MNK kinases regulate multiple TLR pathways and innate proinflammatory cytokines in macrophages

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Rowlett RM, Chrestensen CA, Nyce M, Harp MG, Pelo JW, Cominelli F, Ernst PB, Pizarro TT, Sturgill TW, Worthington MT. MNK kinases regulate multiple TLR pathways and innate proinflammatory cytokines in macrophages. Am J Physiol Gastrointest Liver Physiol 294: G452–G459, 2008. First published November 21, 2007; doi:10.1152/ajpgi.00077.2007.—The MNK kinases are kinases located downstream of both the p38 and ERK MAP kinase pathways and act to increase gene expression. MNK inhibition using the compound CGP57380 has recently been reported to inhibit tumor necrosis factor (TNF) production in macrophage cell lines stimulated with Escherichia coli lipopolysaccharide (LPS). However, the range of receptors that signal through the MNK kinases and the extent of the resultant cytokine response are not known. We found that TNF production was inhibited in RAW264.7 macrophage cells by CGP57380 in a dose-responsive manner. The compound also blocked the production of the proinflammatory cytokines TNF, IL-6, and monocyte chemoattractant protein-1 at 4 h. Taken together, these data demonstrate that MNK kinases signal and respond to LPS stimulation. IL-10 production was higher in mouse model of Crohn’s disease-like ileitis (SAMP1/YitFc strain) mice than in controls, which TNF is known to play an important pathogenic role (23, 29), to determine the role of MNK kinases in regulating macrophage effector cell function.

METHODS

Reagents. CGP57380 was a kind gift from Hermann Gram (Arthritis and Bone Metabolism, Novartis, Basel, Switzerland) and the anti-tristetraprolin (TTP) antibody was a kind gift from Jiahuai Han (Scripps Institute, La Jolla, CA). In experiments in which CGP53870 was used, cell-culture grade DMSO in sealed ampoules (Sigma-Aldrich, no. 2650) was used both to resuspend the drug and in equal amounts without the drug in negative controls. The anti-actin antibody was obtained from ICN; the anti-eIF4E and anti-phospho-eIF4E antibodies were obtained from Cell Signaling. Anti-rabbit and anti-MAP kinase pathways has a significant impact on downstream TNF production and its subsequent biological effects.

The MAP kinase-interacting serine/threonine kinases (MNKs) are kinases located downstream of both the p38 and ERK pathways. The two family members, MNK1 and MNK2, are substrates for activating phosphorylation by both ERK and p38 (46) and are induced by either pathway (11). MNKs are the only kinases known to phosphorylate Ser-209 of the cap-binding eIF4E protein, which acts to increase translation (16, 27, 41, 44). Both MNK1 and MNK2 are autoinhibited by the COOH terminus in the absence of p38 and p42/ERK phosphorylation (18) but are not required for normal development or viability when both mouse genes are inactivated (45). A recent report regarding MNK kinases in the regulation of TNF in response to Escherichia coli lipopolysaccharide (LPS) suggested that this family regulates the innate immune response in macrophages (2). Whether an anti-inflammatory effect of MNK inhibition is a feature unique to LPS or involves cytokines other than TNF is currently unknown. It is also unknown whether MNK inhibition is a viable strategy for the treatment of inflammatory diseases. If so, the lack of a phenotype in an unstressed MNK1/2 dual-knockout mouse suggests that these kinases could be excellent targets for anti-inflammatory drug development (45).

A well-studied agonist of TNF production in macrophages is E. coli LPS (4), which signals through the p38, JNK/SAPK, and ERK pathways to produce TNF (38). In the present study, we used RAW264.7 macrophage-like cells as well as bone marrow-derived macrophages (BMDM) from SAMP1/YitFc mice, a model of spontaneous Crohn’s disease in which TNF is known to play an important pathogenic role (23, 29), to determine the role of MNK kinases in regulating macrophage effector cell function.

TUMOR NECROSIS FACTOR (TNF) plays a pivotal role in the regulation of normal immunity (3) as well as pathogenic inflammatory responses seen in diseases such as rheumatoid arthritis and Crohn’s disease, in which anti-TNF strategies have revolutionized the treatment of patients suffering from these chronic inflammatory disorders (37). It is well known that the synthesis of biologically active TNF is regulated at several different levels and that MAP kinase pathways are critical for its production as well as that of several other proinflammatory cytokines (38). As such, interfering with the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
mouse horseradish peroxidase-conjugated secondary antibodies were obtained from Upstate Bioscience and Novagen, respectively. RAW264.7 cells were obtained from the American Type Culture Collection and grown in RPMI 1640 with 10% heat-inactivated FBS. Phenol-extracted and ion exchange-purified E. coli 055:B5 LPS (no. L4524) and other laboratory chemicals were obtained from Sigma-Aldrich. Endotoxin and RNase-free sterile water was used for all solutions. Endotoxin testing was performed using the Pyrotell Limulus amebocyte lysate assay (Associates of Cape Cod).

Western blotting. RAW264.7 cells were split into 12-well plates at 1.0 × 10^6 cells/well and allowed to attach overnight. Protein gels, transfer blotting, and development were performed as previously described using the ECL Plus Kit (Amersham) (8), except that Ponceau S staining was used to verify the uniformity of transfers. Secondary antibodies were typically used at a 1:5,000 dilution. E. coli LPS was used at 100 ng/ml for stimulation and at 10 ng/ml for overnight incubation designed to induce tolerance. Except for a single image developed by using the STORM scanner (Amersham; fig. 4A), all Western blots were imaged with X-ray film.

Time course of TNF mRNA production. RAW264.7 cells were split into 12-well plates at 1.0 × 10^6 cells/well and allowed to attach overnight. At 30 min prior to stimulation with LPS, the cells were pretreated with CP57380 to 50 μM vs. DMSO control wells, and then LPS was added to 100 ng/ml. For the actinomycin D experiments, the cells were similarly stimulated with LPS for 1 h, except that actinomycin D was added to a 5 μM final concentration with either CP57380 or DMSO control.

RNA methods and real-time PCR. Total cellular RNA was purified by using the Qiagen RNeasy kit and quantified spectrophotometrically, and then 2 μg total RNA were used for reverse transcription using the Invitrogen SuperScript First Strand Synthesis cDNA kit with the accompanying random primers according to the manufacturer’s protocol. Real-time PCR was performed on an ABI Prism SDS 7000 in paired reactions using mouse TNF Taqman primers (Mm00443258_m1, Applied Biosystems) with 12.5 μl of Taqman Universal Mix, 1.25 μl of Taqman primers, 10.25 μl of sterile water, and 1 μl of cDNA. 18S ribosomal RNA was quantified by using the Taqman primer probe set (no. 431-893E, Applied Biosystems), and the amount of TNF gene expression was normalized to 18S by the ΔΔCt (Ct, cycle threshold) method (1). Thermocycling conditions were: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s, 60°C for 1 min. Controls without reverse transcriptase and without template RNA were included.

Animal methods. All mice were housed under specific pathogen-free conditions at the University of Virginia in autoclaved microisolator cages under positive pressure HEPA-filtered air and were given sterile food and water ad libitum. All mice were used and cared for in accordance with protocols approved by the Institutional Animal Care and Use Committee and Association for Assessment of Laboratory Animal Care guidelines.

BMDM were derived from the SAMP1/YitFc (SAMP) mouse strain, which was propagated from brother-sister breeding (over 20 generations) at the University of Virginia. Initial breeding mice (SAMP1/Yit) were generously provided by S. Matsumoto (Yakult Central Institute for Microbiological Research, Tokyo, Japan) and were originally derived from the AKR/J strain (Jackson Laboratories, Bar Harbor, ME). Spontaneous ileitis develops in these animals virtually 100% penetrance by 10 wk of age. Their phenotype shows a culture supernatant was collected and centrifuged, and the cell pellet was resuspended in 5 ml of fresh medium containing 200 U/ml rmGM-CSF and returned to the original plate. On day 10, cells were washed 2× with PBS to remove nonadherent cells, and 10 ml of fresh medium were added. On days 6 and 8, half of the culture supernatant was collected and centrifuged, and the cell pellet was resuspended in 5 ml of fresh medium containing 200 U/ml rmGM-CSF and returned to the original plate. On day 10, cells were washed 2× in PBS, detached by use of 0.02% EDTA in PBS, and collected by centrifugation at 300 g for 5 min at room temperature. Resulting BMDM were split into 12-well plates, seeded at 1.0 × 10^6 cells/well, and allowed to attach overnight. At 30 min prior to the addition of LPS (100 ng/ml), BMDM were pretreated with either 50 μM CGP57380 or vehicle, and supernatants were collected at 0, 4, and 24 h following stimulation.

Immunological methods. The mouse Quantikine TNF ELISA kits were obtained from R&D systems and performed by using the accompanying protocol; 50,000 cells per well in 100 μl of media were used for ELISA assays in 96-well microtiter plates, including experiments performed using the Invivogen Toll-like receptor (TLR) agonist kit (San Diego). Pam3CSK4 was used at 1 μg/ml, heat-killed *Listeria monocytogenes* (HKL) at 10^6 cells/ml, poly(I:C) at 25 μg/ml, Salmonella LPS at 1 μg/ml, flagellin at 1 μg/ml, FSL1 at 1 μg/ml, imiquimod at 10 μg/ml, ssRNA40 at 10 μg/ml, and ODN2006 at 5 μM. The Cytometric Bead Array was used to measure cytokine protein production in supernatants obtained from LPS-stimulated RAW264.7 cells and BMDM from SAMP mice and was performed per the manufacturer’s protocol (BD Biosciences). The previously validated (24) ODN1826 (TLR9 agonist, TCC ATG ACG TTC CTG ACG TT) and ODN1982 (inactive, tcc agg act tct ctc agg tt) were synthesized by Integrated DNA Technologies as RNase-free phosphodiester oligonucleotides.

RESULTS

Macrophages are major effectors of innate immunity, stimulated by a broad variety of bacterial products through specific TLRs on the cell surface to produce proinflammatory cytokines, such as TNF. *E. coli* LPS is a potent stimulus to macrophage gene expression, especially TNF, by engaging the TLR4 membrane signaling complex (15). Andersson and Sundler (2) reported that inhibition with the compound CGP57380 reduced mouse macrophage TNF production, but whether this compound leads to sustained effects to inhibit MKN kinases is not known. We used the macrophage RAW264.7 cell line to pursue these questions. In macrophages, prolonged treatment with the MKN inhibitor CGP57380 abolishes RAW264.7 cell eIF4E phosphorylation after LPS stimulation, without changing total eIF4E levels (Fig. 1). Tolerizing doses of LPS (10 ng/ml overnight, followed by 100 ng/ml stimulation), also led to mild decreases of phospho-eIF4E at early time points (not shown).

Whether TLR bacterial pattern recognition pathways other than for LPS signal through the MKN kinases to promote TNF production is not known. Our first approach to resolve whether active MKN is required to produce TNF in RAW264.7 cells was to use *E. coli* LPS (TLR4 pathway) and a previously validated Cpg DNA agonist, ODN1826 (24) (TLR9 pathway)
TNF was increased in response to and to further establish whether the effect was dose responsive.

whether other TLR receptors would be inhibited by CGP57380 commercial TLR agonist panel on these cells to determine TLR signaling pathways require MNK expression, we used a agonists were combined (Fig. 2A). To determine whether other TLR signaling pathways require MNK expression, we used a commercial TLR agonist kit. RAW264.7 cells were plated overnight in a 12-well tissue culture plate, then incubated in parallel tissue culture wells with either 50 μM CGP57380 (CGP, left) or an equivalent amount of control DMSO vehicle (DMSO, right). The cells were lysed at the times in hours across the top of the figure, and parallel Western blots were performed with the antibodies specific for serine 209 phosphorylated eIF4E (top), total eIF4E (middle), and actin as a loading control (bottom). Results are representative of at least 3 experiments.

The MAP kinase-interacting serine/threonine kinase (MNK) kinase inhibitor blocks RAW264.7 eIF4E phosphorylation in extended culture. RAW264.7 cells were plated overnight in a 12-well tissue culture plate, then incubated in parallel tissue culture wells with either 50 μM CGP57380 (CGP) or an equivalent amount of control DMSO vehicle (DMSO). RAW264.7 cells do not express MNK kinases, which are shown) at 0, 25, and 50 μM CGP57380. Duplicate paired wells were assayed by TNF ELISA. Equivalent amounts of DMSO were added to the wells without CGP57380. Control NT (Control NT) of this treatment show that, even though TNF mRNA is generally unstable, there is a subset of the mRNA (~10%) that remains undegraded after actinomycin D treatment in the mock-treated cells. The TNF mRNA in CGP57380-inhibited cells continues to be degraded at subsequent time points, suggesting that the MNK pathway assists in preventing a component of macrophage TNF mRNA decay. Thus, similar to the role of MNK in T-cell SGs, inhibition of MNK in macrophages allows uninhibited mRNA degradation.
Because of the enhanced degradation of TNF mRNA in response to CGP57380 treatment, we were curious whether MNK inhibition increased the abundance of the TTP protein, which both promotes degradation of TNF mRNA by binding to AU-rich elements in the TNF 3′-untranslated region and is a component of SGs (20, 47). The phenotype of the TTP knockout mouse is one of TNF overexpression, with resultant arthritis, dermatitis, glomerulonephritis, and ds-DNA antibodies (40). TTP can cause both 3′-exonuclease- and micro-RNA-based degradation (19, 25), and increased TTP induced by MNK inhibition would suggest a mechanism for the reduced TNF mRNA production. In T cells, MNK inhibition regulates TNF expression via the 3′-untranslated region of the TNF mRNA (6) and TTP is active in the T-cell regulation of TNF (34). A prior report suggested no effect of CGP57380 on TTP production (5), but those experiments used concentrations that we predict were too low (0.1, 1, and 10 μM) to show significant inhibition of TNF production by interpolation of our graph using TLR agonists (Fig. 2B) and at an earlier time point (2 h after LPS). Since TTP expression has been generally concordant with TNF, the prior conditions may not be relevant.
for TTP regulation by MNKs. RAW264.7 cells were stimulated for 4 h with 100 ng/ml *E. coli* LPS in the presence of 0, 25, and 50 μmol/l CGP57380, with the results shown in Fig. 4A. Rather than being upregulated by CGP57380, TTP protein production was suppressed by MNK inhibition. Thus TTP expression is regulated by the MNK kinases in a coordinate fashion with TNF, one of its major targets. This effect can be seen after the inflammatory response has started; the suppression of TTP protein can be seen 30 min after LPS stimulation (Fig. 4B). As shown in Fig. 4C, TTP is also stimulated by the CpG nucleotide agonist ODN1826, but not a scrambled control nucleotide (ODN1982, “Cont NT”). The induction of TTP by either route (TLR4 vs. TLR9) is blocked by CGP57380.

LPS stimulation of macrophages releases a program of proinflammatory cytokines, including IL-6, IL-10, IL-12, and TNF. To understand how the MNK kinases affect the innate immune response, we stimulated RAW264.7 cells with LPS for 4 h and performed a mouse multiplexed bead immunoassay (Cytometric Bead Array) that measures the proinflammatory cytokines IL12p70, monocyte chemoattractant protein (MCP)-1, TNF, IL-6, and IFN-γ and the anti-inflammatory cytokine IL-10, comparing mock and CGP57380-treated cells. The results, shown in Fig. 5, reveal a striking suppression by MNK inhibition for the proinflammatory cytokines TNF (Fig. 5A) and IL-6 (Fig. 5B), but an increased production of IL-10 (Fig. 5C), suggesting a change from a pro- to an anti-inflammatory cytokine response with inhibition of the MNK pathway in macrophages. IFN-γ, MCP-1, and IL-12p70 were not appreciably produced by these cells (not shown).

We were interested whether the effects observed in the RAW264.7 cell line could also be obtained by using primary cultured macrophages isolated from a murine model of human disease. SAMP1/YitFc (SAMP) mice spontaneously develop discontinuous, ileal-specific lesions with 100% penetrance by 10 wk of age, which closely resembles human Crohn’s disease (23, 36). Histologically, these mice show segmental transmural inflammation, mucosal and submucosal granulomas, altered epithelial cell morphology, and, in a small percentage of mice, perianal disease (36). Importantly, the disease in SAMP mice is responsive to several biologics that are currently used to treat the human condition, including anti-TNF antibodies; in fact, TNF is known to play an important role in the pathogenesis of ileitis in these mice (23, 29). We used BMDM macrophages from SAMP mice that were pretreated with either CGP57380 or vehicle control (DMSO) and stimulated with 100 ng/ml LPS. Supernatants were collected after 0, 4, and 24 h and analyzed of cytokine production via a Cytometric Bead Array. CGP57380 was capable of significantly reducing the amount of TNF produced by SAMP BMDM by more than 84 and 82%, at 4 and 24 h, respectively (P < 0.02 vs. control for both time points) (Fig. 6A). CGP57380 also potently inhibited the production of IL-6 by 93% at 4 h (P < 0.03) and 72.7% at 24 h (Fig. 6B), as well as MCP-1 by 68.7 and 86.9% at 4 and 24 h.

Fig. 5. MNK inhibition changes the cytokine profile of RAW264.7 cells. RAW264.7 cells were incubated with either CGP57380 vs. DMSO control and stimulated with 100 ng/ml *E. coli* LPS for 4 h. Cytometric bead array assays of paired aliquots of media for TNF (A), IL-6 (B), and IL-10 (C) are shown. Error bars are the standard error of the mean. Results are representative of at least 2 experiments.
(P < 0.04 and P < 0.05), respectively (Fig. 6C), in BMDM from SAMP mice. Interestingly, MNK inhibition did not prevent the production of IL-10 from these cells (Fig. 6D). Thus MNK signaling in primary BMDM from Crohn’s disease-like SAMP mice is associated with a proinflammatory cytokine response but does not inhibit production of the anti-inflammatory cytokine, IL-10.

**DISCUSSION**

MNK1 and 2 mediate signals from either the mitogenic (ERK) or stress (p38) MAP kinase pathways (18). They lack the DFG amino acid motif typical for other protein kinases that coordinates a Mg\(^{2+}\) ion and is otherwise critical for activation of \(\gamma\)-ATP residing in the ATP binding pocket. Instead, the central phenylalanine in a DFD motif sterically prevents ATP binding unless a conformational change occurs because of p38 or ERK phosphorylation at their respective sites in the autoinhibitory loop (18). In normal tissues, the MNK1 kinase activity is inducible in response to LPS or insulin stimulation, whereas MNK2 activity is considered constitutive (45). In breast cancer cells, both phosphorylation and activity of the MNKs are constitutive, and this activity correlates with increased HER2 overexpression and is required for optimal colony formation in soft agar, both in vitro correlates of tumorigenicity (9).

Both MNK1 and MNK2, which are 78% homologous in their catalytic domains, bind to eIF4G and phosphorylate eIF4E at Ser209, changing the affinity of the eIF4E for the mRNA cap (42). Ser209 phosphorylation of eIF4E also promotes the nucleocytoplasmic transport of certain transforming mRNAs, like cyclin D1, through a defined motif in the 3′-untranslated region of its mRNA (10, 43). We do not know which MNK isoform (or both) is responsible for our results in this manuscript. In one report, in human embryonal kidney 293 cells, the IC\(_{50}\) for human MNK1 was reported to be ~3 μM, with complete inhibition of eIF4E phosphorylation at ~10 μM (22), whereas in another report maximal inhibition of eIF4E phosphorylation and TNF production from human Jurkat T-cells was 40 μM (6). Our findings are similar to those of Andersson and Sundler (2), who saw maximal inhibition of eIF4E and LPS-induced TNF production in murine RAW264.7 cells above 40 μM CGP57380.

The fact that multiple TLR pathways that cause macrophage TNF production are inhibited by CGP57380 in a dose-responsive manner and that multiple innate, proinflammatory cytokines are affected by MNK inhibition reveals the central role these kinases play in inflammation. The changes in TNF mRNA level and pattern of mRNA degradation were not related to the TNF regulatory protein TTP, since TTP protein production is also inhibited by MNK inhibition. A recent publication suggests that the formation of SGs is prevented when MNK is inhibited (12). SGs are subcellular organelles where untranscribed mRNAs can safely reside undegraded
until the stress is removed. That observation is concordant with the ~10% of LPS-induced TNF mRNA that was stable after actinomycin D treatment without CGP57380 that was degraded when the inhibitor was added to the culture.

The finding that TTP expression requires MNK kinase activation is similar to previous reports that p38 is required for TTP expression. In that case, TTP is a direct target for p38 phosphorylation (8) and p38 promotes TTP degradation via the proteasome (5, 28). In our hands, recombinant TTP was not a substrate for activated, recombinant MNK1 kinase, unlike an eIF4E control (not shown). A previous report suggested that TTP was not affected by MNK inhibition by CGP57380 (5); however, that report used significantly lower (0.1, 1, and 10 μM) concentrations and assayed at an earlier time point (2 h) after LPS stimulation compared with the 4-h time point we used. Consistent with the requirement for a higher concentration to suppress TTP production, from our TLR agonist studies in Fig. 2B, we would not expect doses at those lower concentrations to have significant effects on TNF production.

Several lines of evidence support the concept that cytokines are important mediators of intestinal inflammation and a broad array of cytokines have been implicated in the pathogenesis in inflammatory bowel disease (IBD). In fact, successful strategies that have been recently developed (and are currently in development) to treat IBD are based on intervention of cytokine pathways, with the end goal of designing therapeutics with greater specificity and decreased toxicity for these patients (33). MNK inhibition may represent an example of such a strategy. We have shown that MNK inhibition reduces proinflammatory cytokines known to be important in mucosal innate immune responses and chronic intestinal inflammation, including TNF, IL-6, and MCP-1. In fact, TNF plays a critical role in the pathogenesis of Crohn’s disease as inhibition of TNF using monoclonal antibody therapy dramatically improves disease activity in this patient population (39). Similarly, in the SAMP mouse strain, a single injection of a chimeric anti-murine TNF antibody markedly suppresses the ileal-specific disease in which the mechanism of action involves homeostatic regulation of intestinal mucosal cell apoptosis resulting in a net decrease of chronic gut inflammation (29). In regard to IL-6, high serum levels in quiescent Crohn’s patients are predictive of clinical relapse (26), and an anti-IL-6 monoclonal antibody has recently been shown to hold promise in treating Crohn’s disease (17). The ileitis characteristic of SAMP mice improves, in fact, when signaling through the STAT3 transcription factor is blocked, thereby reducing IL-6 production, and it is worsened by exogenous IL-6 (31). MCP-1 is increased in the intestinal mucosal of IBD patients (30, 35), and the degree of intestinal inflammation in Crohn’s disease is associated with MCP-1 tissue levels; furthermore, there is evidence for different MCP-1 genotypes of a functional single nucleotide polymorphism (G/A) located in the distal regulatory region of the MCP-1 gene and an association with different disease behavior in Crohn’s disease (14). Finally, the release of IL-10 from SAMP BMDM after LPS stimulation and pretreatment with CGP57380 is exciting, suggesting that an anti-inflammatory response is not prevented by MNK inhibition. Therefore, our results would suggest that MNK inhibition promotes the overall dampening of inflammation mediated by macrophages by reducing proinflammatory cytokine production while permitting responses mediated by anti-inflammatory cytokines, such as IL-10.

In summary, we demonstrate that MNK inhibitors may represent promising therapeutic agents in inflammatory diseases in which bacterial products and innate, proinflammatory cytokines play a critical role in their pathogenesis. Initial studies of the MNK 1/2 double knockout mouse appeared to have no obvious phenotype (45), suggesting that pharmacological inhibitors of these kinases might have great potential in treating inflammatory diseases like Crohn’s disease in which TNF and IL-6 have known pathogenic roles and in which the anti-inflammatory effects of IL-10 may limit disease severity. Although our studies show that MNK inhibition can potentially inhibit TLR responses and innate, proinflammatory cytokine production in macrophages, further studies will be required to see whether these effects can be achieved in whole animal models of inflammation.

GRANTS

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