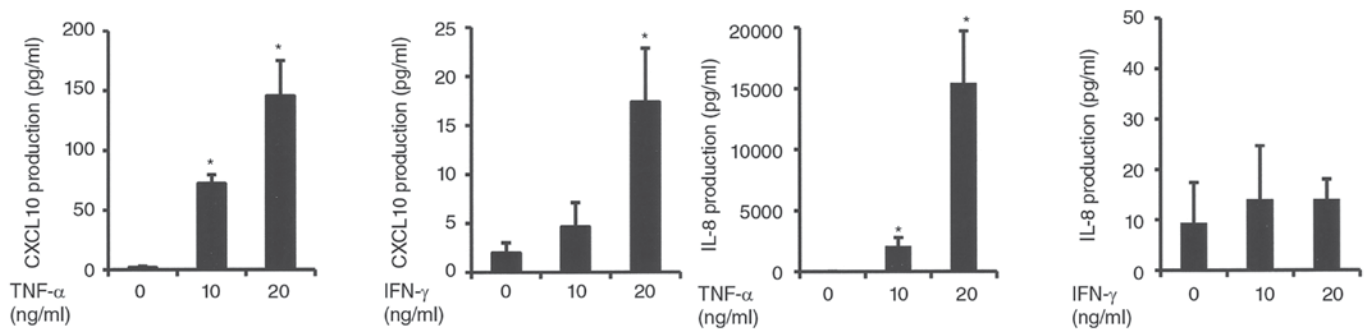


Figure 2. Effects of TNF- $\alpha$  and IFN- $\gamma$  on induction of CXCL10 and IL-8 protein in synovial fibroblasts from temporomandibular joint. Cells were exposed to IFN- $\gamma$  (10, 20 ng/ml) for 24 h, then the levels of CXCL10 and IL-8 in culture supernatant were measured by ELISA. Data are shown as the mean  $\pm$  standard deviation of 3 independent experiments. \*Significant increase as compared to non-treated cells (Student's t-test:  $P < 0.05$ ). TNF, tumor necrosis factor; IFN, interferon; CXCL, C-X-C motif chemokine ligand; IL, interleukin.

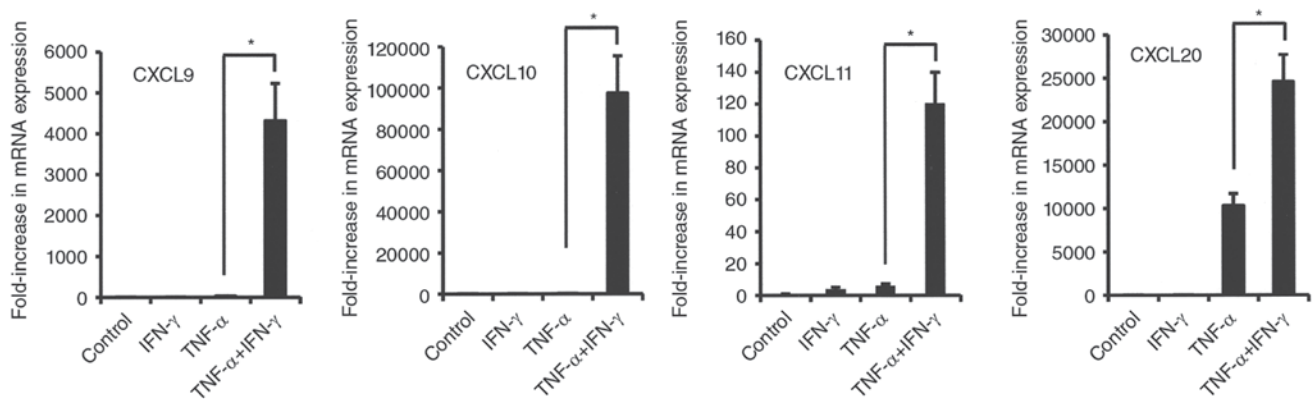


Figure 3. Effects of combination of TNF- $\alpha$  and IFN- $\gamma$  on mRNA expression of CXCL9, CXCL10, and CXCL11 in synovial fibroblasts from temporomandibular joint. Cells were exposed to 20 ng/ml of TNF- $\alpha$ , IFN- $\gamma$ , or those in combination for 12 h, then mRNA expression of the indicated chemokines was examined. Results are shown as relative to  $\beta$ -actin, the internal control. Values are presented as the mean  $\pm$  standard deviation of 3 independent experiments. \*Significantly different as compared to TNF- $\alpha$  ( $P < 0.05$ ). TNF, tumor necrosis factor; IFN, interferon; CXCL, C-X-C motif chemokine ligand.

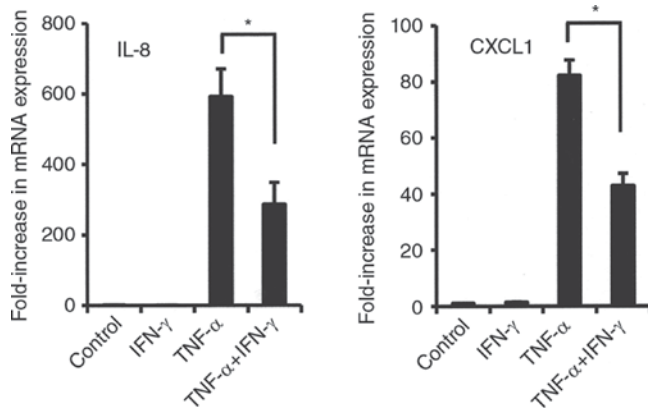


Figure 4. Effects of combination of TNF- $\alpha$  and IFN- $\gamma$  on mRNA expression of IL-8 and CXCL1 in synovial fibroblasts from temporomandibular joint. Cells were exposed to 20 ng/ml of TNF- $\alpha$ , IFN- $\gamma$  or those in combination for 12 h, then mRNA expression of the indicated chemokines was examined. Results are shown as relative to  $\beta$ -actin, the internal control. Values are presented as the mean  $\pm$  standard deviation of 3 independent experiments. \*Significantly different as compared to TNF- $\alpha$  ( $P < 0.05$ ). TNF, tumor necrosis factor; IFN, interferon; CXCL, C-X-C motif chemokine ligand; IL, interleukin.

levels of CXCR2 agonists, IL-8 and CXCL1 as compared to TNF- $\alpha$  alone (Fig. 4).

**Effects of TNF- $\alpha$  on NF- $\kappa$ B activation in synovial fibroblasts from TMJ.** NF- $\kappa$ B, an inducible transcription factor well known for its involvement in inflammatory and immune responses, is activated by phosphorylation of I $\kappa$ B $\alpha$ , then activated NF- $\kappa$ B is translocated to the nucleus, and induces target gene expression (15). We examined the effects of TNF- $\alpha$  and IFN- $\gamma$  on NF- $\kappa$ B activation in synovial fibroblasts from the TMJ. TNF- $\alpha$  increased the phosphorylation of I $\kappa$ B $\alpha$  (Fig. 5A), as well as NF- $\kappa$ B p65 DNA-binding activity in the nucleus (Fig. 5B), indicating that TNF- $\alpha$  participates in NF- $\kappa$ B activation. However, IFN- $\gamma$  did not have an effect on NF- $\kappa$ B activation in the presence or absence of TNF- $\alpha$  (Fig. 5B). To examine CXCL10 and IL-8 expressions mediated by TNF- $\alpha$  via an NF- $\kappa$ B dependent pathway, we investigated the effects of Bay 11-7082, an NF- $\kappa$ B inhibitor, on expressions of these cytokines mediated by TNF- $\alpha$ . Pre-treatment with Bay 11-7082 resulted in an increase of TNF- $\alpha$ -induced IL-8 and CXCL10 protein levels in both the presence and absence of IFN- $\gamma$  (Fig. 5C).

**Effects of IFN- $\gamma$  on STAT1 activation in synovial fibroblasts from TMJ.** STAT1 is a key mediator of gene expression induced by type II interferons, such as IFN- $\gamma$ , and activated STAT1 directly regulates the expression of CXCL10 (16). We examined whether STAT1 phosphorylation in synovial

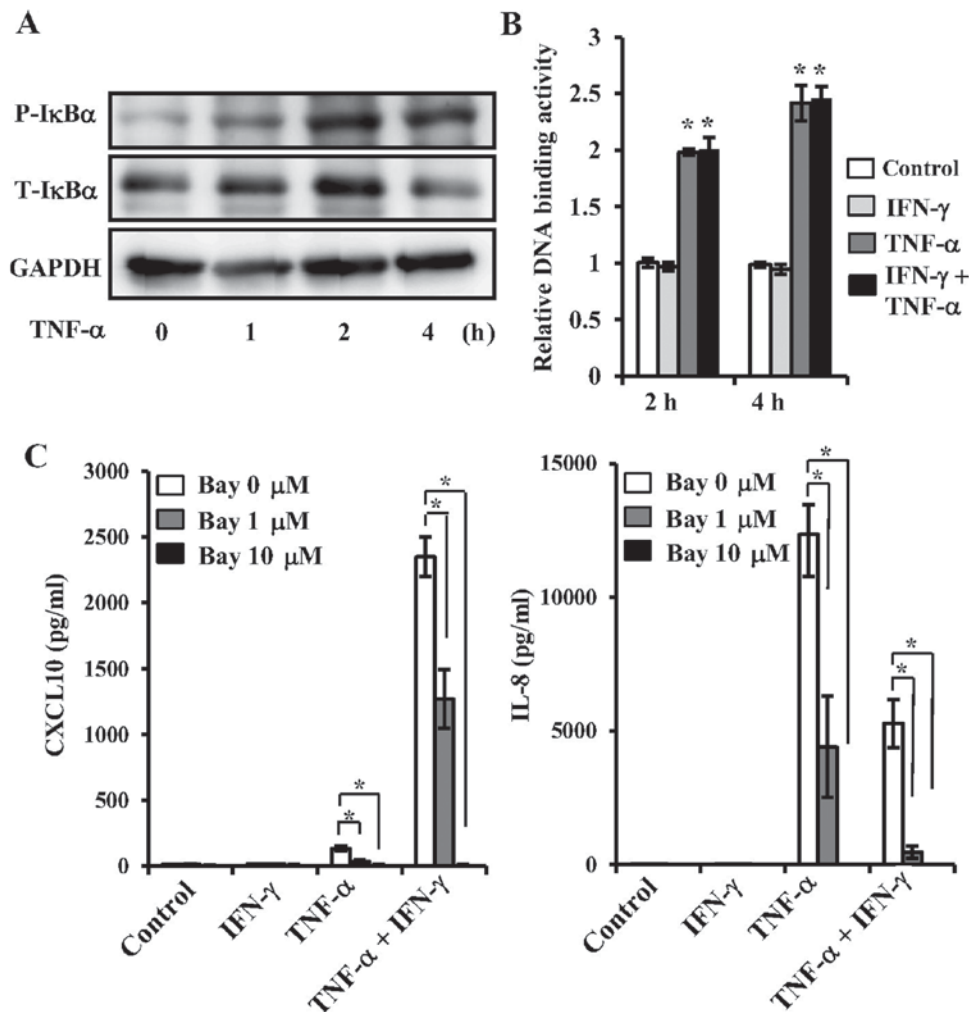


Figure 5. Effects of TNF- $\alpha$  on NF- $\kappa$ B activation, and effects of NF- $\kappa$ B inhibitor on TNF- $\alpha$ -mediated CXCL10 and IL-8 expressions in synovial fibroblasts from temporomandibular joint. (A) Effect of TNF- $\alpha$  on phosphorylation of IκB $\alpha$ . Cells were exposed to TNF- $\alpha$  (20 ng/ml) for various time periods, after which cell extracts were subjected to SDS-PAGE. Phosphorylation of IκB $\alpha$  was examined by western blotting analysis with antibodies against phospho-specific IκB $\alpha$  (P-IκB $\alpha$ ), total IκB $\alpha$  (T-IκB $\alpha$ ), and GAPDH. (B) Effect of TNF- $\alpha$  on NF- $\kappa$ B activation. Cells were exposed to 20 ng/ml of TNF- $\alpha$ , IFN- $\gamma$ , or those in combination for 2 or 4 h, after which nuclear extracts were subjected to NF- $\kappa$ B (p65) transcription factor assays. NF- $\kappa$ B p65 DNA-binding activity was examined and the results are expressed as fold changes relative to the non-treated control. \*Significantly different from non-treated cells ( $P < 0.05$ ). (C) Effect of NF- $\kappa$ B inhibitor on TNF- $\alpha$ -mediated CXCL10 and IL-8 expressions. Cells were pre-incubated with Bay-11-7082 (Bay; 1 or 10  $\mu$ M) for 1 h, then exposed to 20 ng/ml of TNF- $\alpha$ , IFN- $\gamma$  or those in combination for 24 h, after which the levels of CXCL10 and IL-8 in culture supernatants were measured by ELISA. Data are shown as the mean  $\pm$  standard deviation of 3 independent experiments. \*Significant difference as compared to Bay at 0  $\mu$ M (Student's  $t$ -test,  $P < 0.05$ ). TNF, tumor necrosis factor; IFN, interferon; CXCL, C-X-C motif chemokine ligand; IL, interleukin; NF, nuclear factor; T, total; P, phosphorylated.

fibroblasts from the TMJ activated by IFN- $\gamma$ , and IFN- $\gamma$  was increased in a time-dependent manner (Fig. 6A). No effect of TNF- $\alpha$  on IFN- $\gamma$  induced-STAT-1 activation was observed (Fig. 6B). Exposure to the JAK/STAT inhibitor AG490 resulted in partial inhibition of CXCL10 expression when fibroblasts were simultaneously stimulated with IFN- $\gamma$  and TNF- $\alpha$ , while the decrease in TNF- $\alpha$ -induced IL-8 caused by exposure to IFN- $\gamma$  was recovered by addition of AG 490. (Fig. 7).

## Discussion

TNF- $\alpha$ , a pro-inflammatory cytokine produced by immune cells, such as Th1 cells, is thought to be involved in TMJ destruction. Takahashi *et al* (4) reported detection of TNF- $\alpha$  in synovial fluid samples from TMD patients affected by disc derangement with locking or clicking as compared to those from healthy individuals. In addition, Suzuki *et al* (17) demonstrated

that TNF- $\alpha$  was predominantly expressed in cells of the synovial lining and blood vessels in synovial specimens obtained from patients with TMJ internal derangement, while, others have shown that TNF- $\alpha$  increased the production of several chemokines, such as IL-8, CXCL1, and CCL20, in synovial fibroblasts from the TMJ (8,9). On the other hand, TNF- $\alpha$  is also known as a potent inducer of NF- $\kappa$ B, an inflammatory inducible transcription factor. Binding of TNF- $\alpha$  with the cell surface receptors, TNF receptor 1 (TNFR1) and TNFR2 was found to lead to phosphorylation of IκB $\alpha$ , then activated NF- $\kappa$ B translocated to the nucleus to induce the target inflammatory gene (15). In another study, Ke *et al* (18) demonstrated that activation of NF- $\kappa$ B is responsible for TNF- $\alpha$ -induced cyclooxygenase 2 expression in synovial fibroblasts from the TMJ. In the present study, similar to previous reports, TNF- $\alpha$  shown to increase the expression of various chemokines and also active NF- $\kappa$ B. In addition, we found that addition of an

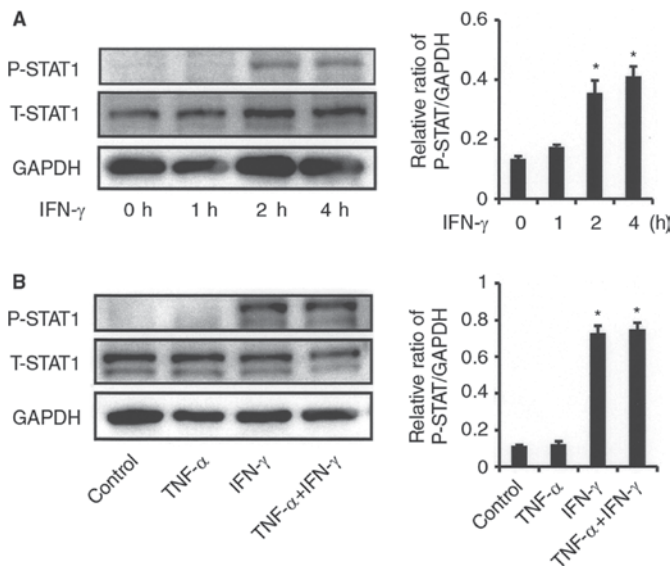


Figure 6. Effects of IFN- $\gamma$  on STAT1 activation in synovial fibroblasts from TMJ. (A) Effect of IFN- $\gamma$  on STAT1 phosphorylation. Cells were exposed to IFN- $\gamma$  (20 ng/ml) for various time periods, after which cell extracts were subjected to SDS-PAGE. Phosphorylation of STAT1 was examined by western blot analysis with antibodies against phospho-specific STAT1 (P-STAT1), total STAT1 (T-STAT1), and GAPDH. Phosphorylation of the proteins was evaluated by comparing the integrated density of the phosphorylated-bands, then the relative ratio of phosphorylated proteins in comparison with GAPDH values was determined. \*Significantly different from non-treated cells ( $P < 0.05$ ). (B) Effect of TNF- $\alpha$  on IFN- $\gamma$  induced-STAT1 phosphorylation. Cells were exposed to 20 ng/ml of TNF- $\alpha$ , IFN- $\gamma$ , or those in combination for 2 h, after which cell extracts were subjected to SDS-PAGE. Phosphorylation of STAT1 was examined by western blotting analysis with antibodies against P-STAT1, T-STAT1, and GAPDH. The relative ratio of phosphorylated proteins in comparison with the GAPDH values was determined. \*Significantly different as compared to non-treated cells ( $P < 0.05$ ). TNF, tumor necrosis factor; IFN, interferon; T, total; P, phosphorylated; STAT, signal transducer and activator of transcription.

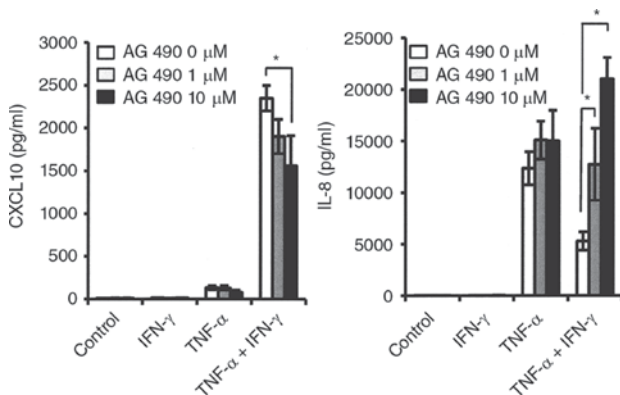


Figure 7. Effects of JAK/STAT inhibitor on TNF- $\alpha$ -mediated CXCL10 and IL-8 expressions in synovial fibroblasts from TMJ. (C) Effect of AG490 on TNF- $\alpha$ -mediated expression of CXCL10 and IL-8. Cells were pre-incubated with AG490 (1 or 10  $\mu$ M) for 1 h, then exposed to 20 ng/ml of TNF- $\alpha$ , IFN- $\gamma$  or those in combination for 24 h, after which the levels of CXCL10 and IL-8 in culture supernatants were measured by ELISA. Data are shown as the mean  $\pm$  standard deviation of 3 independent experiments. \*Significant difference as compared to 0  $\mu$ M of AG490 (Student's t-test,  $P < 0.05$ ).

NF- $\kappa$ B inhibitor to the cultures resulted in dramatic decreases in TNF- $\alpha$ -mediated IL-8 and CXCL10 expressions in synovial fibroblasts from the TMJ. Therefore, NF- $\kappa$ B plays an

important role in regulation of TNF- $\alpha$ -mediated expression of various inflammatory chemokines in synovial fibroblasts in the TMJ.

IFN- $\gamma$  produced by activated T cells is known to induce CXCR3 chemokines (13), such as CXCL10, CXCL9, and CXCL11, which share an approximately 40% amino acid sequence identity and bind to the chemokine receptor CXCR3, which is mainly expressed by activated T cells (11). IFN- $\gamma$ -induced CXCR3 chemokines have been found in synovial fluid from rheumatoid arthritis (RA) patients, and are thought to contribute to development of Th1 immune responses in the joints (19,20). Also, high levels of CXCR3 chemokines and CD4 $^{+}$  T cells expressing the CXCR3 receptor were found in inflamed synovial tissues from RA patients (21), and the serum level of CXCL10 in RA patients was reported to be correlated with disease activity (22). In the present study, stimulation with IFN- $\gamma$  alone slightly increased the expression of CXCR3 chemokines in synovial fibroblasts obtained from the TMJ, while that in combination with TNF- $\alpha$  led to dramatic increases in expression of those chemokines. A few reports have noted the contribution of T-cell-induced inflammation to the pathogenesis of TMD, including a study that showed the presence of CD45RO $^{+}$  T cells and CD68 $^{+}$  macrophages in samples from patients with generalized osteoarthritis and rheumatoid arthritis of TMD (23). We speculate that the synergistic effect of IFN- $\gamma$  and TNF- $\alpha$  on induction of CXCR3 chemokines, such as CXCL10, mobilizes a large number of T cells toward the site of inflammation, which may promote and shape the pathological condition of the TMJ.

IL-8 and CXCL1 are functional homologues, and have been shown to be primarily associated with neutrophil recruitment and inflammation (24). IL-8 binds to both the CXCR1 and CXCR2 receptors, which are found on the surface of neutrophils, while CXCL1 binds only to the CXCR2 receptor (25). In an *in vivo* study that used rabbit models of TMJ arthritis, TNF- $\alpha$  and IL-8 expressions were observed in immune cells and synovial fibroblasts from the TMJ, while IL-8 was shown to be mainly produced in infiltrating inflammatory cells and synovial cells during the acute stage (26). In the present study as well, IL-8 expression in synovial fibroblasts from the TMJ was dramatically increased by TNF- $\alpha$ . In contrast, some investigators have reported that IFN- $\gamma$  inhibited TNF- $\alpha$ -mediated inflammatory responses in various cell types. Kohara *et al* (27) found that IFN- $\gamma$  directly inhibited induction of osteoclastogenesis in bone marrow macrophages and another showed that IFN- $\gamma$  inhibited TNF- $\alpha$ -induced collagenase expression in chondrocytes (28). Our results revealed that IFN- $\gamma$  inhibited increases in IL-8 and CXCL1 caused by TNF- $\alpha$ . In addition, Kristense *et al* (29) reported that TNF- $\alpha$  was consistently detected in healthy young individuals and high levels were associated with a high level of IFN- $\gamma$ , which was sporadically found in those subjects. Therefore, IFN- $\gamma$  has both pro-inflammatory and anti-inflammatory properties, while IFN- $\gamma$  and TNF- $\alpha$  may control the pro-/anti-inflammatory balance of homeostatic levels of both cytokines under normal TMJ conditions.

IFN- $\gamma$  has been shown to trigger prolonged activation of the transcription factor STAT1 via the IFN- $\gamma$  receptor and JAK1/2, which induces expression of various genes, such as

CXCL10 (30,31). Activation of STAT1 involves phosphorylation of tyrosine and serine residues, which are required for the protein to exert its function (31,32), while the JAK2 inhibitor AG490 prevents site-specific phosphorylation of STAT1 by JAK kinase. It was also reported that most IFN- $\gamma$ -inducible genes expressed in the synovium of RA patients are likely targets of STAT1 (30). Kasperkovits *et al* (33) investigated STAT1 expression in synovial tissues of RA patients using immunohistochemistry, and found elevated levels of total STAT1 protein, with both its activated tyrosine and serine phosphorylated forms seen in RA synovium specimens as compared with the control group. Although it remains unknown whether activation of STAT1 is associated with the pathogenesis of TMD, IFN- $\gamma$  increased STAT1 phosphorylation in synovial fibroblasts from the TMJ in the present study. Furthermore, AG490 partially decreased the combined effect of IFN- $\gamma$  and TNF- $\alpha$  on induction of CXCL10 expression, and recovered the decrease in IL-8 induced by that combination. It is possible that IFN- $\gamma$  participates in differential regulation of those TNF- $\alpha$ -induced chemokines via JAK/STAT signaling in synovial fibroblasts, and also contributes to modulation of the inflammatory process in the TMJ.

In the present study, we used human synovial cells derived from a patient with condyle bone hypertrophy without TMJ disease, because it was difficult to obtain human synovial cells from the TMJ of a healthy donor. Although these synovial fibroblasts are considered to have characteristics similar to those of normal synovial fibroblasts from the TMJ, comparisons of response to TNF- $\alpha$  and IFN- $\gamma$  by synovial cells between those from healthy controls and subjects with TMJ diseases *in vivo models* may be needed in the future to more clearly elucidate the factors involved.

In summary, our results demonstrated that the expression of several chemokines including CXCL10 were increased by TNF- $\alpha$  and IFN- $\gamma$  in synovial fibroblasts obtained from the TMJ. In addition, TNF- $\alpha$ -mediated IL-8 and CXCL10 production was associated with NF- $\kappa$ B signaling. Also, IFN- $\gamma$  was shown to differentially regulate IL-8 and CXCL10 production induced by TNF- $\alpha$  via JAK/STAT signaling. We concluded that TNF- $\alpha$  and IFN- $\gamma$  cooperatively regulate the expressions of several chemokines including CXCL10 in synovial fibroblasts from the TMJ, and may contribute to its pathological condition of the TMJ.

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