MAPK Kinase 3 Potentiates Chlamydia HSP60-Induced Inflammatory Response through Distinct Activation of NF-κB

Yanhua Kang, Fang Wang, Zhe Lu, Hangjie Ying, Hang Zhang, Wen Ding, Cuili Wang and Liyun Shi

*J Immunol* 2013; 191:386-394; Prepublished online 31 May 2013; doi: 10.4049/jimmunol.1300481

http://www.jimmunol.org/content/191/1/386

References

This article cites 53 articles, 28 of which you can access for free at: http://www.jimmunol.org/content/191/1/386.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
MAPK Kinase 3 Potentiates Chlamydia HSP60-Induced Inflammatory Response through Distinct Activation of NF-κB

Yanhua Kang,*† Fang Wang,*† Zhe Lu,* Hangjie Ying,*† Hang Zhang,* Wen Ding,* Cuili Wang,*† and Liyun Shi*

Chlamydia pneumoniae (C. pneumoniae) remains one of the leading causes of bacterial pneumonia and has been implicated in the pathogenesis of some inflammation-related diseases, such as asthma, chronic obstructive pulmonary disease, and vascular diseases. Heat shock protein 60 is one of the pathogenic components of C. pneumoniae that is closely associated with the inflammatory disorders. However, the molecular basis for the immunopathologic property of chlamydial heat shock protein (cHSP60) has not been elucidated. In this article, we report that MAPK kinase 3 (MKK3) is essential for cHSP60-induced lung inflammation, because MKK3-knockout mice displayed significantly reduced lung neutrophil accumulation and decreased production of proinflammatory mediators, correlating with the alleviated inflammatory response in lung tissues. Mechanistically, p38 kinase was selectively activated by MKK3 in response to cHSP60 and activated NF-κB by stimulating the nuclear kinase, mitogen- and stress-activated protein kinase 1. The specific knockdown of mitogen- and stress-activated protein kinase 1 in macrophages resulted in a defective phosphorylation of NF-κB/RelA at Ser276 but had no apparent effect on RelA translocation. Furthermore, TGF-β-activated kinase 1 was found to relay the signal to MKK3 from TLR4, the major receptor that sensed cHSP60 in the initiation of the inflammatory response. Thus, we establish a critical role for MKK3 signaling in cHSP60 pathology and suggest a novel mechanism underlying C. pneumoniae–associated inflammatory disorders. The Journal of Immunology, 2013, 191: 386–394.
In addition, cHSP60 stimulated dendritic cells to up-regulate the phenotypic molecules and T cell–stimulatory capability. The fact that pulmonary inflammation can be induced by purified cHSP60 in mice further supported its proinflammatory property (16). Moreover, a recent investigation indicated that *Mycobacterium* with a cpn60 mutant was unable to induce the inflammatory response and reduce the granulomatous response in an animal model, reinforcing the pathogenic role of this agent in infectious diseases (17). Despite these findings, the molecular basis for the inflammation-promoting effects of cHSP60 have not been elucidated.

Previous studies indicated that cHSP60 engages with TLR4 (and likely TLR2) to produce cytokines, including TNF-α and IL-12p40, and the chemokines KC and MIP-2 in macrophages (16, 18, 19). The subordinate activator MyD88 is activated by cHSP60 and, in turn, activates NF-κB–dependent transcriptional activity (20). However, the optimized activation of NF-κB involves the integration of the various signaling pathways triggered by a given stimuli and is controlled by multiple steps, such as the selection of genomic targets, binding kinetics, and the phosphorylation of RelA/NF-κB and its cytoplasmatic shuttling; thus, details of the activation module of cHSP60 have to be explored (21). Additionally, some studies indicated that MAPKs, a fundamental mechanism governing the cellular response to extracellular stimuli, are also involved in cHSP60 activity. MAPKs are proline-directed serine/threonine-specific protein kinases and include at least four distinct classes of kinases: ERKs, JNK, p38 MAPK, and ERK5 (22). In addition to the mitogenic actions of ERK induced by cHSP60 in human vascular cells, p38 in particular seems to play an essential role in chlamydial pathogenesis (15). It was shown that p38 is potentially activated by *Chlamydia* or cHSP60 in human airway epithelial cells. The activation of p38, but not of ERK, c-Jun kinase/JNK, or PI3K, was required for the activation of NF-κB and the release of GM-CSF (23). A recent report indicated that the inhibition of p38 MAPK led to a 70–90% inhibition in IFN-β expression induced by *Chlamydia muridarum*. This finding suggests a prominent role for p38 in chlamydial infection and immunity (24). However, the mechanism driving p38 signaling upon cHSP60 stimulation and the in vivo relevance of this signaling, particularly in the lung, have not been defined. Because mice with a genetic deletion of p38 are not available as a result of the associated embryonic lethality (19–22), we sought to determine whether MAPK kinase 3 (MKK3), which is a presumed upstream kinase of p38, might be involved in cHSP60–associated inflammatory disorders.

MKK3 is one of the MAPK kinases that are hypothesized to phosphorylate p38 at the Thr-Gly-Tyr site. Previous studies demonstrated that MKK3 is rapidly activated by proinflammatory cytokines, such as TNF-α, IL-1β, and type I IFN, and is responsible for full activation of the p38 pathway (25). The activation of p38 and its subordinate kinase and the production of IL-8, IL-6, and matrix metalloproteinase 3 are significantly impaired in MKK3–deficient cells. Therefore, the pathway is critically involved in inflammatory diseases, such as inflammatory arthritis and diabetic nephropathy (26, 27). More importantly, MKK3 has been implicated in the lung inflammation and injury caused by a broad range of innocuous insults, such as endotoxin, ventilator, oxidant, and infectious agents (28–31).

In this study, we used MKK3-knockout mice to show that MKK3-mediated signaling is essential for the inflammatory response induced by cHSP60 in vivo or in vitro. p38 kinase was selectively activated and further stimulated mitogen- and stress-activated protein kinase (MSK1) kinase to activate RelA/NF-κB in a distinct manner, which was dependent on the phosphorylation of RelA/p65 at Ser720, but not at Ser536, and independent of RelA translocation. Moreover, TGF-β–activated kinase 1 (TAK1), which is downstream of TLR4, was found to initiate and deliver the signaling cascade. Thus, we not only defined a critical role for the MKK3/p38 pathway in the cHSP60–induced response, we also revealed, for the first time to our knowledge, a novel action module of the pathogenic factor that is likely integral to chlamydial inflammatory diseases.

### Materials and Methods

#### Mice and reagents

MKK3-knockout mice were generated as previously described (32) and maintained under specific pathogen–free conditions. All of the animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval and monitoring of the Animal Care and Use Committee of the School of Medicine at Hangzhou Normal University. All of the Abs, unless otherwise indicated, were obtained from Cell Signaling. The Ab for β-actin was obtained from Sigma-Aldrich. The plR-LK plasmids were obtained from Promega. The MAPK inhibitors U0126 (ERK1/2) and SB203580 (p38) were purchased from Calbiochem. Polymyxin B1 was obtained from Sigma.

#### Preparation of recombinant cHSP60

The cHSP60 gene (1467608) was cloned from the genomic DNA of *C. pneumoniae* (ATCC 53592) into the expression vector pET-28a (Novagen) with a hexahistidine tag attached to the C terminus. The recombinant protein was overexpressed in *Escherichia coli* BL21 cells and induced by isopropyl 1-thio-β-D-galactopyranoside. The recombinant protein was purified by affinity chromatography using Ni-NTA-agarose (QIAGEN) and ion exchange with HiTrap Q ion exchange (33). To circumvent the effects of any possible endotoxin contamination in the recombinant protein, cHSP60 was pretreated with Polymyxin B (20 μg/ml) or Endotoxin Removal Resin (Pierce). Endotoxin levels in the cHSP60 protein remained ≤5 EU/ml, as measured by a *Limulus* amoebocyte lysate kit (33).

#### Animal experiments

The mice were anesthetized i.p. with ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). A total volume of 50 μl normal saline (NS) or cHSP60 (2 μg/g body weight) was instilled intratracheally (i.t.) (16, 34).

#### Cell culture and generation of peritoneal macrophages

The RAW264.7 cell lines were obtained from the American Type Culture Collection and grown in RPMI 1640 medium (Life Technologies) containing 10% (v/v) heat-inactivated FCS. To prepare murine peritoneal macrophages, 8–20-wk-old mice were injected i.p. with 3% thioglycollate broth. After 72 h, the peritoneal cells were harvested, and the macrophages were harvested by quick adhesion.

#### RNA isolation and quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen), following the manufacturer’s protocol. SYBR Green PCR Master Mix (Bio-Rad) was used to detect mRNA levels, and relative expression levels were determined by applying the ΔΔCt method using β-actin as the endogenous control. The following primers were used in the tests: TNF-α, forward 5′-AAGGCCCAGGG-TGGTCCTGGAG-3′ and reverse 5′-AGGCCAGTGTTGACAGCCT-3′; IL-6, forward 5′-CCACTTCAAGGTGGAGCTCTTATGA-3′ and reverse 5′-AGTGCATCATCAGGTGTTCATATCC-3′.

#### Bronchoalveolar lavage, cell differentiation, and histological analysis

Briefly, the trachea was exposed through a midline incision and cannulated with a sterile 22-gauge needle. Bronchoalveolar lavage fluid (BALF) was used to detect mRNA levels, and reverse expression levels were determined by applying the ΔΔCt method using β-actin as the endogenous control. The following primers were used in the tests: TNF-α, forward 5′-AAGGCCCAGGG-TGGTCCTGGAG-3′ and reverse 5′-AGGCCAGTGTTGACAGCCT-3′; IL-6, forward 5′-CCACTTCAAGGTGGAGCTCTTATGA-3′ and reverse 5′-AGTGCATCATCAGGTGTTCATATCC-3′.

#### Bronchoalveolar lavage, cell differentiation, and histological analysis

Briefer, the trachea was exposed through a midline incision and cannulated with a sterile 22-gauge needle. Bronchoalveolar lavage fluid (BALF) was obtained by flushing three times with 1 ml 0.5 mmol/l EDTA/PBS. After centrifugation, the supernatants were stored at 80˚C until use. Total cell numbers in BALF were counted with a hemocytometer, and differential cell counts were determined on cytospin preparations with Diff-Quick staining (MEB) (34). Alternatively, neutrophils and macrophages in the BALF were assessed by immunostaining and subsequent flow cytometry (35). For the histological analysis, mouse lung samples were harvested thoroughly in PBS, fixed in 4% (w/v) formalin, and embedded in paraffin. Then, 5-μM sections were stained with H&E using standard procedures.
Plasmid constructs, transfection, and luciferase reporter assays

For the IL-6 reporter plasmids, DNA sequences (−300 to +1) were amplified from RAW264.7 genomic DNA, and the products were inserted into the KpnI–HindIII sites of the pGL3-Basic vector (Promega). Mutations in the putative AP-1 (−61 to −55), C/EBP (−161 to −147), or NF-κB (−73 to −64) binding sites in the mouse IL-6 promoter were generated using the Quik-Change Site-Directed Mutagenesis kit (Stratagene) (36). All of the constructs were confirmed by DNA sequencing. To test NF-κB–driven IL-6 transcriptional activity, the mouse IL-6 reporter plasmid was transfected in macrophages using Jet-ENDO transfection reagents (Polyplus). Twenty-four hours later, the cells were stimulated with cHSP60 (10 μg/ml), the vehicle, or heated cHSP60, as indicated, collected, and then lysed for the luciferase test. The luciferase activity was measured through dual-luciferase assays (Promega).

RNA interference

Small interfering RNA (siRNA) targeting mouse TAK1 or MSK1, as well as the scramble siRNA, were synthesized by GIMA (Shanghai, China) (37). siRNA duplexes were transfected into macrophages using INTERFERin-HTS, according to the standard protocol (Polyplus). The knockdown efficacy was determined by RT-PCR or Western blotting.

Immunofluorescence staining and confocal microscopy

Wild type (WT) and MKK3−/− peritoneal macrophages were seeded onto slides at 30% confluence and stimulated with cHSP60 or vehicle for the indicated time. The cells were then collected, fixed with 100% methanol, washed, and permeabilized in 0.2% saponin. After blocking with 5% bovine serum, the cells were stained with primary rabbit anti-p65 overnight at 4˚C and then stained with goat anti-rabbit IgG conjugated to Texas Red (Invitrogen). Finally, after the nuclei were labeled with DAPI (Invitrogen), the cells were mounted in VECTASHIELD and analyzed using fluorescence confocal microscopy (LSM confocal microscope; Carl Zeiss) (38).

Determination of cytokines and myeloperoxidase levels

The levels of TNF-α, IL-6, KC, and MIP-2 were measured in the culture supernatants or BALF by ELISA (R&D Systems). Lung myeloperoxidase (MPO) levels were determined using mouse MPO ELISA (Hycult Biotech), following the manufacturer’s instructions.

Western blotting

Western blots were performed following standard protocols. Total cell lysates were prepared, and the protein concentration was determined by a BCA protein assay (Thermo Fisher Scientific). Cell extracts were then subjected to SDS-PAGE, transferred onto a nitrocellulose membrane, and blotted as previously described. Targeted proteins were visualized using an ECL Western blotting kit (ECL Amersham Biosciences).

Statistical analysis

All of the data, unless otherwise indicated, are presented as the mean ± SD of independent experiments. The statistical significance of the differences between two groups was analyzed with the Student t test. Differences with a p value ≤0.05 were considered statistically significant. All of the calculations were performed using the Prism software program for Windows (GraphPad Software).

Results

Disruption of MKK3 prevents cHSP60-induced pulmonary inflammation

The correlation between high cHSP60 deposition and Chlamydia inflammatory disorders suggests that cHSP60 has an inflammation-promoting effect (13, 14, 16, 39). To determine whether MKK3 signaling is involved in cHSP60 activity in vivo, pulmonary inflammation was induced in WT and MKK3−/− mice through i.t. inoculation of purified cHSP60. The results showed that MKK3

![FIGURE 1](http://www.jimmunol.org/) MKK3 deficiency leads to an alleviated pulmonary inflammatory response induced by cHSP60. Age- and sex-matched WT and MKK3−/− mice (n = 5) were administered 50 μl of cHSP60 (2 μg/g body weight) or NS i.t. Six hours later, the animals were euthanized for histological and other functional analysis. (A) Representative H&E staining of lung tissues collected from WT and MKK3−/− mice (original magnification ×200). (B) Total cell and neutrophil counts in BALF. (C) MPO activity in the lung tissues. (D) Levels of IL-6, TNF-α, MIP-2, and KC in BALF from WT and MKK3−/− mice. All results are mean ± SD. *p < 0.05, **p < 0.01 versus WT controls.
deficiency led to alleviated pulmonary inflammatory responses, as evidenced by histological analysis (Fig. 1A). The numbers of total cells and neutrophils in BALF were remarkably decreased in MKK3 \(^{-/-}\) mice compared with those in their WT littermates (Fig. 1B). The level of MPO in lung tissues, an indicator of neutrophil activity, was diminished by MKK3 deficiency in mice (Fig. 1C). This finding might explain, at least in part, the diminished recruitment of proinflammatory cells upon cHSP60 challenge in MKK3 \(^{-/-}\) mice (Fig. 1D). These results indicate that MKK3 plays an essential role in the cHSP60-initiated inflammatory response.

MKK3 mediates cHSP60-induced NF-κB activation in macrophages

We then examined the mechanism used by MKK3 to drive cHSP60 activity in the macrophages. We first examined the activation of MKK3 by cHSP60 in RAW cells and found that a profound phosphorylation of MKK3 was markedly induced and sustained for >2 h (Fig. 2A). To explore the relevance of MKK3 in the cHSP60-initiated reaction, peritoneal macrophages from WT and MKK3-deficient mice were subjected to cHSP60 stimulation, and the production of proinflammatory cytokines in these cells was compared. As shown in Fig. 2B and 2C, cHSP60-mediated induction of IL-6 and TNF-α production was significantly attenuated in MKK3-deficient macrophages, at both the mRNA and protein levels.

Because NF-κB is one of the principal transcription factors that regulates proinflammatory gene expression, we tested cHSP60-driven NF-κB activity using a luciferase reporter assay. The assay revealed that NF-κB-dependent promoter activation was greatly induced by cHSP60; however, it was repressed by the loss of MKK3, indicating that this factor is required for optimal NF-κB activation upon cHSP60 stimulation (Fig. 2D). Strikingly, phosphorylation of p65 at Ser276 was markedly inhibited, whereas no effect or even a slight increase in phospho-Ser38 was observed in MKK3 \(^{-/-}\) macrophages compared with WT controls (Fig. 2E).

Given that the release of RelA/NF-κB from its IκB restraint and the translocation of RelA into the nuclear compartment constitute the determinant step for the initiation of gene transcription, we then examined the effect of MKK3 on RelA translocation induced by cHSP60. As shown in Fig. 2F, a robust translocation of RelA into the nucleus was induced by cHSP60, peaking at 30 min postexposure. Unexpectedly, no apparent difference in the nuclear abundance of RelA was observed between WT and MKK3 \(^{-/-}\) cells during the course of the reaction, suggesting that MKK3 had no effect on the RelA nuclear translocation. Taken together, these results indicate a pivotal role for MKK3 in the cHSP60-initiated inflammatory response that is largely dependent on a distinct activation of the NF-κB transcriptional program.

p38 MAPK is critically involved in the cHSP60-induced response

It is known that MAPKs, particularly p38, are regulated by MKK3 and linked to the activation of NF-κB (22, 40). Therefore, we evaluated the involvement of MAPKs in MKK3-mediated cHSP60

---

**FIGURE 2.** MKK3 \(^{-/-}\) macrophages display a defective response to cHSP60. (A) Western blot analysis of p-MKK3 and total MMK3 in RAW cells treated with cHSP60 (10 μg/ml) for the indicated lengths of time. The expression of IL-6 and TNF-α at the mRNA level (B) and protein level (C) by cHSP60 stimulation was assessed by quantitative real-time PCR and ELISA, respectively, in WT and MKK3 \(^{-/-}\) peritoneal macrophages. NF-κB–driven promoter activity (D) and phosphorylation of p65 (Ser276 or Ser38) (E) in response to cHSP60 (or heated cHSP60 or LPS, as indicated). (F) Confocal analysis of RelA nuclear translocation upon stimulation with cHSP60. p65 immunoreactivity is shown in red, and the nuclei are stained with DAPI (blue). Scale bar, 5 μm. Data are mean ± SD of three independent experiments. Representative results from three independent experiments are depicted in (E) and (F). \(*p < 0.05, **p < 0.01\) versus WT or vehicle-treated controls.
activity in vitro. We first examined the activation of MAPKs by cHSP60 and found that ERK1/2 and p38 were strongly phosphorylated in the primary macrophages. Interestingly, deletion of MKK3 led to a specific inhibition of p38 phosphorylation and had a marginal effect on phospho-ERK1/2 (Fig. 3A). Consistently, cHSP60-induced production of IL-6 and TNF-α was significantly reduced by the inhibitor for p38 but not by the ERK inhibitor (Fig. 3B). This finding suggests a specific requirement of MKK3/p38 in the cHSP60-induced inflammatory response. To elucidate the molecular mechanism by which p38 regulated the gene expression in this context, we focused our attention on the expression of IL-6, a classic proinflammatory effector molecule. The result showed that inhibition of p38 kinase significantly repressed cHSP60-induced IL-6 promoter activity (Fig. 3C). Thus, we identified a critical role for p38 kinase, which acts downstream of MKK3, in the cHSP60-initiated response.

**MSK1 is essential for NF-κB activation upon cHSP60 stimulation**

p38 MAPK can regulate gene expression via direct activation of transcription factors or by stimulation of nuclear kinases and other transcriptional components (41, 42). MSK1 is one of the substrates of p38 and was shown to activate NF-κB in response to various stimuli (43, 44). We next examined whether MSK1 participated in the cHSP60-triggered signaling pathway; we found that it was rapidly phosphorylated in RAW cells and that this effect was almost completely abrogated upon p38 inhibition (Fig. 4A). In agreement with the activation of p38 by MKK3 shown above, downregulation of MSK1 activation was observed in MKK3−/− macrophages (Fig. 4B). Moreover, the specific interference of MSK1 led to diminished phosphorylation of p65/RelA (Ser276) and significantly lower NF-κB activity than did the other mutated constructs. This result indicates a predominant role for the NF-κB element in the cHSP60 response. As we demonstrated above, knockdown of MSK1 significantly decreased NF-κB activity, which caused the reduced generation of inflammatory mediators in response to cHSP60. Nevertheless, because MSK1 is known to activate other transcription factors, such as AP-1 and C/EBPs, in addition to NF-κB, we constructed IL-6 promoter reporter plasmids mutated at NF-κB element in the cHSP60 response.

**Predominant role for NF-κB in the MKK3-mediated inflammatory response**

As we demonstrated above, knockdown of MSK1 significantly decreased NF-κB activity, which caused the reduced generation of inflammatory mediators in response to cHSP60. Nevertheless, because MSK1 is known to activate other transcription factors, such as AP-1 and C/EBPs, in addition to NF-κB, we constructed IL-6 promoter reporter plasmids mutated at NF-κB element in the cHSP60 response.

![Graphs and images showing phosphorylation and promoter activity](http://www.jimmunol.org/)

**FIGURE 3.** p38 is selectively activated by MKK3. (A) The phosphorylation of p38 and ERK1/2 was analyzed in WT and MKK3−/− primary macrophages in response to cHSP60 treatment. The immunoblots are representative of three independent experiments. IL-6 and TNF-α expression (B) and IL-6 promoter activity (C) induced by cHSP60 were determined in RAW cells pretreated with DMSO (DM), the MEK inhibitor U0126 (U0; 10 μM), or the p38 inhibitor SB203580 (SB; 20 μM). Data are mean ± SD of at least three independent experiments. *p < 0.05, **p < 0.01 versus vehicle-treated controls.

**FIGURE 4.** MSK1 mediates NF-κB–dependent transcriptional activity. (A) Phosphorylation of MSK1 by cHSP60 was analyzed in RAW cells pretreated or not with the p38 inhibitor SB203580 (SB; 20 μM). (B) Time-dependent activation of MSK1 by cHSP60 was analyzed in macrophages from WT and MKK3−/− mice. (C) The MSK1 level was tested by immunoblotting in RAW cells transfected with MSK1-siRNA (siM) or nonspecific siRNA (siN), and cHSP60-induced p65 phosphorylation (Ser276) was analyzed in siRNA-treated cells. cHSP60-induced NF-κB promoter activity (D) and IL-6 and TNF-α expression (E) were analyzed in RAW cells transfected with siM or siN. Data are mean ± SD of at least three independent experiments. The immunoblots are representative of three independent experiments. *p < 0.05, **p < 0.01 versus siN-treated controls.

data demonstrate that MSK1 is a critical component in the MKK3/p38 signaling cascade that drives the NF-κB–dependent cHSP60 activity.

As we demonstrated above, knockdown of MSK1 significantly decreased NF-κB activity, which caused the reduced generation of inflammatory mediators in response to cHSP60. Nevertheless, because MSK1 is known to activate other transcription factors, such as AP-1 and C/EBPs, in addition to NF-κB, we constructed IL-6 promoter reporter plasmids mutated at NF-κB, AP-1, and C/EBP sites to dissect their respective contributions to cHSP60 activity. As shown in Fig. 5B, a promoter with mutations at the NF-κB site exhibited a more profound downregulation of luciferase activity than did the other mutated constructs. This result indicates a predominant role for the NF-κB element in the cHSP60 response. Accordingly, the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) generated a defective expression of proinflammatory cytokines in macrophages (Fig. 5C). The macrophages from RelA−/− mice exhibited a diminished cytokine response to cHSP60 (Fig. 5D). Therefore, we concluded that the transcriptional activity...
of RelA/NF-κB is essential for the MKK3-mediated proinflammatory activity of cHSP60.

**TLR4 participates in the MKK3-promoted cHSP60-signaling pathway**

The data from our study support the importance of MKK3/p38 signaling in cHSP60 activity. However, we are still unaware of the upstream signaling pathways activated and the initial events in the response. Some previous studies indicated that TLR4 senses cHSP60 and initiates the response, whereas other investigators showed that TLR2 is responsible for the reaction (19, 39). Thus, we sought to evaluate the roles of these receptors in cHSP60 activity using primary macrophage cells from TLR2−/− and TLR4−/− mice. As shown in Fig. 6A and 6B, cHSP60-induced IL-6 and TNF-α production were markedly repressed in TLR4−/− macrophages compared with WT cells. In contrast, TLR2 deficiency exerted much lower inhibitory effects on the expression of these cytokines. Interestingly, cells deficient in both TLR2 and TLR4 exhibited a more profound reduction in cHSP60 response compared with WT counterparts, as evidenced by downregulation of the generation of proinflammatory cytokines, reduced neutrophil infiltration and decreased MPO levels in the lung tissues, and the compromised inflammatory reaction observed through histological analysis (Fig. 8). The MKK3−/− and TLR4−/− phenotypes share many key features, suggesting that TLR4 and downstream MKK3 signaling...
constitute the essential mechanistic machinery responsible for cHSP60-initiated pathological activity.

**Discussion**

It was suggested that many *Chlamydia*-associated diseases, either localized or systemic, share a common pathology because of the deposition of cHSP60. cHSP60 is highly expressed at the site of infection and/or the circulation, and this expression is concurrent with the onset and development of *Chlamydia* inflammatory diseases. Despite the close link between cHSP60 and *Chlamydia*-associated disorders, the action module of cHSP60 and the related molecular mechanism have not been elucidated. In this study, we identified that MKK3/p38 is a critical signaling pathway that regulates the cHSP60-induced IL-6 and TNF-α (D, E) were analyzed in RAW cells transfected with siT or siN. Data are mean ± SD of at least three independent experiments. The immunoblots are representative of three independent experiments. *p < 0.05, **p < 0.01 versus siN-treated controls.

**FIGURE 8.** Attenuated pulmonary inflammation induced by TLR4 deficiency following cHSP60 challenge. Age- and sex-matched WT and TLR4−/− mice (*n* = 5) were administered cHSP60 (2 μg/g body weight) or NS i.t. The animals were euthanized 6 h postadministration and subjected to the analysis. Neutrophil counts in BALF (A), MPO activity in the lungs (B), and IL-6 and TNF-α levels in BALF (C) were analyzed. Data are mean ± SD. (D) Representative H&E staining of lung tissues collected from WT and TLR4−/− mice 6 h after treatment with cHSP60 or vehicle (original magnification ×200). *p < 0.05, **p < 0.01 versus WT controls.

Our studies are based on in vivo and in vitro observations using a recombinant cHSP60 produced from *E. coli*. This raises the concern that the effects that we observed are derived from contaminated bacterial components, most likely from *E. coli* LPS. Indeed, the strategy of using recombinant cHSP60 or other pathogenic proteins has been widely adopted in functional studies (33, 47, 48). In this study, to eliminate the trace endotoxin from the preparation, cHSP60 was pretreated with LPS eliminator, Polymyxin B, throughout the experiments. To confirm that the recombinant protein, and not the contaminated LPS, caused the effects reported, we found that the rapid induction of inflammatory cytokines was completely abrogated when cHSP60 was inactivated through a preheating treatment. Moreover, it was found that the *Mycobacterium* devoid of the cpn60 homolog failed to induce an inflammatory response in mice, further confirming the inflammation-promoting effect of cHSP60. Interestingly, a report showed that LPS-stimulated p38 phosphorylation, NF-κB activation, and IL-6 production remained intact in MKK3-deficient fibroblast-like synoviocytes, suggesting that action of LPS is different from that of cHSP60, at least in terms of MKK3 signaling (26).

CHSP60 is one of the chlamydial major Ags involved in a variety of biological activities, such as cell proliferation, reactive oxygen species production, mitochondria stress, and the inflammatory and immune responses. Multiple key mediators participate in cHSP60-induced signaling transduction, which exhibits preferential activation modules in distinct contexts. In this study, we...
established the importance of MKK3 and downstream p38 signaling in the cHSP60-induced inflammatory response. This finding is consistent with the previous observation that p38 signaling is essential in Chlamydia pathology (15, 23, 24). Using a combination of genetic-deletion and pharmacologic-blocking approaches, we defined a specific requirement for p38 in the activation of the nuclear kinase MSK1 and, thereby, NF-κB transcriptional activity. p38 kinase is a stress-activated protein kinase with multivalent functions. This kinase can directly phosphorylate transcription factors or activate several downstream protein kinases, such as MAPK-interacting kinases, MAPK-activated kinases, and MSKs (22, 41, 49). Among these, MSK1 was shown to be activated by proinflammatory or infectious stimuli; thus, it serves as a mediator that delivers the upstream signal to the transcription machinery. In the current study, we revealed that MSK1 was rapidly activated by p38 kinase upon cHSP60 stimulation. Knockdown of MSK1 in macrophages resulted in defective phosphorylation of RelA/p65 at Ser276, but not at Ser536, which was associated with a significant downregulation of NF-κB–driven promoter activity. This result, consistent with what we initially observed in MKK3−/− macrophages, reflects a unique regulatory module of NF-κB activation in response to cHSP60. It seems that the potential of MKK3 to mediate cHSP60 activity is not dependent on the translocation of RelA or on the phosphorylation of RelA at Ser536, the two critical steps in the classical activation of NF-κB.

NF-κB is generally composed of a heterodimer of p65 and p50 subunits, which are sequestered in the cytoplasm by the inhibitor IκBα. Upon stimulation, IκBαs become phosphorylated and degraded. This degradation results in the release of NF-κB and facilitates its entry into the nucleus. Simultaneously, RelA/p65 becomes phosphorylated in a stimulus-dependent manner at multiple sites, including Ser276, Ser292, and Ser536 (50, 51). In the current study, we provide compelling data suggesting that profound phosphorylation of p65 at Ser276 caused by MKK3 signaling is indispensable for optimized NF-κB transcriptional activity and full acquisition of the cHSP60 effect. This finding was evidenced by the remarkable downregulation of NF-κB–driven promoter activity and effector cytokine expression in MKK3-deficient macrophages and by the reduced susceptibility of MKK3−/− mice to cHSP60 challenge. To support this conclusion, it was shown that Ser276 phosphorylation of RelA constitutes the transcriptional machinery by enhancing the binding of RelA to the κB elements, which promotes the phospho-acetylation of histones and the recruitment of the coactivator p300/CREB and PoII (52).

Nevertheless, it should be noted that cHSP60 also induced the phosphorylation of RelA at Ser536 and the cytoplasmic shuttling of RelA, as indicated in our results. Therefore, we suggest an action model of cHSP60 in macrophages that includes at least two pathways. The canonical pathway is mediated by the nuclear translocation of p65 and the phosphorylation of p65 at Ser536 upon cHSP60 stimulation. In contrast, MKK3, by activating the subordinate kinase p38 and MSK1, induced a distinct model of NF-κB activation. Although the contribution of these two pathways in cHSP60 activity has yet to be addressed, it is likely that these two pathways act in concert to give rise to the full action of cHSP60. In support of this hypothesis, a recent study indicated that the constitutive intestinal level of NF-κB was insufficient to trigger destructive inflammation in a mouse model of enteritis. Additional activation of MAPK, in combination with the already active NF-κB, was necessary to induce significantly enhanced TNF-α production and led to widespread inflammation and tissue damage (53). The novel mechanistic configuration underlying cHSP60 activity that we provide in this article seems to be of particular interest, given the prevalence of cHSP60-associated inflammatory pathology. Thus, inhibition of MKK3 signaling may have potential benefits for the treatment of such disorders.

Another important finding from our study is the identification of TAK1 as an essential component in the MKK3/p38-signaling cascade induced by cHSP60. Using the specific gene interference, we demonstrated that TAK1 is essential for the activation of MKK3 and the downstream signaling molecules p38 and MSK1. Macrophages with the TAK1 gene silenced exhibited repressed MKK3/p38 activation and decreased NF-κB activation, correlating with the downregulation of IL-6 and TNF-α production following cHSP60 exposure. Importantly, the activation of TAK1 and downstream MKK3 was profoundly impaired in cells deficient in TLR4, the putative sensor for the cHSP60 signal. This finding indicates a critical role for TAK1 in the relay of signaling from the PRR to the downstream-signaling pathways. In addition, TAK1 was proven to directly activate IκB kinase complex through Lys63-linked polyubiquitin, which causes IκBα phosphorylation and results in NF-κB activation (46). Therefore, TAK1 might also participate in the MKK3-independent pathway that we suggested above and might serve as the converging point of the two pathways underlying cHSP60 activity. Thus, further dissection of TAK1 function in Chlamydia pathology might be warranted. Interestingly, an increasing amount of data suggests that TAK1 itself can be targeted directly by several pathogenic molecules, such as Helicobacter pylori Caga, HIV gp41, and T-lymphotropic virus-I protein TAX (54–56). Through a physical interaction with or modification by the pathogenic proteins, TAK1 is exploited by the respective microorganism to enhance NF-κB activation and expression of the effector mediators, which are critically involved in the development of infection and inflammation. Therefore, whether cHSP60 secreted from the intracellular bacteria can directly induce NF-κB activation by targeting TAK1 is worthy of investigation.

Taken together, our findings establish a critical role for MKK3/p38 signaling in the cHSP60-induced inflammatory response and unveil a previously unknown activation module of this pathogenic agent. Thus, this study may open new opportunities to protect against chlamydial inflammatory diseases.

Acknowledgments

We thank Dr. Deborah A. Quinn and Dr. Hang Zhao (Massachusetts General Hospital, Harvard Medical School, Boston, MA) for MKK3−/− and TLR-deficient mice and Dr. Eugene Y. Chin (Department of Surgery, Brown University, Providence, RI) for the pGL3−NF-κB plasmid. We also thank Hongping Ying (Central Laboratory of Medical Research, Hangzhou Normal University) for histological analysis of lung tissues.

Disclosures

The authors have no financial conflicts of interest.

References


