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Apoptosis Signal-Regulating Kinase 1 Is Mediated in TNF-α-Induced CCL2 Expression in Human Synovial Fibroblasts

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ABSTRACT

Tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine with a critical role in osteoarthritis (OA), was primarily produced by monocytes/macrophages and plays a crucial role in the inflammatory response. Here, we investigated the intracellular signaling pathways involved in TNF-α-induced monocyte chemoattractant protein 1 (MCP-1)/CCL2 expression in human synovial fibroblast cells. Stimulation of synovial fibroblasts (OASF) with TNF-α induced concentration- and time-dependent increases in CCL2 expression. TNF-α-mediated CCL2 production was attenuated by TNFR1 monoclonal antibody (Ab). Pretreatment with an apoptosis signal-regulating kinase 1 (ASK1) inhibitor (thioredoxin), JNK inhibitor (SP600125), p38 inhibitor (SB203580), or AP-1 inhibitor (curcumin or tanshinone IIA) also blocked the potentiating action of TNF-α. Stimulation of cells with TNF-α enhanced ASK1, JNK, and p38 activation. Treatment of OASF with TNF-α also increased the accumulation of phosphorylated c-Jun in the nucleus, AP-1-luciferase activity, and c-Jun binding to the AP-1 element on the CCL2 promoter. TNF-α-mediated AP-1-luciferase activity and c-Jun binding to the AP-1 element were inhibited by TNFR1 Ab, thioredoxin, SP600125, and SB203580. Our results suggest that the interaction between TNF-α and TNFR1 increases CCL2 expression in human synovial fibroblasts via the ASK1, JNK/p38, c-Jun, and AP-1 signaling pathway. J. Cell. Biochem. 113: 3509–3519, 2012.

KEY WORDS: TNF-α; CCL2; ASK1; OA

Osteoarthritis (OA) is a chronic joint disorder characterized by slow progressive degeneration of articular cartilage, subchondral bone alteration, and variable secondary synovial inflammation. In response to macrophage-derived proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), OA synovial fibroblasts (OASF) produce chemokines that promote inflammation, neovascularization, and cartilage degradation via activation of matrix-degrading enzymes such as matrix metalloproteinases [Mor et al., 2005; Shen et al., 2011]. Although the pathogenesis of the disease remains elusive, there is increasing

Abbreviations used: ASK1, apoptosis signal-regulating kinase 1; MKKK, MAPK kinase kinase; OA, osteoarthritis; OASF, osteoarthritis synovial fibroblasts; RA, rheumatoid arthritis; TNF, tumor necrosis factor; siRNA, small interference RNA; qPCR, quantitative real time PCR; ELISA, enzyme-linked immunosorbent assay.

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evidence indicating that leukocyte migration plays an important role in the perpetuation of inflammation in synovium [Choy and Panayi, 2001; Sakkas and Platsoucas, 2007]. This process of leukocyte migration is mediated largely by chemokines and chemokine receptors that are produced or expressed by particular inflammatory cell subsets [Coelho et al., 2008; Levinger et al., 2011]. For example, monocyte chemoattractant protein 1 (MCP-1)/CCL2 and macrophage inflammatory protein 1β (MIP-1β)/CCL4 are key chemokines involved in synovium.

Chemokines are low molecular weight secretory proteins that can regulate the chemotaxis and metabolic activity of specific leukocyte subsets. CCL2, a ligand of CCR2, is chemotactic for monocyte/macrophages and activated T cells [Maghazachi et al., 1994; Szekanecz et al., 2003]. It was reported that the levels of CCL2 are increased in the blood, synovial fluid, and synovial tissue of patients with OA and rheumatoid arthritis (RA) [Koch et al., 1992; Levinger et al., 2011]. Injection of MCP-1 into rabbit joints resulted in marked macrophage infiltration in the affected joint [Akahoshi et al., 1993]. Treatment with CCL2 antagonist before disease onset in an MRL/lpr mouse model of arthritis was shown to prevent the onset of arthritis [Gong et al., 1997].

Elevated levels of proinflammatory cytokines including TNF-α in the synovial fluid have been detected in OA and RA patients [Di Giovine et al., 1988; Saxne et al., 1988]. TNF-α is synthesized as a transmembrane protein with a molecular mass of 26 kDa and the pro-peptide is secreted as a soluble 17-kDa molecule on cleavage by TNF-α-converting enzyme [Cross et al., 2006]. TNF-α acts as a potent stimulus of inflammatory responses through up-regulation of many genes, including those for cytokines, chemokines, proteinases, cyclooxygenase, and adhesion molecules [Tracey and Cerami, 1993; Liang et al., 2011]. Although CCL2 has been shown to mediate local and systemic inflammatory reactions in OA and RA, the detail mechanisms underlying TNF-α-induced CCL2 expression in human synovial fibroblasts were needed defining.

Apoptosis signal-regulating kinase 1 (ASK1), a member of the MAPK kinase kinase (MKKK) family, is a serine/threonine protein kinase involved in regulating diverse cellular responses and is an upstream activator of JNK and p38 MAPK [Ichijo et al., 1997; Yang et al., 2010]. ASK1 has been reported to be activated in response to many stress signals, including H_2O_2, endoplasmic reticular stress, and amyloid β peptide [Ichijo et al., 1997; Hsu et al., 2007; Yang et al., 2011]. To regulate ASK1 activity, there are multiple steps, including dimerization, phosphorylation, and protein–protein interactions [Hwang et al., 2005]. Phosphorylation of the Ser^{967} residue in ASK1 is required for formation of the ASK-14-3-3 complex, which maintains ASK1 inactive [Goldman et al., 2004]. Following stimulation, phosphorylation of Thr^{845}, which is essential for ASK1 activation, triggers the dissociate 14-3-3 from Ser^{967}, resulting in enhancement of ASK1 catalytic activity [Goldman et al., 2004; Hwang et al., 2005]. However, the role of ASK1 in regulating TNF-α-mediated CCL2 expression in human synovial fibroblasts is still unknown. In this study, we explored the intracellular signaling pathway involved in TNF-α-induced CCL2 production in human synovial fibroblasts. The results show that TNF-α activates TNFR1 and causes the activation of the ASK1, JNK/p38, c-Jun, and AP-1 pathways, leading to up-regulated of CCL2 expression.

MATERIALS AND METHODS

MATERIALS

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, ASK1, p-JNK, JNK, p-p38, p38, p-c-Jun, c-Jun, P84, and the small interfering RNAs (siRNAs) against TNFR1, ASK1, c-Jun, and a control for experiments using targeted siRNA transfection (each consists of a scrambled sequence that does not lead to specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies specific for ASK1 phosphorylated at Thr^{845} and Ser^{967} were purchased from Cell Signaling and Neuroscience (Danvers, MA). Tanshinone IIA was purchased from BIOMOL (Butler Pike, PA). Mouse monoclonal antibodies specific for TNFR1 and CCL2 were purchased from R&D Systems (Minneapolis, MN). The CCL2 enzyme immunoassay kit was purchased from Cayman Chemical (Ann Arbor, MI). Recombinant human TNF-α was purchased from PeproTech (Rocky Hill, NJ). The AP-1 luciferase plasmid was purchased from Stratagene (La Jolla, CA). The p38 dominant negative mutant was provided by Dr. J. Han (University of Texas South-western Medical Center, Dallas, TX). The JNK dominant negative mutant was provided by Dr. M. Karin (University of California, San Diego, CA). The pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, WI). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

CELL CULTURES

After approval by the local ethics committee, human synovial fibroblasts were isolated using collagenase treatment of synovial tissues obtained from knee replacement surgeries of 25 patients with OA. Fresh synovial tissues were minced and digested in a solution of collagenase and DNase. Isolated fibroblasts were filtered through 70-μm nylon filters. The cells were grown on plastic cell culture dishes in 95% air/5% CO_2 in RPMI 1640 (Life Technologies) that was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (pH adjusted to 7.6). Fibroblasts from passages four to nine were used for the experiments [Tang et al., 2007; Chiu et al., 2009].

MEASUREMENT OF CCL2 PRODUCTION

Human synovial fibroblasts were cultured in 24-well culture plates. After reaching confluency, cells were treated with CCL2 and then incubated in a humidified incubator at 37°C for 24 h. To examine the downstream signaling pathways involved in TNF-α treatment, cells were pretreated with various inhibitors for 30 min (the inhibitors were left in the culture medium) before addition of TNF-α (1 ng/ml) administration. After incubation, the medium was removed and stored at −80°C until the assay was performed. CCL2 in the medium was assayed using CCL2 enzyme immunoassay kits, according to the procedure described by the manufacturer.

QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from synovial fibroblasts with a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA (in 2 μl RNase-free water) that

was reverse transcribed into cDNA with an MMLV RT kit (Promega) and following the manufacturer's recommended procedures [Hsieh et al., 2003; Wang et al., 2003]. cDNA synthesis was performed in a final volume of 20 μl containing 4 μl of 5× buffer, 1 μl of dNTPs (mixture of dATP, dCTP, dGTP, and dTTP), 1 μl of oligo (dT), 20 U of RNasin ribonuclease inhibitor, 2 μg of template, 200 U of MMLV, and DEPC-treated water. The reverse transcription reaction mixture was incubated at 37°C for 60 min and then at 70°C for 5 min to inactivate MMLV. Quantitative real time PCR (qPCR) analysis was carried out with TaqMan® one-step PCR Master Mix (Applied Biosystems, Foster City, CA). cDNA template (2 μl) was added to each 25-μl reaction with sequence-specific primers and TaqMan® probes. All target gene primers and probes were purchased commercially (β-actin was used an internal control) (Applied Biosystems). qPCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions were: 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T). CCL2 mRNA levels were normalized to β-actin mRNA levels and expressed relative to control using the ΔΔCt method.

WESTERN BLOT ANALYSIS

Cellular lysates were prepared as described [Huang et al., 2003; Tseng et al., 2003]. Proteins were resolved using SDS–PAGE and transferred to Immobilon polyvinyldifluoride membranes. The membranes were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit antibodies against human p-JNK, JNK, p-p38, p38, p-c-Jun, or c-Jun (1:1,000) for 1 h at room temperature. After three washes, the blots were incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (Ab, 1:1,000) for 1 h at room temperature. The blots were visualized with enhanced chemiluminescence on Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

PREPARATION OF NUCLEAR EXTRACTS

Cells were harvested and suspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice and vortexed for 10 s. Nuclei were pelleted by centrifugation at 12,000*g* for 20 s. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was suspended in buffer C (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4 M NaCl) for 30 min on ice. The supernatants containing nuclei proteins were collected by centrifugation at 12,000*g* for 20 min and stored at −70°C.

TRANSFECTION AND REPORTER GENE ASSAY

Human synovial fibroblasts were co-transfected with 0.8 μg AP-1 luciferase plasmid and 0.4 μg β-galactosidase expression vector. OASF cells were grown to 80% confluency in 12-well plates and then transfected on the following day with Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then added to the cells. After 24 h of transfection, the cells were incubated with the indicated reagents. After a further 24 h of incubation, the medium was removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to the transfection efficiency, which was monitored by activity of the co-transfected β-galactosidase expression vector.

CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation analysis was performed as described previously [Chiu et al., 2009]. DNA immunoprecipitated with an anti-c-Jun Ab was purified and extracted with phenol–chloroform. The purified DNA pellet was subjected to PCR. PCR products were then resolved by 1.5% agarose gel electrophoresis and visualized with UV light. The primers 5′-TAAGGGGAGAGGAGGGAAAAAT-3′ and 5′-TTCATGCTGGAGGCGAGAGTGC-3′ were utilized to amplify across the CCL2 promoter region (−486 to +6).

STATISTICS

Data were expressed as means ± SEM. For statistical evaluation, we used the Mann–Whitney *U*-test for non-Gaussian parameters. The difference was considered significant if the *P*-value was <0.05.

RESULTS

TNF-α INDUCES CCL2 PRODUCTION IN HUMAN SYNOVIAL FIBROBLASTS

TNF-α is involved in the pathology of OA [Di Giovine et al., 1988; Saxne et al., 1988]. The typical pathology of OA includes chronic inflammation of the synovium that is characterized by infiltration of inflammatory cells and synovial hyperplasia, especially of fibroblast-like synoviocytes. Therefore, we used human synovial fibroblasts to investigate the signaling pathways of TNF-α in the production of CCL2. Treatment of OASF with TNF-α (0.3–1 ng/ml) for 24 h induced CCL2 production in a concentration-dependent manner (Fig. 1B), and this induction occurred in a time-dependent manner (Fig. 1D). After TNF-α (1 ng/ml) treatment for 24 h, the amount of CCL2 released had increased in OASF cells (Fig. 1D). In addition, stimulation of cells with TNF-α also led to increased expression of CCL2 mRNA and protein in a concentration-dependent manner (Fig. 1A,C). It has been reported that TNF-α exerts its effects through interaction with a specific receptor TNFR1 [Bianchi and Meier, 2009]. Pretreatment of OASF cells with TNFR1 mAb reduced TNF-α-increased CCL2 expression (Fig. 1E,F). Transfection of cells with TNFR1 siRNA reduced TNFR1 protein expression (Fig. 1E). In addition, transfection of cells with TNFR1 siRNA reduced TNF-α-increased CCL2 expression (Fig. 1E,F). Therefore, an interaction between TNF-α and TNFR1 is very important for CCL2 production in human synovial fibroblasts.

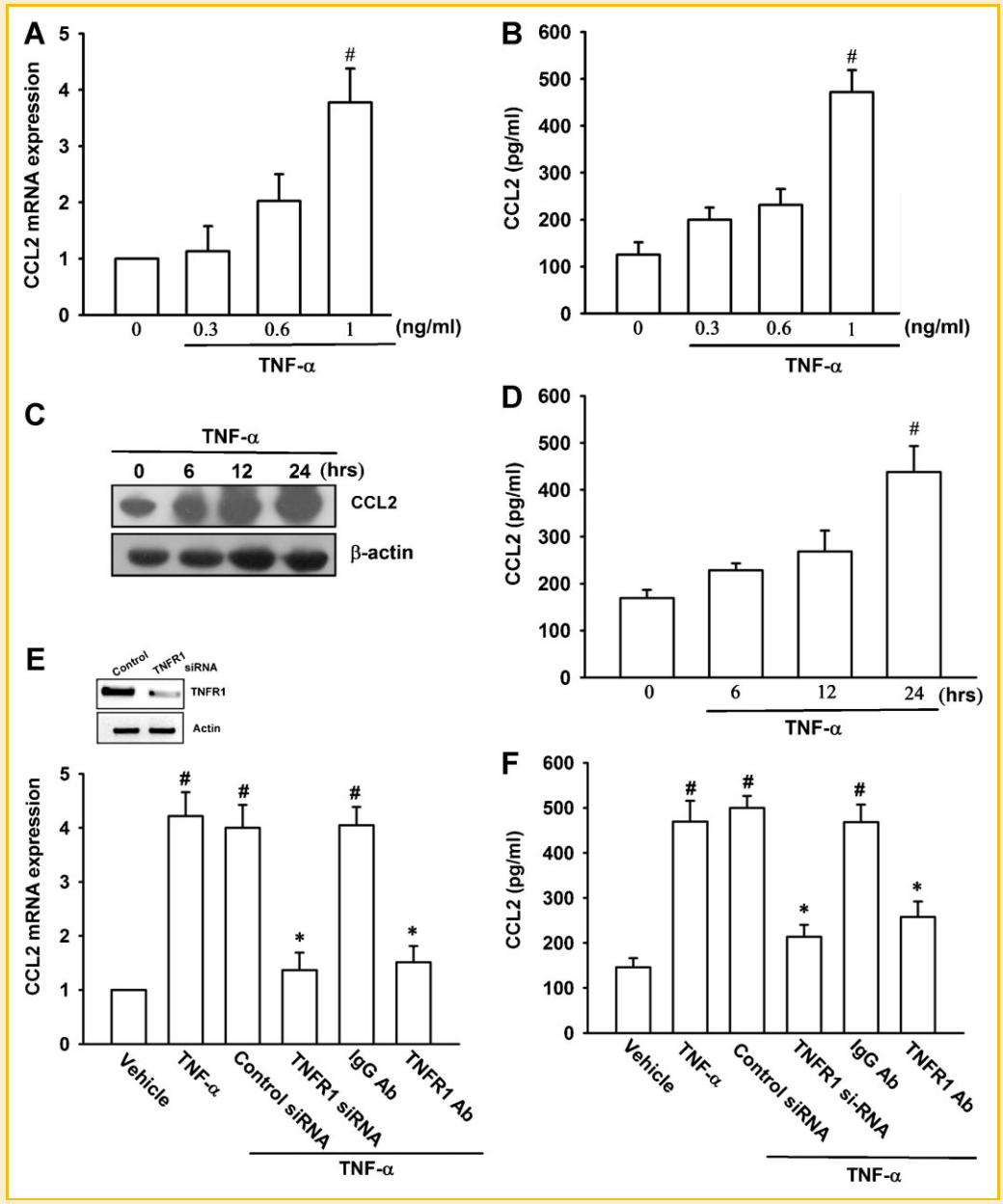

Fig. 1. Concentration- and time-dependent increases in CCL2 production by TNF-α. A: OASF cells were incubated with TNF-α for 24 h, and CCL2 mRNA was examined by qPCR. OASF were incubated with various concentrations of TNF-α for 24 h (B) or with TNF-α (1 ng/ml) for 6, 12, or 2 h (D). Media were collected to measure CCL2. (n = 5). (C) OASF cells were incubated with TNF-α (1 ng/ml) for 6, 12, or 24 h, and CCL2 protein expression was examined by Western blotting. (E; upper panel) OASF cells were transfected with TNFR1 or control siRNA for 24 h, the protein levels of TNFR1 was determined by Western blotting. E and F: Cells were pretreated for 30 min with TNFR1 Ab (1 μg/ml) or transfected with TNFR1 siRNA for 2 h followed by stimulation with TNF-α for 24 h, and CCL2 expression was examined by qPCR and ELISA (n = 5). $^{*}P < 0.05$ as compared with basal level. $\#P < 0.05$ as compared with TNF-α-treated group.

INVOLVEMENT OF ASK1 IN TNF-α-INDUCED CCL2 EXPRESSION

Previous studies have shown that ASK1 plays a crucial role in regulating the expression of genes [Matsuzawa and Ichijo, 2008; Yu et al., 2009]. To determine whether ASK1 is involved in TNF-α-triggered CCL2 production, OASF cells were pretreated with thioredoxin, an ASK1 inhibitor, for 30 min and then incubated with TNF-α for 24 h. As shown in Figure 2A–C, pretreatment with thioredoxin reduced TNF-α-induced CCL2 production and expression, suggesting that ASK1 may play a role in TNF-α-induced CCL2 expression in OASF. Transfection with ASK1 siRNA specifically blocked protein expression of ASK1 (Fig. 2A). In addition, ASK1 siRNA also reduced TNF-α-induced CCL2 expression (Fig. 2A,B). We then directly measured phosphorylation of ASK1 in response to TNF-α. ASK1 activation, as indicated by phosphorylation at the activation loop Thr^{845} and dephosphorylation at Ser^{967}, was assessed by immunoblot analysis. Treatment of OASF cells with TNF-α significantly increased phosphorylation at Thr^{845} concomitant reduction of phosphorylation at Ser^{967} (Fig. 2D). Dissociation of

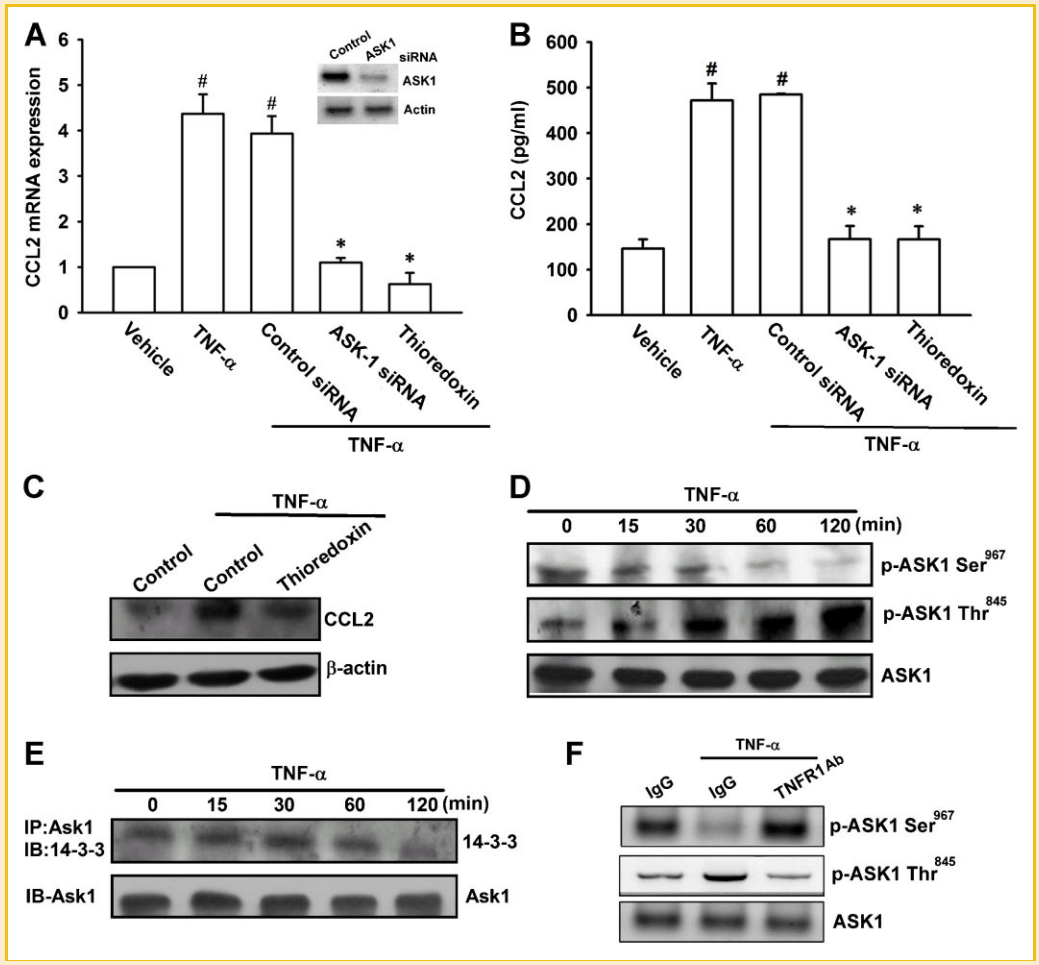

Fig. 2. ASK1 is involved in TNF-α-induced CCL2 expression. OASF cells were pretreated for 30 min with thioredoxin (200 ng/ml) or transfected with ASK1 siRNA followed by stimulation with TNF-α for 24 h. Media and total RNA were collected, and the expression of CCL2 was analyzed with ELISA and qPCR (A and B). OASF cells were transfected with ASK1 or control siRNA for 24 h, the protein levels of ASK1 was determined by Western blotting (A; upper panel). OASF cells were pretreated for 30 min with thioredoxin followed by stimulation with TNF-α for 24 h, and CCL2 protein expression was examined by Western blotting (C). Cells were incubated with TNF-α for indicated time intervals, and ASK1 phosphorylation was examined by Western blotting (D). Cells were incubated with TNF-α for different times and then immunoprecipitated (IP) with anti-ASK1. The IP complexes were subjected to immunoblotting (IB) with anti-14-3-3 (E). Cells were pretreated for 30 min with TNFR1 Ab for 30 min, followed by stimulation with TNF-α for 30 min, and ASK1 phosphorylation was determined by Western blotting (F). $^{*}P < 0.05$ as compared with basal level. $\#P < 0.05$ as compared with TNF-α-treated group.

ASK1 from 14-3-3 (an inhibitory protein) was previously reported to lead to ASK1 activation [Zhang et al., 1999]. Therefore, we used coimmunoprecipitation to determine whether TNF-α induced ASK1 dephosphorylation was accompanied by dissociation of the ASK1-14-3-3 complex. As shown in Figure 2E, TNF-α rapidly induced ASK1 dissociation from 14-3-3. Pretreatment of cells with TNFR1 Ab also reduced TNF-α-induced ASK1 phosphorylation of Thr^{845} and dephosphorylation of Ser^{967} (Fig. 2F). Based on these results, TNF-α appears to act through a TNFR1- and ASK1-dependent signaling pathway to enhance CCL2 production in human synovial fibroblasts.

THE JNK AND P38 SIGNALING PATHWAYS ARE INVOLVED IN THE POTENTIATING ACTION OF TNF-α

ASK1 belongs to the MAPKKK family and activates the p38 and JNK pathways via MKK3/6 and MKK4/7, respectively [Ichijo et al., 1997]. We thus investigated the role of JNK and p38 in mediating TNF-α-induced CCL2 expression using the specific JNK inhibitor SP600125 and p38 inhibitor SB203580. As shown in Figures 3 and 4A–C, TNF-α-induced CCL2 expression was markedly attenuated by pretreatment of cells for 30 min with SP600125 and SB203580 or transfection of cells for 24 h with JNK and p38 mutant. To directly confirm the crucial role of JNK and p38 in CCL2 expression, we measured the level of JNK and p38 phosphorylation in response to TNF-α. Indeed, treatment of fibroblasts with TNF-α resulted in a time-dependent phosphorylation of JNK and p38 (Figs. 3 and 4D). We next evaluated the relationship among TNFR1, ASK1, and JNK/p38 in the TNF-α-mediated signaling pathway and found that pretreatment of cells for 30 min with TNFR1 Ab, or thioredoxin markedly inhibited the TNF-α-induced JNK and p38 phosphorylation (Figs. 3 and 4E). Based on these results, TNF-α appears to act via TNFR1 receptor and the ASK1- and JNK/p38-dependent

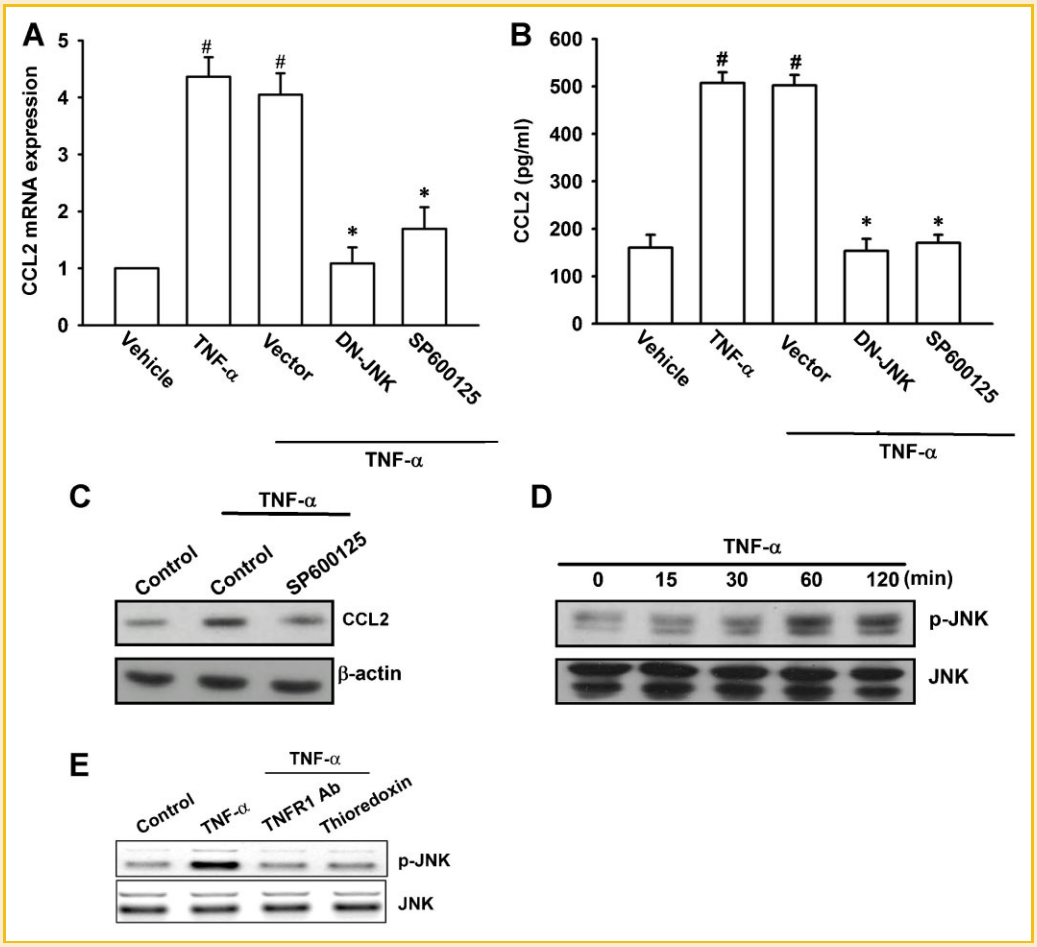

Fig. 3. JNK is involved TNF-α-induced CCL2 expression. OASF cells were pretreated for 30 min with SP600125 (3 μg/ml) or transfected with JNK mutant followed by stimulation with TNF-α for 24 h. Media and total RNA were collected, and the expression of CCL2 was analyzed with ELISA and qPCR (A and B). OASF cells were pretreated for 30 min with SP600125 followed by stimulation with TNF-α for 24 h, and CCL2 protein expression was examined by Western blotting (C). Cells were incubated with TNF-α for indicated time intervals, and JNK phosphorylation was examined by Western blotting (D). Cells were pretreated for 30 min with TNFR1 Ab, or thioredoxin for 30 min, followed by stimulation with TNF-α for 30 min, and JNK phosphorylation was determined by Western blotting (E). $^{*}P < 0.05$ as compared with basal level. $\#P < 0.05$ as compared with TNF-α-treated group.

signaling pathway to enhance CCL2 production in human synovial fibroblasts.

INVOLVEMENT OF AP-1 IN TNF-α-INDUCED CCL2 EXPRESSION

The promoter region of human CCL2 contains AP-1 binding site [Lin et al., 2004]. To examine the role of the AP-1 binding site in TNF-α-mediated CCL2 expression, the AP-1 inhibitor curcumin and tanshinone IIA were used. Pretreatment of cells with curcumin or tanshinone IIA reduced TNF-α-enhanced CCL2 expression (Fig. 5A–C). AP-1 activation was further evaluated by analyzing the accumulation of phosphorylated c-Jun in the nucleus as well as by a chromatin immunoprecipitation assay. Treatment of cells with TNF-α resulted in a marked accumulation of phosphorylated c-Jun in the nucleus (Fig. 5D). Transfection of cells with c-Jun siRNA suppressed the expression of c-Jun (Fig. 5A). TNF-α-induced CCL2 expression was also inhibited by c-Jun siRNA but not by control siRNA (Fig. 5A,B). We next investigated whether c-Jun binds to the AP-1 element on the CCL2 promoter after TNF-α stimulation. The in vivo recruitment of c-Jun to the CCL2 promoter (−468 to +6) was assessed via chromatin immunoprecipitation assay [Lin et al., 2008]. In vivo binding of c-Jun to the AP-1 element of the CCL2 promoter occurred after TNF-α stimulation (Fig. 6A). The binding of c-Jun to the AP-1 element by TNF-α was attenuated by TNFR1 Ab, thioredoxin, SP600125, and SB203580 (Fig. 6A). To further confirm that the AP-1 element is involved in TNF-α-induced CCL2 expression, we performed transient transfection with AP-1 promoter-luciferase constructs. Synovial fibroblasts incubated with TNF-α showed a 2.5-fold increase in AP-1 promoter activity. The increase in AP-1 activity by TNF-α was antagonized by TNFR1 Ab, thioredoxin, SP600125, and SB203580 or TNFR1 and ASK1 siRNA or JNK and p38 mutant (Fig. 6B,C). Taken together, these data suggest that the activation of the TNFR1, ASK1, JNK/p38, c-Jun, and AP-1 pathway is required for the TNF-α-induced increase in CCL2 in human OASF cells.

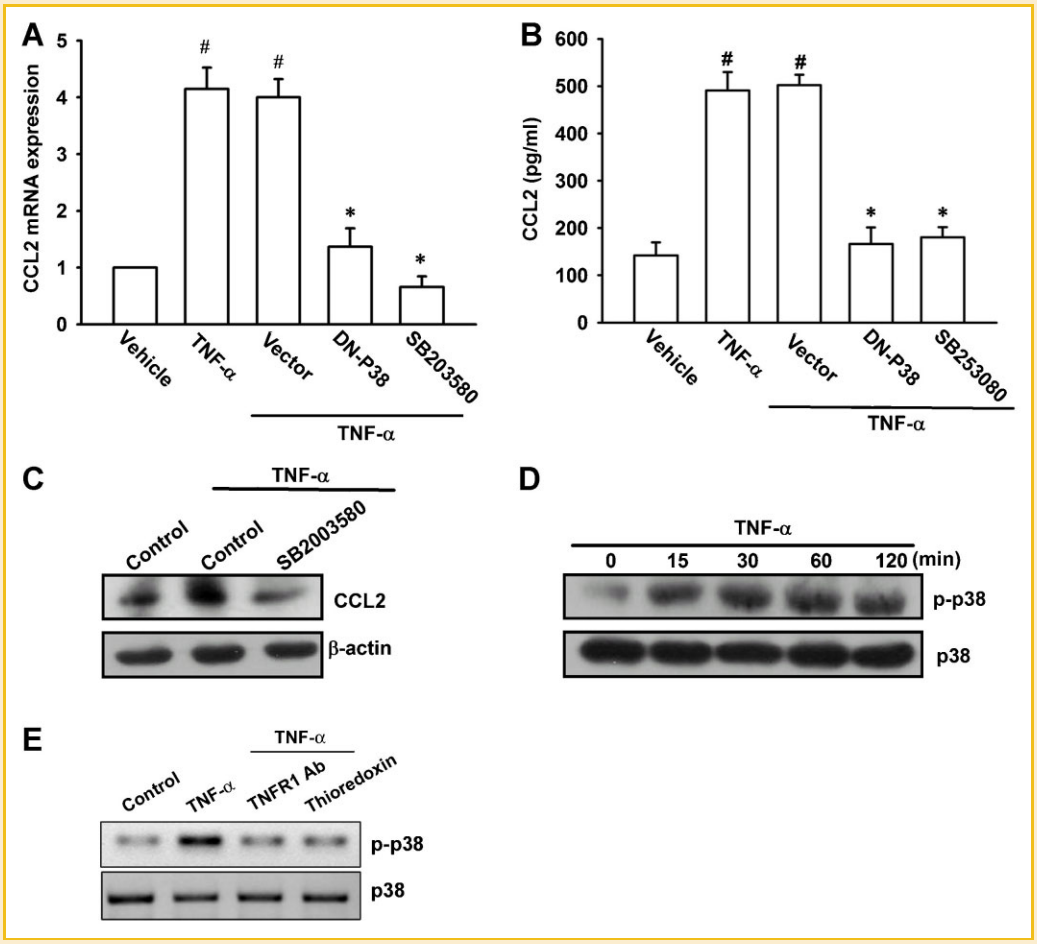

Fig. 4. p38 is involved in TNF-α-mediated CCL2 production in synovial fibroblasts. OASF cells were pretreated for 30 min with SB203580 (10 μg/ml) or transfected with p38 mutant followed by stimulation with TNF-α for 24 h. Media and total RNA were collected, and the expression of CCL2 was analyzed with ELISA and qPCR (A and B). OASF cells were pretreated for 30 min with SB203580 followed by stimulation with TNF-α for 24 h, and CCL2 protein expression was examined by Western blotting (C). Cells were incubated with TNF-α for indicated time intervals, and p38 phosphorylation was examined by Western blotting (D). Cells were pretreated for 30 min with TNFR1 Ab, or thioredoxin for 30 min, followed by stimulation with TNF-α for 30 min, and p38 phosphorylation was determined by Western blotting (E). $^{*}P < 0.05$ as compared with basal level. $\#P < 0.05$ as compared with TNF-α-treated group.

DISCUSSION

OA is a heterogeneous group of conditions associated with defective integrity of articular cartilage as well as related changes in the underlying bone. The chronic inflammatory process is mediated through a complex cytokine network. It is not yet completely understood which factors are responsible for initiating the degradation and loss of articular tissues. TNF-α is considered to be the major ones contributing to the process of development of OA [Pelletier et al., 2001]. Here we further identified CCL2 as a target protein for the TNF-α signaling pathway that regulates the cellular inflammatory response. We showed that potentiation of CCL2 by TNF-α requires activation of the TNFR1 receptor, ASK1, JNK/p38, c-Jun, and AP-1 signaling pathways.

TNF-α, a pro-inflammatory cytokine with a critical role in OA, is primarily produced by monocytes/macrophages and expressed as a bioactive 26 kDa precursor transmembrane molecule or a secreted mature 17 kDa cytokine [Wajant et al., 2003]. The biological activity of TNF-α is mediated by binding to two distinct but related receptors of TNFR1 and TNFR2. It has been reported that TNF-α-induced MAPK activation through TNFR1 but not TNFR2 receptor in OA and RA synovial fibroblasts [Kunisch et al., 2007]. Here, we confirmed that TNFR1 receptor is required for TNF-α-induced CCL2 expression. Pretreatment of cells with TNFR1 Ab reduced TNF-α-induced CCL2 expression. This was further confirmed by the result that the TNFR1 siRNA inhibited the enhancement of CCL2 production by TNF-α. Therefore, the interaction between TNF-α and TNFR1 is very important for CCL2 production by human synovial fibroblasts.

ASK1 activity is regulated by multiple mechanisms including phosphorylation and interactions with various proteins. Phosphorylation at Ser^{967} is essential for ASK1 association with 14-3-3 protein, which attenuates ASK1 activity [Zhang et al., 2003]. A previous study showed that ROS induces dephosphorylation of Ser^{967} as well as phosphorylation of Thr^{845} in the ASK1 activation loop, both of which are correlated with ASK1 activity [Valko et al., 2006]. Here, we found that TNF-α enhanced dephosphorylation at

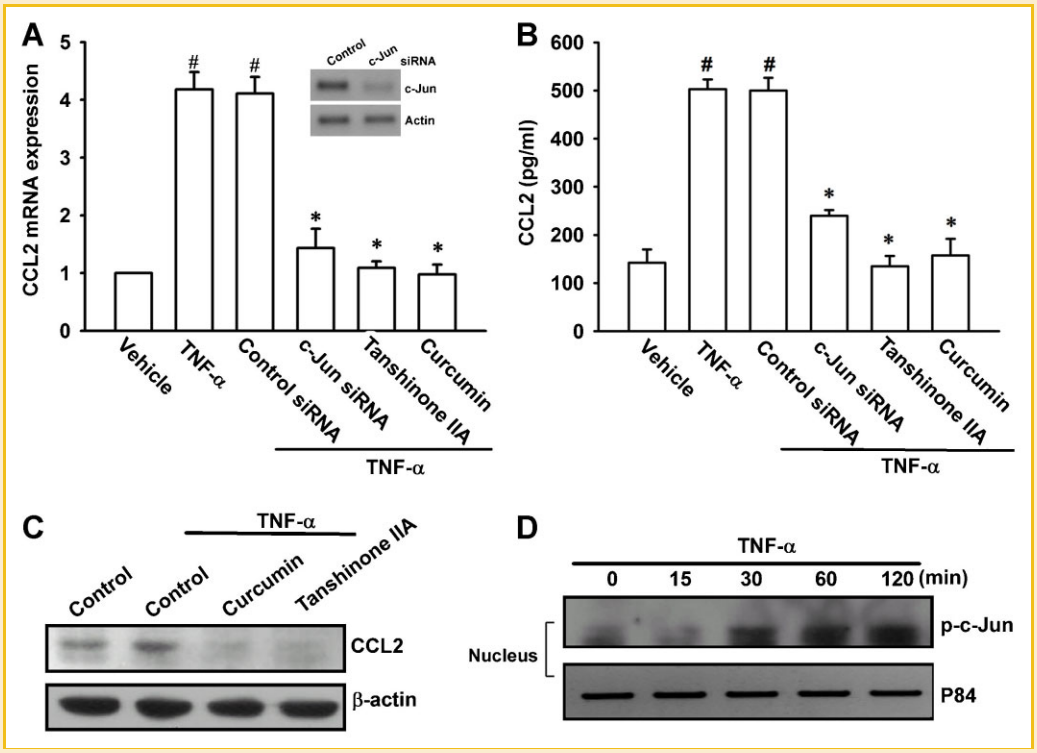

Fig. 5. AP-1 is involved in the potentiation of CCL2 production by TNF-α. OASF cells were pretreated for 30 min with curcumin and tanshinone IIA or transfected with c-Jun siRNA followed by stimulation with TNF-α for 24 h. Media and total RNA were collected, and the expression of CCL2 was analyzed with ELISA and qPCR (A and B). OASF cells were transfected with c-Jun or control siRNA for 24 h, the protein levels of c-Jun was determined by Western blotting (A; upper panel). OASF cells were pretreated for 30 min with curcumin and tanshinone IIA followed by stimulation with TNF-α for 24 h, and CCL2 protein expression was examined by Western blotting (C). OASF cells were incubated with TNF-α for indicated time intervals, and c-Jun phosphorylation in nucleus were determined by Western blotting (D). $^{*}P < 0.05$ as compared with basal level. $\#P < 0.05$ as compared with TNF-α-treated group.

Ser^{967} and phosphorylation at Thr^{845} of ASK1. However, we did not examine the role of ROS on TNF-α enhanced induced ASK1 activation. Which signaling pathways are upstream molecules in TNF-α enhanced induced ASK1 activation in synovial fibroblasts needs further examination. Furthermore, ASK1 inhibitor or siRNA inhibited TNF-α induced CCL2 expression. Therefore, ASK1 activation is required for TNF-α induced CCL2 expression in human synovial fibroblasts.

ASK1 is an upstream molecule of JNK and p38, which have been shown to be involved in the regulation of genes expression [Takeda et al., 2008]. We showed in present study that TNF-α increased the p38 and JNK phosphorylation. Pretreatment of cells with p38 and JNK inhibitor antagonized the TNF-α-induced CCL2 expression. This was further confirmed by the result that p38 and JNK mutant inhibited the enhancement of CCL2 by TNF-α. On the other hand, TNFR1 Ab and thioredoxin reduced TNF-α-mediated JNK and p38 phosphorylation. Taken together, our results provide evidence that TNF-α up-regulates CCL2 in human synovial fibroblasts via the TNFR1, ASK1, and JNK/p38 signaling pathway.

There are several binding sites for a number of transcription factors including NF-κB, Sp-1, and AP-1 in the 5′ region of the CCL2 gene [Kok et al., 2009]. Recent studies of the CCL2 promoter have demonstrated that CCL2 induction by several transcription factors occurs in a highly stimulus-specific or cell-specific manner [Lin et al., 2004; Kok et al., 2009]. The results of our current study show that AP-1 activation contributes to TNF-α-induced CCL2 expression in synovial fibroblasts. Pretreatment of cells with an AP-1 inhibitor curcumin or tanshinone IIA reduced TNF-α-increased CCL2 expression. Therefore, the AP-1 binding site is likely to be the most important site for TNF-α-induced CCL2 production. The AP-1 sequence binds to members of the Jun and Fos families of transcription factors. These nuclear proteins interact with the AP-1 site as Jun homodimers or Jun–Fos heterodimers formed by protein dimerization through their leucine zipper motifs. The results of our study show that TNF-α induced c-Jun nuclear accumulation. In addition, c-Jun siRNA abolished TNF-α-induced CCL2 expression in OASF cells. Therefore, c-Jun activation mediates by TNF-α-increased CCL2 expression. Furthermore, TNF-α increased the binding of c-Jun to the AP-1 element within the CCL2 promoter, as shown by a chromatin immunoprecipitation assay. Binding of c-Jun to the AP-1 element was attenuated by TNFR1 Ab, thioredoxin, SP600125, and SB203580. Using transient transfection with AP-1-luciferase as an indicator of AP-1 activity, we also found that TNF-α induced an increase in AP-1 activity. In addition, TNFR1 Ab, thioredoxin, SP600125, and SB203580 or TNFR1 and ASK1 siRNA or JNK and p38 mutant reduced TNF-α-increased AP-1 promoter

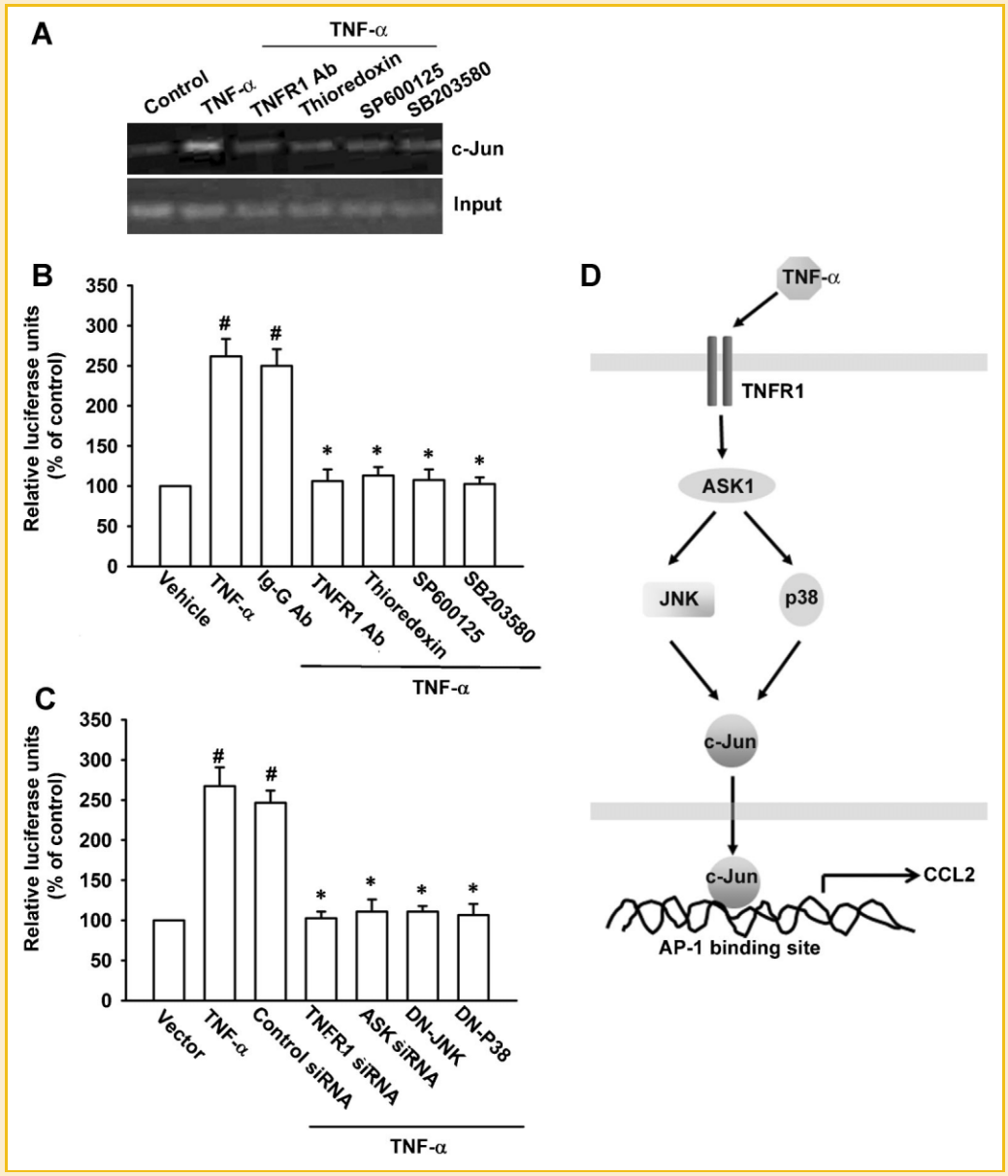

Fig. 6. The TNFR1, ASK1, and JNK/p38 pathway is involved in TNF-α-induced AP-1 activation. A: OASF cells were pretreated with TNFR1 Ab, thioredoxin, SP600125, or SB203580, and then stimulated with TNF-α for 120 min. A chromatin immunoprecipitation assay was then performed. The chromatin was immunoprecipitated with anti-c-Jun. One percent of the precipitated chromatin was assayed to verify equal loading (input). OASF cells were transfected with the AP-1-luciferase expression vector and then pretreated with TNFR1 Ab, thioredoxin, SP600125, and SB203580 or cotransfected with TNFR1, ASK1, and c-Jun siRNA or JNK and p38 mutant before incubation with TNF-α for 2 h (B and C). Luciferase activity was then assayed. $^{*}P < 0.05$ as compared with basal level. $\#P < 0.05$ as compared with TNF-α-treated group. D: Schematic diagram of the signaling pathways involved in TNF-α-induced CCL2 expression in synovial fibroblasts. TNF-α increases CCL2 expression by binding to the TNFR1 receptor and activating ASK1 and JNK/p38, which enhances binding of c-Jun to the AP-1 site. This results in the transactivation of CCL2 expression.

activity. These results indicate that the interaction between TNF-α may act through the TNFR1, ASK1, JNK/p38, c-Jun, and AP-1 pathway to induce CCL2 activation in human OASF cells.

In conclusion, we explored the signaling pathway involved in TNF-α-induced CCL2 expression in human synovial fibroblasts. We found that TNF-α increased CCL2 expression by binding to the TNFR1 receptor and activating ASK1 and JNK/p38, which enhanced binding of c-Jun to the AP-1 site and resulted in the transactivation of CCL2 expression (Fig. 6D). These findings may provide a better understanding of the mechanisms of OA pathogenesis.

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