Role of MAPK Kinase 6 in Arthritis: Distinct Mechanism of Action in Inflammation and Cytokine Expression

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Development of p38α inhibitors for rheumatoid arthritis has been hindered by toxicity and limited efficacy. Therefore, we evaluated whether MKK6, an upstream kinase that regulates p38 isoforms, might be an alternative therapeutic target in inflammatory arthritis. Wild-type (WT), MKK6−/−, and MKK3−/− mice were administered K/B×N serum to induce arthritis. Articular expression of activated kinases and cytokines was determined by Western blot, qPCR, ELISA, and multiplex analysis. Immunoprecipitation and confocal microscopy experiments were performed to determine the subcellular location of MKK6, p-p38, and MAPKAPK2 (MK2). Arthritis scores were significantly lower in MKK6−/− mice compared with WT mice. Joint destruction and osteoclast differentiation were lower in MKK6−/−, as were articular IL-6 and matrix metalloproteinase-3 expression. Phospho-p38 levels were modestly decreased in the joints of arthritic MKK6−/− mice compared with WT but were significantly higher than MKK3−/− mice. P-MK2 was low in MKK6−/− and MKK3−/− mice. Uncoupled p38 and MK2 activation was also observed in cultured, MKK6−/− FLS and confirmed using kinase assays. Immunoprecipitation assays and confocal microscopy showed that P-p38 and MK2 colocalized in activated WT but not MKK6−/− FLS. Distinct patterns of cytokine production were observed in MKK6−/− and MKK3−/− cells. MKK6 deficiency suppresses inflammatory arthritis and joint destruction, suggesting it might be a therapeutic target for inflammation. Although MKK3 and MKK6 activate the p38 pathway, they regulate distinct subsets of proinflammatory cytokines. MKK6 appears mainly to facilitate p38 and MK2 colocalization in the nucleus rather than to phosphorylate p38.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial cytokine production, joint destruction, and increased mortality. Biologic therapies that target individual cytokines have improved clinical outcomes. However, they only provide substantial benefit to a minority of patients and require parenteral administration (1, 2). Alternative approaches that modulate proinflammatory cytokine production are being explored, especially by targeting signal transduction pathways. Of these, the p38 MAPK pathway has emerged as an attractive possibility because of its central role in regulating cytokine and metalloproteinase (MMP) expression in diseases like RA (3, 4). However, clinical development of selective p38α inhibitors has been plagued by toxicity, especially liver, skin, and CNS, and limited efficacy (5, 6).

We have proposed that upstream MAP kinase kinases (MKK) that modulate p38 function could be targeted as an alternative by broadly inhibiting several p38 isoforms (7). Two of these upstream kinases, namely MKK3 and MKK6, activate p38 via dual phosphorylation at Thr180 and Tyr182. Unlike p38α−/− mice, MKK3−/− and MKK6−/− mice are viable and do not have evidence of organ toxicity associated with p38 inhibitors (8). Our previous studies showed that MKK3 deficiency suppresses passive K/B×N serum-induced arthritis (9). However, the role of MKK6 in arthritis is unknown and the two upstream kinases might not be redundant in the p38 signaling pathway and have different functions (8, 10). Thus, we studied the role of MKK6 in inflammatory arthritis and the molecular mechanisms by which MKK3 and MKK6 regulate inflammatory responses.

In this study, we show for the first time that MKK6 deficiency suppresses inflammatory arthritis. These studies also demonstrated that MKK3 and MKK6 have distinct mechanisms of action and distinct profiles of cytokine regulation. Mechanistic studies suggest that MKK3 plays a critical role in p38 phosphorylation. Although MKK6 can also activate p38 directly, it contributes to the colocalization of p38 with its substrate MAPKAPK2 (MK2). Based on its ability to regulate key proinflammatory cytokines, we propose that MKK6 is a novel therapeutic target for inflammatory diseases like RA.

Materials and Methods

Mice

MKK3−/− and MKK6−/− generated by Dr. Richard Flavell (Yale University, CT) were back-crossed for 10 generations onto the C57BL/6 background. KRN-TCR transgenic mice were a gift of Drs. D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) and the Institut de Génétique et de Biologie Moléculaire et Cellulaire (11) and were maintained on a C57BL/6 background (K/B). Arthritic mice were obtained by crossing...
K/B with NOD/Lt (N) animals (K/B × N). C57BL/6 mice were purchased from Charles River Laboratories. All experimental protocols involving animals were reviewed and approved by the University of California San Diego Institutional Animal Care and Use Committee (IACUC).

Passive K/B × N mouse serum transfer model of arthritis
Recipient mice were injected with 100 μl i.p. of pooled adult K/B × N mice serum on days 0 and 2. Arthritis was evaluated for each swollen paw using a semiquantitative scoring system (0 – 4 per paw; maximum score of 16) as previously described (12). For tissue protein assays, snap-frozen ankle joints were homogenized in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2.5 mM MgCl2, 1.0 mM EDTA (pH 8.0), 20 mM β-glycerophosphate, 10 mM NaF, 1 mM Na3VO4, 10 μg/ml aprotinin, 1 μM pepstatin A, 1 mM PMSF) and assayed by ELISA or immunoblotting. For tissue mRNA assays, RNA was isolated, and quantitative real-time PCR was performed. For histologic analysis, paws were fixed in 4% formalin and then decalcified in 14% EDTA (Sigma-Aldrich) with pH adjusted to 7.2. H&E staining was performed on 2-μm sections for quantification of inflammation and bone erosion and toluidine blue staining for determination of cartilage breakdown were performed. Quantitative morphometric analysis of synovial inflammation, bone erosion and cartilage proteoglycan loss was performed using a microscope (Zeiss Axioskop 2; Zeiss) and the OsteoMeasure System (OsteoMetrics).

Fibroblast-like synoviocytes (FLS) and culture conditions
Synovia from MKK6−/−, MKK3−/−, and wild-type (WT) mice were microdissected from ankles, minced, and digested with 1 mg/ml collagenase (13). The cell suspension was cultured in DMEM supplemented with 10% FBS, penicillin, streptomycin, and l-glutamine in a humidified 5% CO2 atmosphere. At confluence, cells were trypsinized, split at a 1:3 ratio, and recultured in medium. FLS were used from passages 4 – 6. Cells were synchronized in 0.1% FBS for 48 h before addition of stimulating cytokines.

Reagents
Anti-phospho (P)-p38 (Thr-180/Tyr-182), anti-p38, anti-P-MAPKAPK-2 (P-MK2), anti-MAPKAPK-2 (MK2), anti-P-mitogen-, and stress-activated protein kinase (P-MSK1), anti-MSK1 Ab and ATF2-fusion protein were purchased from Cell Signaling Technology. Recombinant human TNF-α and mouse IL-1β proteins were purchased from R&D Systems. LPS was obtained from Sigma-Aldrich. SB203580-HCl was obtained from Calbiochem.
Cytokine and MMP assay
For immunofluoroscopy, tissue protein extracts in modified RIPA buffer were assayed by ELISA (R&D Systems). For multiplex analysis, cells were treated with medium, 50 ng/ml TNF, or 1 μg/ml LPS for 24 h. Supernatants were assayed for CCL3 (macrophage inflammatory protein 1α [MIP1α]), CCL2 (MCP-1), mouse IL-8 (KC), CCL5 (RANTES), and CXCL10 (induced protein-10 [IP-10]) (Linco, Millipore) and analyzed by Bio-Plex System (Bio-Rad). Total RNA was harvested to assay MMP3, IL-6, and HPRT mRNA expression. Quantitative real-time PCR was performed using the GeneAmp 7300 sequence detection system (Applied Biosystems) as previously described (14).

Western blot analysis
Western blot analysis was performed as previously described (15). Synoviocytes were treated with IL-1β (2 ng/ml) or medium. Fifty micrograms of lysates were loaded and separated by SDS-PAGE, and then Western blot analysis was performed. Visualization and quantitation were performed using Versadoc Imaging system and Quantity One software (Bio-Rad).

In vitro kinase assays
IL-1β-treated FLS were lysed in modified RIPA buffer (16). One hundred micrograms of lysate was incubated with agarose-conjugated rabbit anti-MKK2 Ab (1/100; Upstate Biotechnology) overnight at 4°C on a rotator. The immunoprecipitates were washed four times and incubated with kinase buffer (50 mM HEPES [pH 7.4], 1 mM MgCl2, 5 mM KCl, 0.2 mM Na3VO4, 50 mM NaF, 10 μg/ml aprotinin, 1 mM pepstatin A, and 1 mM PMSF) containing 25 μCi of [γ-32P]ATP (PerkinElmer), 25 μM ATP, and 2 μg Hsp25 (Genway Biotech) at 30°C for 30 min. The nonradioactive p38 kinase assay kit (CST) was used according to manufacturer’s instructions. Reactions were run on a 4–12% Bis-Tris gel (Invitrogen) and visualized by autoradiography.

Nuclear extract preparation and immunoprecipitation
FLS were serum starved for 0.1% FCS/DMEM for 48 h and stimulated with IL-1β (2 ng/ml) for 0.5, or 15 min at 37°C. The cells were washed with PBS and incubated in lysis buffer (20 mM HEPES, 1.5 mM MgCl2, 5 mM KCl, 0.2 mM Na3VO4, 50 mM NaF, 10 μg/ml aprotinin, 1 mM pepstatin A, and 1 mM PMSF) containing 25 μCi of [γ-32P]ATP (PerkinElmer), 25 μM ATP, and 2 μg Hsp25 (Genway Biotech) at 30°C for 30 min. The nonradioactive p38 kinase assay kit (CST) was used according to manufacturer’s instructions. Reactions were run on a 4–12% Bis-Tris gel (Invitrogen) and visualized by autoradiography.

Confocal microscopy
FLS were cultured in eight-chamber poly t-lysine coated glass slides (Nunc) (16,000 cells/well). The cells were serum starved for 24 h and then stimulated with IL-1β (2 ng/ml) for 15 min at 37°C. Cells were then washed, fixed with 4% formaldehyde, and permeabilized with 0.1% Triton X-100/PBS. The samples were blocked with 2.5% donkey serum/0.05% Tween 20/1%BSA/PBS for 1 h at room temperature. The cells were incubated with mouse anti-P-p38 (Abcam) and rabbit anti-MKK2 Abs in blocking solution overnight at 4°C. Appropriate IgG control Abs were used according to manufacturer’s instructions (DakoCytomation). Cells were then washed and incubated with donkey anti-rabbit AlexaFluor 488, donkey anti-mouse AlexaFluor 648 (Molecular Probes) for 2 h at room temperature. The FLS were counterstained with Hoechst 33342 at 1 μg/ml, and mounted with Prolong Gold anti-fade reagent (Invitrogen), air dried, and analyzed using Zeiss LSM510 Laser Scanning Confocal Microscope. At least 10 cells/condition were analyzed and quantified using Zeiss LSM viewer and Colocalizer Express software and expressed as percent yellow pixels ± SEM.

Statistical analysis
Data are expressed as mean ± SEM. Student’s t test was used for comparisons between two groups and ANOVA for repeated measures using Kaleidograph 4 software (Synergy Software).

Results
MKK6 deficiency attenuates arthritis, cartilage destruction, and bone erosion
To determine whether MKK6 contributes to synovial inflammation and joint destruction, MKK6−/−, MKK6−/−, MKK3−/−, and WT mice were studied in the K/B×N serum transfer model of arthritis. MKK6 deficiency significantly decreased arthritis severity throughout the course of the model (Fig. 1A; p < 0.0001, n = 6/group) and was comparable to MKK3 deficiency. In contrast, MKK6−/− arthritis severity was similar to WT. Ankle histopathology on day 12 (Fig. 1B) showed significantly less fibrosis (p = 0.04), cartilage destruction (p = 0.0001), and bone erosion (p = 0.0007) in MKK6−/− mice compared with WT. Osteoclasts were undetectable in arthritic MKK6−/− mice, but were easily demonstrated in WT mice (Fig. 1C). IL-6 and MMP3 protein were evaluated in joint extracts of MKK6−/−, MKK6−/−, and WT mice at day 12 and were significantly lower in MKK6−/− mice (Fig. 2A; p = 0.001, n = 6/group). Articular IL-6 and MMP3 mRNA levels were also significantly lower in MKK6−/− mice (Fig. 2B; p <
Serum IL-6, KC (mouse IL-8 homologue), and G-CSF levels were significantly decreased in arthritic MKK6−/− joints compared with WT (data not shown). Therefore, MKK6 is required for full expression of passive K/BN arthritis, synovial cytokine and MMP expression, and osteoclast maturation.

**p38 and MK2 activation in vivo**

Initial experiments to assess p38 and MK2 activation were performed using joint extracts of arthritic WT, MKK6−/−, and MKK3−/− mice (n = 6/group). Western blot analysis showed that P-p38 levels in MKK6−/− mice were only modestly decreased compared with WT mice after K/BN serum administration but that P-p38 was nearly undetectable in the MKK3−/− mice (p = 0.0003 for MKK6−/− vs MKK3−/− t test; Fig. 3A). A second experiment confirmed that P-p38 levels in the joints of arthritic WT and MKK6−/− mice were similar (Fig. 3A). Despite the presence of P-p38 in the arthritic joints of MKK6−/− mice, phosphorylation of p38 substrates MK2 and MSK1 was significantly lower in MKK6−/− mice joints compared with WT mice, as were other p38 substrates such as P-p65 and P-Hsp25 (Fig. 3B; p < 0.0001, ANOVA with repeated measures). B, Activation of two p38 substrates, MK2 and MSK1, was significantly decreased in MKK3−/− and MKK6−/− compared with WT (MK2: WT: 2.6 ± 0.7, MKK6−/−: 0.7 ± 0.2, MKK3−/−: 0.6 ± 0.1, p = 0.004; MSK1: WT: 0.53 ± 0.04, MKK6−/−: 0.29 ± 0.04, MKK3−/−: 0.15 ± 0.01, p < 0.0001). C, Phosphorylation of two additional substrates, p65 and Hsp27, was also significantly decreased in MKK3−/− and MKK6−/− mice compared with WT (n = 6). * represents p < 0.05.

***FIGURE 3.*** Effect of MKK6 deficiency on synovial kinase phosphorylation. Joint lysates obtained from K/B×N-treated WT, MKK6−/−, MKK3−/− and untreated WT mice were evaluated by Western blot analysis. A, P-p38 expression was significantly lower in MKK6−/− and MKK3−/− compared with WT mice (WT: 5.1 ± 1.4, MKK6−/−: 1.2 ± 0.2, MKK3−/−: 0.3 ± 0.01; p < 0.0001, ANOVA with repeated measures). B, Activation of two p38 substrates, MK2 and MSK1, was significantly decreased in MKK3−/− and MKK6−/− compared with WT (MK2: WT:2.6 ± 0.7, MKK6−/−: 0.7 ± 0.2, MKK3−/−: 0.6 ± 0.1, p = 0.004; MSK1: WT: 0.53 ± 0.04, MKK6−/−: 0.29 ± 0.04, MKK3−/−: 0.15 ± 0.01, p < 0.0001). C, Phosphorylation of two additional substrates, p65 and Hsp27, was also significantly decreased in MKK3−/− and MKK6−/− mice compared with WT (n = 6). * represents p < 0.05.
Phosphorylation of p38 and MK2 in cultured synoviocytes. Cultured fibroblast-like synoviocytes were stimulated with IL-1 and evaluated for kinase activation. Data are shown as the ratio of arbitrary densitometry units as mean ± SEM. *, represents p < 0.05. A. Western blot analysis of WT, MKK6−/−, and MKK3−/− synoviocytes stimulated with IL-1 for various times showed that P-p38 expression was comparable between WT and MKK6−/− cells but reduced in MKK3−/− synoviocytes. B. After 15 min of IL-1 stimulation, P-p38 expression was significantly reduced in MKK3−/− (p = 0.004, n = 3 lines/group) but unaffected in MKK6−/− FLS compared with WT (p = 0.18). C. Despite normal P-p38 levels, P-MK2 expression was significantly reduced in MKK3−/− and MKK6−/− FLS (p = 0.027, ANOVA). D. FLS extracts were immunoprecipitated using anti-P-p38 Ab and in vitro kinase assays were performed using anti-P-p38 immunoprecipitates with ATF2 as substrate (n = 4). P38 kinase function was in the MKK6−/− FLS even though it does not activate MK2 in FLS. E. MK2 kinase function was determined using anti-MK2 immunoprecipitates with Hsp25 as substrate (n = 3 lines). The data show that MK2 activity is significantly decreased in MKK6−/− cells despite normal p38 activity.

was similar to WT and significantly increased compared with MKK3−/− cells (WT: 0.72 ± 0.19, MKK3−/−: 0.40 ± 0.04 (p = 0.004), MKK6−/−: 0.85 ± 0.08, n = 3/group). Despite increased P-p38 levels in IL-1 stimulated MKK6−/− FLS phosphorylation of MK2 was significantly decreased in both MKK3−/− and MKK6−/− FLS (Fig. 4C, P-MK2/MK2 ratio WT: 1.35 ± 0.22, MKK3−/−: 0.64 ± 0.06, MKK6−/−: 0.85 ± 0.05, p = 0.027, n = 3 separate lines/group). To compare p38 and MK2 activity of MKK6−/− and WT cells, in vitro kinase assays were performed using anti-P-p38 immunoprecipitates. Fig. 4D shows that the function of p38 was normal in MKK6−/− cells because immunoprecipitated P-p38 was able to phosphorylate ATF2 normally in intact FLS (results of four independent experiments). Immunoprecipitated MK2 function was significantly reduced as determined by in vitro kinase assays using Hsp25 as a substrate despite normal p38 activity in IL-1 stimulated MKK6−/− FLS (Fig. 4E, results of three independent experiments). These data indicate that MKK6 deficiency does not suppress p38 function in FLS, but that the resultant P-p38 generated in MKK6−/− FLS does not efficiently activate its substrate MK2.

p38 isoform expression and function in FLS

We then evaluated whether differential p38 and MK2 activation in MKK6−/− cells might be due to altered expression and/or activation of the p38 isoforms. To address this possibility, we evaluated the four p38 isoforms in WT, MKK3−/−, and MKK6−/− FLS. Western blot analysis showed that all p38 isoforms were expressed in MKK3−/− and MKK6−/− FLS (data not shown). Previous studies indicate that MKK6 activates all four p38 isoforms, while MKK3 only phosphorylates p38α, γ, and δ (17, 18). To assay p38 activation, IL-1β-stimulated FLS lysates were immunoprecipitated with an anti-P-p38 Ab and Western blot analysis was performed with anti-p38 isoform-specific and anti-P-p38 Abs. Phosphorylation of p38α and γ isoforms in response to IL-1 was decreased in MKK3−/− cells compared with WT (Fig. 5). p38β phosphorylation was decreased in both MKK3−/− and MKK6−/− FLS. p38β phosphorylation was not induced in MKK3−/− cells and was decreased in MKK6−/− FLS.

Potential role of MKK6 in p38 and MK2 complex formation

Our data suggest that MKK3 is mainly responsible for p38 phosphorylation and MKK6 is necessary for optimal MK2 phosphorylation. Therefore, we considered the hypothesis that MKK6 might facilitate colocalization of activated p38 and MK2 in the nucleus, allowing efficient MK2 phosphorylation. To test this possibility, WT and MKK6−/− FLS nuclear extracts were immunoprecipitated...
MKK6 and MKK3 differentially regulate cytokine production by FLS

Because MKK6 and MKK3 differentially regulate p38 activation, we considered whether they might have functional differences such as the regulation of proinflammatory cytokines. Cultured synoviocytes from MKK6−/−, MKK3−/−, and WT mice were stimulated with medium, TNF or LPS. Supernatants were assayed for inflammatory mediators (Fig. 7). CCL3 (MIP1α) and KC production was significantly inhibited in TNF- and LPS-stimulated MKK6-deficient cells compared with WT (MIP1α 90 ± 7% inhibition, p = 5 × 10−4; KC 56 ± 4% inhibition p = 5 × 10−3, n = 3). In contrast, CCL5 (RANTES) and CXCL10 (IP-10) production was lower in the TNF-stimulated MKK3 deficient cells (RANTES 35 ± 6%, p = 1 × 10−4; IP-10 49 ± 2%, p = 0.009, n = 3). MCP-1 expression was not regulated by either MKK3 or MKK6. These data show that MKK6 and MKK3 regulate distinct cytokine profiles.

Discussion

The mitogen-activated protein kinases JNK, ERK, and p38 are examples of signaling molecules that have been implicated in RA (19). p38 MAP kinase, in particular, has been evaluated as a therapeutic target in RA because p38 inhibitors are effective in animal models of arthritis (20, 21). Several of these compounds have been evaluated in human clinical trials, but in many cases toxicity and limited efficacy has interfered with clinical development (5).

An alternative to direct inhibition of an individual p38 isoform is to target one of its upstream activating kinases, such as MKK3 or MKK6. These MKKs are structurally related kinases that mediate p38 activity in response to stress and cytokines. For instance, both MKK3 and MKK6 regulate TNF-stimulated p38 activation in vivo (8); MKK3 is also required for TNF-mediated IL-6 production in murine embryonic fibroblasts (22). We recently showed that MKK3 deficiency suppresses inflammatory arthritis and decreases synovial cytokine production (9). However, there is no information on the contribution of MKK6, which, like MKK3, is expressed and activated in the rheumatoid synovium (15).

Our data show that MKK6 deficiency markedly suppresses arthritis in a passive arthritis model, with beneficial effects on clinical scores, osteoclast formation, and histologic evidence of joint damage. This is the first demonstration that MKK6 plays a role in the pathogenesis of a chronic inflammatory disease. Production of proinflammatory mediators, like IL-6 and MMP3 were also significantly lower in the joints of arthritic MKK6−/− mice. The protective effect of MKK6 deficiency in arthritis was similar to that...
MKK6 functions as a scaffold protein that facilitates the formation of p38-MKK complexes in the nucleus, leading to MK2 phosphorylation. Previous studies by Posas et al. (36) demonstrated that MKKs in yeast act as scaffold proteins to activate specific MAPKs in response to stress. They showed that Pbs2p MAPKK functions as a scaffold protein in activating Hog1p MAPK in response to hyperosmotic stress. Additional studies are in progress to determine whether MKK6 functions as a scaffold protein that facilitates MK2 phosphorylation.

We then measured the functional effects of MKK6 deficiency on the expression of key inflammatory mediators. These studies showed that MKK3 and MKK6 regulate distinct profiles of proinflammatory genes in activated synoviocytes. Although MKK6 deficiency reduces TNF- and LPS-induced MIP1α and KC production, MKK3 deficiency suppresses TNF-induced RANTES and IP-10. Neither MKK affected MCP-1 expression, even though MKK6 deficiency inhibits TNF-induced MCP-1 in endothelial cells (37). These patterns of cytokine production indicate that MKK6 deficiency can potentially preserve some TLR-mediated response. Dissecting the cytokine profiles raises the possibility that one might potentially select a therapeutic target based on the pathogenesis of specific diseases and the genes regulated by a particular kinase.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


