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Synoviocyte Innate Immune Responses: I. Differential Regulation of Interferon Responses and the JNK Pathway by MAPK Kinases

Toshio Yoshizawa, Deepa Hammaker, Susan E. Sweeney, David L. Boyle, and Gary S. Firestein

JNK is a key regulator of matrix metalloproteinase production in rheumatoid arthritis. It is regulated by two upstream kinases known as MKK4 and MKK7. Previous studies demonstrated that only MKK7 is required for cytokine-mediated JNK activation and matrix metalloproteinase expression in cultured fibroblast-like synoviocytes (FLS). However, the functions of MKK4 and MKK7 in synoviocyte innate immune responses have not been determined. TNF, peptidoglycan (PGN), and LPS stimulation led to higher and more prolonged MKK7 phosphorylation compared with MKK4 in FLS. However, this pattern was reversed in poly(I-C) stimulated cells. siRNA knockdown studies showed that TNF, PGN, and LPS-induced JNK and c-Jun phosphorylation are MKK7 dependent, while poly(I-C) responses require both MKK4 and MKK7. Poly(I-C)-induced expression of IP-10, RANTES, and IFN-β mRNA was decreased in MKK4- or MKK7-deficient FLS. However, MKK4 and MKK7 deficiency did not affect phosphorylation of IκB kinase-related kinases in the TLR3 signaling pathway. MKK7, but not MKK4 deficiency, significantly decreased poly(I-C)-mediated IRF3 dimerization, DNA binding, and IFN-sensitive response element-mediated gene transcription. These results were mimicked by the JNK inhibitor SP600125, indicating that JNK can directly phosphorylate IRF3. In contrast, deficiency of either MKK4 or MKK7 decreased AP-1 transcriptional activity. Therefore, JNK is differentially regulated by MKK4 and MKK7 depending on the stimulus. MKK7 is the primary activator of JNK in TNF, LPS, and PGN responses. However, TLR3 requires both MKK4 and MKK7, with the former activating c-Jun and the latter activating both c-Jun and IRF3 through JNK-dependent mechanisms. The Journal of Immunology, 2008, 181: 3252–3258.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation, and erosion of cartilage and bone. The MAPKs, including ERK, and p38 MAPK, and JNK, play a critical role in this process by regulating production of cytokines and proteases that contribute to disease pathogenesis (1–3). JNK, in particular, enhances extracellular matrix degradation in the inflamed joint through the production of matrix metalloproteinases (MMPs) (4, 5), largely by activating the transcription factor AP-1. Although JNK is an attractive therapeutic target, blocking the kinase directly might adversely affect cell survival, homeostasis, and host defense (6, 7). One possible way to modulate this pathway in a more site- and event-specific manner is to target its upstream MAPK kinases, MKK4 and MKK7 (8, 9). All three components of the signaling cascade (JNK, MKK4, and MKK7) are expressed and activated in rheumatoid arthritis synovium (10), and understanding their functions could help determine which therapeutic target has a favorable risk-benefit ratio.

The relative hierarchy of MKKs that integrate responses to extracellular stimuli is cell lineage and stimulus-dependent. For instance, our previous studies demonstrated that MKK7, but not MKK4, is essential for IL-1-mediated JNK activation in cultured fibroblast-like synoviocytes (FLS). However, both MKK4 and MKK7 participate in anisomycin-mediated FLS responses (11). The relative contributions of MKKs to innate immune responses in the synovium have not been defined. Therefore, we examined how MKK4 and MKK7 regulate TLR responses in cultured FLS. These studies show that only MKK7 is required for JNK activation by TLR2 and TLR4, whereas both MKK4 and MKK7 participate in TLR3 responses. The two MKKs use distinct mechanisms involving differential phosphorylation of c-Jun and IRF3.

Materials and Methods

Reagents

Anti-phospho-MKK4 (Ser-80), anti-MKK4, anti-phospho-MKK7, anti-MKK7, anti-phospho-JNK (Thr-183/Tyr-185), and anti-JNK Abs were purchased from Cell Signaling Technology. Anti-phospho-IκB kinase (IKK)ε (Thr-501) was purchased from Rockland. Anti-phospho-TRAF family member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) (Ser-172) was purchased from BD Biosciences. Anti-IRF3 and anti-phospho-TRAF family member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) (Ser-172) was purchased from BD Biosciences. Anti-IRF3 and anti-β-actin Abs were obtained from Santa Cruz Biotechnology. Recombinant human TNF was purchased from R&D Systems. LPS and poly(I-C) were obtained from Sigma-Aldrich. Peptidoglycan (PGN) was purchased from Fluka. SP600125 was obtained from Calbiochem.

Fibroblast-like synoviocytes

FLS were isolated from synovial tissues obtained with informed consent from RA patients at the time of joint replacement as described previously (12). The diagnosis of RA conformed to the American College of Rheumatology 1987 revised criteria (13). Synovial tissues were minced and incubated with 0.5 mg/ml collagenase VIII (Sigma-Aldrich) in serum-free RPMI 1640 (Mediatech) for 1.5 h at 37°C, filtered through a 0.22 μm cell strainer, extensively washed, and cultured in DMEM supplemented with

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FIGURE 2. Kinetics of TLR-induced of MKK4 and MKK7 phosphorylation. FLS were treated with TNF (50 ng/ml), LPS (1 μg/ml), PGN (50 μg/ml), or poly(I-C) (20 μg/ml) for up to 90 min. P-MKK4 and P-MKK7 expression was normalized to total MKK4 and MKK7, respectively. Data are presented as means ± SEM from three independent experiments. TNF, LPS, and PGN induced rapid phosphorylation of MKK4, which returned to a baseline at 30 min. Phosphorylation of MKK7 was sustained for over an hour. This pattern was reversed with poly(I-C) stimulation.

10% FCS (endotoxin content <0.006 ng/ml; Gemini Biosciences), penicillin, streptomycin, gentamicin, and t-glutamine in a humidified 5% CO2 incubator. After overnight culture, nonadherent cells were removed, and adherent cells were trypsinized, split at a 1:3 ratio, and cultured. Synovio-cytes were used from passage 4 through 9, when FLS were a homogeneous population with <1% CD11b, <1% phagocytic, and <1% FcRII positive cells. Cells were synchronized in 0.1% FBS for 48 h before stimulation.

siRNA transfection
Three × 10^5 cells FLS (passages 4 to 6) were transfected with 3 μg of MKK4, MKK7 or scrambled negative control Smartpool small interfering RNA (siRNA; Dharmacon), according to the manufacturer’s instruction (Amaxa). In brief, 75–95% decrease in protein expression is achieved by using this method (11).

Western blot analysis
FLS were treated with TNF (50 ng/ml), LPS (1 μg/ml), PGN (50 μg/ml), or poly(I-C) (20 μg/ml). Cell lysates were loaded on 10% SDS-PAGE, and Western blot analysis was performed as previously described (14).

FIGURE 1. Nuclear extract preparation and native PAGE
Nuclear extracts were obtained from 0.5–1 × 10^6 cells by using the nuclear extraction kit (Panomics), according to the manufacturer’s instruction. The protein concentration was measured by using a protein quantification assay (Bio-Rad). For detecting IRF3 dimers, native PAGE was performed (15). The nuclear extracts (5 μg) were separated using a 6% nondenaturing gel. The samples in the gel were electrophoresed at 120 V for 30 min after pre-running (at 120 V for 30 min). The gel was soaked in SDS electrophoresis buffer (25 mM Tris (pH 8.3), 250 mM glycine, 0.1% SDS) for 30 min at room temperature, before the membrane transfer. Membrane transfer was performed as described for Western blot analysis.

ECSAs
After siRNA transfection, FLS were cultured in 10 cm dishes in DMEM with 10% FCS at 37°C for 24 h. The cells were incubated in fresh medium for 48 h and subsequently serum starved (0.1% FCS/DMEM) for 48 h. FLS were then treated with either medium or poly (I-C) (20 μg/ml) for 60 min. Nuclear extracts were prepared as described above, and EMSAs to detect IFN-sensitive response element (ISRE) and ATF2 binding were performed with the gel-shift assay kit (Panomics), according to the manufacturer’s instruction.

Reporter gene assays
After siRNA transfection, FLS were incubated for 3 days and subsequently 3 × 10^6 cells were transfected with 3 μg of reporter plasmid DNA, ISRE-luc, which has five repeats of the ISRE sequence from ISG15 promoter (16) and 0.3 μg of Renilla reniformis luciferase construct as internal control for transfection efficiency (16) (a gift of Dr. Michael David, University of California San Diego, La Jolla, CA). Sixteen hours after transfection, cells were stimulated with 20 μg/ml poly (I-C) for 24 h. AP-1 reporter assays used AP-1-luc, which contains AP-1 responsive promoter (Clontech). Luciferase activity was measured using a dual luciferase assay kit (Promega),
Quantitative real-time PCR

Total RNA was harvested to assay IP-10, IFN-β, RANTES, MMP3, and GAPDH mRNA expression. Quantitative real-time PCR was performed by using the GeneAmp 7300 sequence detection system (Applied Biosystems), as previously described (17).

Statistical analysis

Data are expressed as mean ± SEM. Comparisons between two groups were performed using Student’s t test. A comparison was considered statistically significant if \( p < 0.05 \).

Results

Kinetics of MKK4 and MKK7 phosphorylation

Previous studies demonstrated that IL-1 and TNF-induced JNK activity in FLS is MKK7-dependent and does not require MKK4 (11, 18, 19). To explore the regulation of MKKs after TLR ligation, we first evaluated the kinetics of MKK4 and MKK7 activation in FLS stimulated with TNF or TLR ligands. As shown in Fig. 1, TNF, LPS (TLR4 ligand), and PGN (TLR2 ligand) induced a rapid increase in MKK7 phosphorylation, but the duration and intensity of MKK4 activation were lower. Surprisingly, this pattern was reversed after TLR3 ligand poly (I-C) stimulation, with more prolonged MKK4 activation compared with MKK7.

Differential use of MKK4 and MKK7 for JNK activation

The kinetics of MKK4 and MKK7 phosphorylation for TLR3 led us to focus on this pathway and determine their relative contributions to JNK activation. MKK4, MKK7, or nontargeting scrambled siRNA–transfected FLS were stimulated with TNF, LPS, PGN, or poly (I-C). P-JNK and P-c-Jun levels were then determined by Western blot analysis. The results for LPS and PGN were similar to TNF because MKK4 deficiency had no effect while MKK7 knockdown significantly decreased responses (Fig. 2, A and B).

FIGURE 3. Effect of MKK4 and MKK7 deficiency on IFN-induced gene expression. MKK4, MKK7, or scrambled (Sc) siRNA-treated FLS were stimulated with poly(I-C) (20 \( \mu \)g/ml) for 24 h. IP-10, IFN-β, and RANTES (A) and MMP3 (B) gene expression was determined by quantitative PCR and normalized to GAPDH. Data are shown as relative expression units (mean ± SEM of three independent experiments). Gene expression of IP-10, IFN-β, and RANTES was significantly decreased in the absence of MKK4, MKK7, or both MKK4 and MKK7 (MKK4/7) compared with Sc control. MMP3 gene expression was significantly decreased in the absence of MKK7 but not MKK4, compared with Sc control.

FIGURE 4. Regulation of IRF3 activation by MKK4 and MKK7. A, The effect of MKK4 and MKK7 deficiency on IKKε and TBK1 activation was determined by Western blot analysis. No changes in IKK-related kinase phosphorylation were observed in MKK-deficient cells. B, MKK4, MKK7, or scrambled (Sc) siRNA-treated FLS were stimulated with poly(I-C) (20 \( \mu \)g/ml) for 1 h. Nuclear extracts were separated by native PAGE, followed by Western blot analysis with an Ab specific to the native conformation of IRF3. Some cells were pretreated with 20 \( \mu \)M of SP600125 (JNK inhibitor) for 1 h before stimulation. The arrows show the monomeric and dimeric forms of IRF3. MKK7 deficiency or SP600125 decreased IRF3 dimerization. C, Amino acid sequences of GST-IRF3 peptides used for kinase assays. D, Anti-JNK Ab immunoprecipitates from poly(I-C) stimulated FLS were used to evaluate kinase activity. The immunoprecipitates phosphorylated wild type GST-IRF3 and mutant IRF3 (385/386A), but not the peptides with alanines at the other indicated sites.
FIGURE 5. Effect of MKK4 and MKK7 on IRF3 and AP1 binding and function. A. EMSAs. Nuclear extracts prepared from siRNA transfected FLS were treated for 1 h with poly(I-C) (20 μg/ml) and analyzed by EMSA using oligonucleotides for IRF3 (A) or ATF2 (B). The arrows indicate the protein/DNA complexes (representative of three separate experiments). Poly(I-C)-induced IRF3 binding was decreased in MKK7-deficient FLS (p = 0.01) or SP600125-treated FLS (p = 0.008), but not by MKK4 deficiency. ATF2 binding activity was not decreased in MKK4- or MKK7-deficient FLS or SP600125-treated FLS. C. Luciferase reporter constructs. MKK4, MKK7, or scrambled (Sc) siRNA-treated FLS were cotransfected with MKK4, MKK7, or scrambled (Sc) siRNA-treated FLS were cotransfected with plasmids with ISRE (A) or AP-1 (D) luciferase constructs. The cells were stimulated with poly(I-C) (20 μg/ml) for 24 h or pretreated with 20 μM of SP600125 (JNK inhibitor) for 1 h before stimulation. The cell lysates were assayed for luciferase activity and normalized to R. reniformis luciferase. Unstimulated cells transfected with Sc siRNA were used as control. The values are the mean ± SEM from three independent experiments. ISRE promoter activity was decreased by MKK7 siRNA or SP600125. AP-1 promoter activity was decreased in MKK4- and MKK7-deficient FLS or by SP600125.

Surprisingly, poly (I-C) required both MKK4 and MKK7 for JNK and c-Jun activation (Fig. 2, A and B). MKK7 deficiency was more effective than MKK4 knockdown in inhibiting JNK and c-Jun phosphorylation.

Decreased TLR3-induced gene expression in MKK4 and MKK7 deficient FLS

We then determined whether the differences in MKK-regulated JNK activation were reflected in TLR3-induced IFN response gene expression. Fig. 3A shows that IP-10, IFN-β, and RANTES expression is significantly lower in FLS that are deficient in either MKK4 or MKK7. Combined MKK4 and MKK7 knockdown did not lead to additional suppression indicating that both are required (Fig. 3A). In contrast, poly (I-C)-induced MMP3 gene expression was decreased in MKK7 but not MKK4-deficient cells (Fig. 3B).

Regulation of IRF3 DNA binding by MKK7-JNK pathway

We then evaluated the effect of MKK4 and MKK7 on IRF3 DNA binding by EMSA using ISRE oligonucleotide containing the IFN-stimulated gene (ISG15) promoter. A significant decrease in IRF3 binding was observed in the nuclear extracts of MKK7-deficient FLS and SP600125 treated FLS compared with control and MKK4 siRNA transfected cells (Fig. 5A). Surprisingly, ATF2/c-Jun binding to the upstream AP-1-like region of the ISG15 promoter was not decreased by either MKK4 or MKK7 deficiency (Fig. 5B).

Role of MKK4 and MKK7 on TLR3-driven gene transcription

The data thus far suggested that MKK7 regulates TLR3-mediated gene expression through activation of JNK followed by IRF3 phosphorylation, dimerization, and nuclear binding. However, the role of MKK4 was uncertain. Because MKK4 induces c-Jun phosphorylation in poly(I-C) stimulated cells, we considered whether MKK4 deficiency affected the function of upstream non-IRF3 elements of the ISG15 promoter. We first confirmed that MKK7 but not MKK4 regulates IRF3-driven gene transcription using an ISRE
FIGURE 6. Regulation of IFN response genes by TLR3 signaling. MKK7 regulates TLR3-mediated gene expression by activating both IRF3 and c-Jun through a JNK-dependent pathway. In contrast, MKK4 only regulates c-Jun. Both AP-1 and ISRE are required for IFN-β gene expression.

Discussion

RA is a systemic inflammatory disease characterized by synovial hyperplasia and joint destruction (1). The disease is perpetuated by cytokines that, in turn, increase production of MMPs that remodel the extracellular matrix (22, 23). The innate immune response in the synovium can also contribute to the pathogenesis of RA. TLR3 and TLR7 are highly expressed in RA synovium and can synergize with other signaling pathways such as TNF to produce proinflammatory cytokines (24, 25). TLR-mediated signaling activates the MAPKs ERK, JNK, and p38 (25), which transduce environmental signals and regulate proinflammatory mediator release (3, 26). JNK has been suggested as a potential target for therapy in RA because it plays a pivotal role in MMP production (4, 5).

JNK activation is regulated by two upstream kinases known as MKK4 and MKK7. We recently showed both MKK4 and MKK7 are expressed and activated in the rheumatoid synovium, especially in the synovial interstitial lining (10). Of interest, MKK7, but not MKK4, regulates IL-1 and TNF responses in cultured FLS while both MKK4 and MKK7 contribute to FLS activation by anisomycin (11). However, the relative contributions of MKK4 and MKK7 to TLR signaling have not been evaluated in FLS. The goal of the present study was to determine the functional hierarchy of MKK4 or MKK7 in synoviocyte innate immune responses. The data demonstrate that TLR3 signaling is unique because both upstream kinases are required.

Although MKK7 is the primary activator of JNK in FLS after TNF or IL-1 activation, numerous studies show that the relative hierarchy of MKK4 and MKK7 depends on the cell lineage and the mode of stimulation. T cell activation due to costimulation is impaired in the absence of MKK4 (27, 28), whereas thymocytes and B cells lacking MKK7 hyperproliferate in response to growth factors or Ag-receptor stimulation (29). Cytokine-induced JNK activation is abolished in MKK7-deficient mast cell (29, 30). In contrast, LPS-stimulated JNK function correlates with MKK4 function in microglia (31). IL-1 responses in murine embryonic fibroblasts depend on MKK7 and UV light responses involve both MKK4 and MKK7 (8). This signaling diversity suggests that JNK can potentially be modulated in a stimulus-specific manner by targeting upstream kinases.

Our initial studies evaluated the kinetics of MKK4 and MKK7 phosphorylation after stimulating FLS with TLR ligands. PGN and LPS stimulation, like TNF, led to prominent MKK7 phosphorylation compared with MKK4. In contrast, poly(I-C) induced a more prominent MKK4 response. Prolonged MKK4 phosphorylation has also been documented in lymphoid cells after TNF stimulation (32). JNK and c-Jun phosphorylation in response to poly(I-C) was dependant on both MKK4 and MKK7; although MKK7 deficiency decreased JNK and c-Jun phosphorylation more effectively than MKK4. It is not clear yet how the preference for a particular upstream kinase translates into severity of disease in vivo, where TLR3 ligation occurs in the context of numerous proinflammatory cytokines. Future studies to evaluate the contribution of MKK4 and MKK7 in vivo will be a critical component to validating either one as a potential therapeutic target in inflammatory arthritis. The functional relevance of pronounced MKK4 activation after TLR3 signaling in FLS was confirmed in gene expression studies demonstrating that MKK4 deficiency suppresses expression of the IFN response genes, IP-10, IFN-β, and RANTES. Expression of these genes was not blocked completely in the absence of MKK4 and MKK7, suggesting that other molecules such as IKKe/TBK1 might also be involved. Poly(I-C)-induced MMP3 gene expression required MK7 but not MKK4, indicating that non-IFN response genes activated by TLR3 require MKK7. These data suggest that...
the hierarchy of MKK use for poly(I-C) stimulation differs from TNF, LPS, or PGN, which are strictly dependent on MKK7.

To evaluate how MKKs regulate TLR3 function, we systematically examined steps leading to IFN-response gene expression. Type I IFN regulation by TLR3 is tightly controlled by several transcription factors, including ATF2/cJun and IRF3 (33, 34). Two IKK homologues, IKKe and TBK1, are intimately involved in this process through IRF3 phosphorylation, dimerization, and nuclear translocation (18, 35). We first showed that MKK4 or MKK7 deficiency does not significantly alter the activation pattern of the IKK-related kinases. Similar results with the JNK inhibitor SP600125 suggested that JNK itself might participate, and this concept was supported by a previous report showing that JNK can directly phosphorylate IRF3 (36). However, the mechanism by which JNK activates IFN signaling was unclear. Thus, we set out to determine whether MKK4 and MKK7 affect IRF3 activation using native gel electrophoresis to determine dimerization. These experiments showed that dimerization was not blocked in MKK4 deficiency but was MKK7 dependent.

C-terminal phosphorylation of IRF3 is also a prerequisite for IRF3 dimerization, nuclear translocation, and activation of IFN gene transcription (37, 38). Several IRF3 serine and threonine residues can be phosphorylated, suggesting IRF3 can be targeted by multiple signaling kinases (39, 40). In vitro kinase assays using synoviolyn lysates confirmed that JNK can activate IRF3 directly and demonstrated the key phosphoacceptors in FLS that are activated by TLR3 ligand (41, 42). Therefore, MKK7 and the JNK pathway appear to lead directly to IRF3 phosphorylation and dimerization rather than requiring an intermediate step. The mechanism by which poly(I-C) activates IRF3 via MKK4 was still uncertain at this point in our studies. The EMSA data supported the concept that MKK7-JNK affects IRF3 binding to the ISRE promoter. MKK7 is also required for ISRE-mediated gene transcription. We surmised that MKK4 influences a different transcription factor for IFN-mediated signaling because ISRE DNA binding activity was not decreased in MKK4 deficient cells.

Subsequent investigations sought to determine whether MKK4 regulates IRF3-driven gene expression. Transcriptional activation of the IFN-β gene requires assembly of an enhancerosome, which depends critically on cooperative interactions among the DNA-binding domains of ATF2/c-Jun and IRF3 (36, 43). First, we examined whether MKK4 affects the ATF2 binding to AP1 promoter. The ATF2/c-Jun heterodimer binds the sequence 5′-TGACATAG-3′, located of upstream of ISG15, in the positive regulatory domain IV of the IFN-β enhancer (44). In particular, the 5′-TGAC-3′ consensus site is important for ATF2 binding (44). Surprisingly, ATF2/c-Jun binding was not decreased by either MKK4 or MKK7 deficiency. This observation led us to investigate whether ATF2/c-Jun is functionally activated, presumably by phosphorylation, in MKK4 or MKK7 deficiency by using an AP-1 luciferase containing 5′-TGAC-3′ sequence. Deficiency of either upstream kinase decreased AP-1 promoter function after poly(I-C) stimulation. Therefore, MKK4 regulates TLR3-mediated gene expression via AP-1 while MKK7 can regulate both AP-1 and ISRE-driven transcription. Both functions are required for full activation of the IFN response in FLS. It is not clear how the MKKs direct JNK to distinct substrates, but the mechanism could involve unique scaffold proteins and signaling complexes (45).

In conclusion, JNK responses are differentially regulated by upstream kinases in FLS and depend on the stimulus. MKK7 is sufficient for TNF receptor, TLR2, and TLR4 responses. However, TLR3-mediated activation of the IFN response genes requires both MKK4 and MKK7. This is especially interesting in light of the recent observation that a subset of RA patients display an IFN signature in their PBMC (46). Future studies identifying the mechanisms of IFN regulation in these patients might lead to individualized therapies that target specific pathways that are relevant in these individuals. The two MKKs have distinct mechanisms, with MKK4 contributing to JNK-mediated activation of c-Jun and the ATF2/c-Jun complex. In contrast, MKK7-activated JNK can phosphorylate both c-Jun and IRF3. By differentially activating the MAPK cascade, cellular responses integrate cellular signals and engage the most appropriate stress response. This information can potentially provide a rational basis for targeting individual upstream kinases in inflammatory diseases and potentially preserve some JNK functions.

Disclosures
The authors have no financial conflict of interest.

References


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MAPK HIERARCHY IN THE JNK PATHWAY