

# MEKK3 is essential for lipopolysaccharide-induced interleukin-6 and granulocyte–macrophage colony-stimulating factor production in macrophages

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## Introduction

Lipopolysaccharide (LPS) is a potent inducer of inflammation by stimulating immune cells to produce pro-inflammatory cytokines, proteases and reactive oxygen and nitrogen species.<sup>1</sup> LPS acts by binding to its cognate receptor, toll-like receptor 4 (TLR4), a member of the evolutionarily conserved interleukin-1 receptor (IL-1R)-TLR supergene family of proteins that are essential for mounting a host defence against infectious pathogens.<sup>2–5</sup> Upon stimulation, IL-1R-TLRs activate four intracellular protein kinase cascades, the I $\kappa$ B $\alpha$  kinase (IKK) nuclear factor- $\kappa$ B (NF- $\kappa$ B), extracellular signal-regulated kinase (ERK), c-Jun NH<sub>2</sub>-terminal kinase (JNK) and p38 cascades, which are essential for the induction of genes encoding

## Summary

Mitogen-activated protein/ERK kinase kinase 3 (MEKK3) is a Ser/Thr protein kinase belonging to the MEKK/STE11 subgroup of the MAP3K family. Recently, we found that MEKK3 plays a critical role in interleukin-1 (IL-1) receptor and Toll-like receptor 4 signalling using established primary mouse embryonic fibroblast (MEF) cell lines. However, the function of MEKK3 in immune cells has not been studied because germ-line MEKK3 knockout mice are embryonically lethal between embryonic days 10 and 11. In this study, we used small interference RNA to the mouse *Mekk3* gene to specifically knock down MEKK3 expression in the macrophage line Raw264.7. We found that the lipopolysaccharide-induced IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) production was dramatically decreased in MEKK3 knockdown cells whereas the tumour necrosis factor- $\alpha$  and IL-1 $\beta$  production were not affected. We also observed that the ERK1/2, p38 and JNK MAPK induction in MEKK3 knockdown cells were moderately inhibited within the first 60 min of stimulation, while the ERK and p38 were more severely inhibited after 2–4 hr of stimulation. Degradation of I $\kappa$ B $\alpha$  was also partially blocked in MEKK3 knockdown cells. Notably, the impairment in IL-6 and GM-CSF production in the MEKK3 knockdown cells was restored by reintroducing a human *Mekk3* cDNA that could not be targeted by mouse *Mekk3*-siRNAs. In conclusion, this study showed that MEKK3 is a crucial and specific regulator of the proinflammatory cytokines IL-6 and GM-CSF in macrophages and provided a novel method for investigating MEKK3 function in other immune cells.

**Keywords:** Cytokine; MAPK; MEKK3; Macrophages; TLR signalling

proinflammatory cytokines.<sup>5–7</sup> The IL-1R-TLR signals are mediated by a conserved Toll/IL-1 receptor (TIR) domain that recruits and assembles intracellular signalling molecules, including MyD88, IL-1R-associated protein kinase, TIR domain-containing adaptor protein/MyD88-adaptor-like and tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6), into complexes.<sup>5,8,9</sup> Biochemical and genetic studies have confirmed the essential roles of these signalling molecules in the activation of downstream kinase cascades and in IL-1R- and TLR-mediated cytokine gene expression.

IL-1R-TLR-mediated signal transduction to the downstream protein kinase cascades requires members of the mitogen-activated protein kinase (MAPK) kinase kinase (MAP3K) family. Studies from many laboratories have

shown that the following members of the MAP3K family are involved in this process: transforming growth factor- $\beta$ -activating kinase 1 (TAK1),<sup>10,11</sup> mitogen-activated protein/ERK kinase kinase 1 (MEKK1),<sup>12–14</sup> MEKK2,<sup>15</sup> MEKK3,<sup>15,16</sup> apoptosis signal-regulating kinase 1 (ASK1), NF- $\kappa$ B-inducing kinase<sup>17</sup> and tumour progression locus 2.<sup>18</sup> However, the underlying molecular mechanism responsible for their regulation, specificity and function in each distinctive IL-1R-TLR signalling pathway remains largely unknown.

Of particular interest is MEKK3, a Ser/Thr protein kinase belonging to the MEKK/STE11 subgroup of the MAP3K family<sup>19,20</sup> that is ubiquitously expressed in multiple tissues and is a potent activator of the NF- $\kappa$ B and MAPK pathways.<sup>19–21</sup> *In vitro*, MEKK3 activates the MAPKs JNK, ERK1/2, p38 and ERK5/big MAPK.<sup>19,22–25</sup> It was recently demonstrated that MEKK3 plays a critical role in IL-1R and TLR4 signalling in studies performed in established primary mouse embryonic fibroblast (MEF) cell lines. In particular, it was found that MEKK3 is essential for IL-1-induced and LPS-induced IL-6 production via the IKK-NF- $\kappa$ B and JNK-p38 MAPK pathways. However, because the germ-line MEKK3 knockout in mice is embryonically lethal between embryonic days 10 and 11, its role has not been studied in immune cells such as macrophages, which are critical for promoting inflammation and innate immunity.

In this study, we developed an alternative strategy for verifying these results by using a lentiviral vector to deliver small interference RNA (siRNA) to knock down *Mekk3* gene expression in the macrophage line Raw264.7. We found that this knocked-down *Mekk3* expression in Raw264.7 cells specifically impaired IL-6 production induced by LPS, which was consistent with our previous findings in MEKK3-deficient MEFs.<sup>26</sup> Importantly, we also show that MEKK3 is required for the production of another important cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), but not for TNF- $\alpha$  and IL-1 $\beta$  production, in the Raw264.7 cells. Together, our findings showed that MEKK3 is a crucial and specific regulator of the production of the proinflammatory cytokines IL-6 and GM-CSF but not of TNF- $\alpha$  and IL-1 $\beta$  in macrophages. This study also provides a novel method for investigating MEKK3 function in other immune cells.

## Materials and methods

### Reagents

LPS was purchased from Sigma (St. Louis, MO), anti-ERK2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-p38 antibody used for immunoblotting was purchased from Cell Signaling (Boston, MA). Anti-MEKK3 was produced by immunizing rabbits with a mouse MEKK3 peptide (amino acids 22–41

of the N terminus) and was affinity purified. Anti- $\beta$ -actin was purchased from Sigma. The IL-6, GM-CSF, IL-1 $\beta$ , and TNF- $\alpha$  enzyme-linked immunosorbent assay kits were purchased from R and D Systems (Minneapolis, MN).

### Vector construction

Construction of the pBS-SKII-huU6 vector has been described previously.<sup>27</sup> Briefly, a *BbsI* site was introduced at the 3' end, which allowed insertion of the siRNA sequences at the +1 position of the U6 transcript. To construct the hairpin siRNA expression cassette, two complementary DNA oligonucleotide sequences (see description below) were synthesized, annealed, and inserted between the *BbsI* and *XhoI* sites immediately downstream from the U6 promoter: 5'-accg(n)<sup>19</sup>tcaagaga(n)<sup>19</sup>cttttc-3'; 3'-(n)<sup>19</sup>aagttct(n)<sup>19</sup>-gaaaagagct-5'. The (n)<sup>19</sup> in the oligonucleotide sequence stands for the 19-nucleotide sense and reverse complementary targeting sequences as described below. The siRNA cassette also features a TTCAAGAGA loop situated between the sense and reverse complementary targeting sequences and a TTTT terminator at the 3' end. The *Mekk3*-siRNA15 contains the sense targeting sequence of *ggatcctatacagcatca*, corresponding to the 894–912 nucleotide positions whereas the *Mekk3*-siRNA35 has the targeting sequence of *gcgagaacatgggtgtag*, corresponding to the 1272–1290 nucleotide positions of the mouse *Mekk3* coding sequence (GenBank accession no. Q61084). The targeting sequence for luciferase-siRNA (*Luci*-siRNA) is *gcgagaacatgggtgtag*, which is directed to the 153–172 region of the firefly gene. The FG12 lentiviral vector derived from the vector FUGW has been described previously.<sup>28</sup> To construct the siRNA-expressing lentiviral vectors, the siRNA expression cassette was digested out from the pBS-SKII-huU6-(siRNA) and subcloned into FG12 between the *XbaI* and *XhoI* sites. Restriction enzyme digestion and DNA sequencing confirmed the resulting plasmid.

### Cell culture and lentiviral vector transduction

Macrophage Raw264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum containing antimycotic antibiotics. The expression of green fluorescent protein (GFP) in the Raw264.7 cells was confirmed in a fluorescence-activated cell sorter (FACS) analysis. The cells were transduced with concentrated lentiviral vector stocks at a multiplicity of infection (MOI) of 10–25 (note that Raw264.7 cells have a considerably lower transducing efficiency) in the presence of 8  $\mu$ g/ml Polybrene (hexadimethrine bromide; Sigma). The transduced cells were harvested 7 days later and analysed by FACS. Briefly,  $1 \times 10^3$  cells were centrifuged with various lentiviral vectors at an MOI of 10 for 1 hr 30 min at 1000 g in the presence of 8  $\mu$ g/ml Polybrene. After

centrifugation, virus supernatants were removed and replaced with 1.5 ml fresh DMEM plus 10% fetal calf serum containing antibiotics. The cells were incubated for 24 hr and then transduced again with lentiviral vectors, as described above. After the cells were incubated for an additional 40–48 hr, the rate of infection was determined by FACS analysis and the data were analysed using CELLQUEST (FACSCaliber, BD Biosciences, Sparks, MD).

The lentiviral vector stocks were produced by calcium phosphate-mediated transient transfection with appropriate amounts of vector plasmid, the HIV-1 lentiviral packaging constructs pRSVREV and pMDLg/pRRE,<sup>29</sup> and the VSV-G expression plasmid pHCMVG of HEK-293 T cells, as described previously.<sup>30</sup> The viruses were collected from the culture supernatant on days 2 and 3 after transfection.

#### *Enzyme-linked immunosorbent assay for IL-6, GM-CSF, IL-1 $\beta$ and TNF- $\alpha$*

Cells were plated at a density of  $2 \times 10^4$  cells in a 48-well culture plate and incubated for 5 hr. The siRNA-transduced Raw264.7 cells were stimulated with various concentrations of LPS (0, 0.1, 0.5  $\mu\text{g/ml}$ ) in 300  $\mu\text{l}$  DMEM containing 5% fetal bovine serum for 24 hr. The medium was collected, and the levels of IL-6, GM-CSF, IL-1 $\beta$  and TNF- $\alpha$  released into the medium from siRNA-infected Raw264.7 cells were measured by an enzyme-linked immunoassay kit (ELISA; R and D Systems) specific for each protein, according to the manufacturer's instructions. Each sample was tested in triplicate.

#### *Immunoblotting analysis*

Raw264.7 cells cultured in 60-mm tissue culture dishes were stimulated, and cell lysates were prepared and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and in immunoblot studies. Raw264.7 cell lysates (10  $\mu\text{g}$  cell lysate protein per lane) were electrophoresed on a 10% SDS–PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Non-specific binding was blocked by a 2-hr incubation of membranes at room temperature with 5% non-fat dried milk and 0.05% Tween-20 in triethanolamine-buffered saline (pH 7.6). The membranes were then probed with a 1/1000 dilution of anti-phospho-JNK, anti-JNK, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, anti-p38 MAPK antibodies (Cell Signaling), anti-MEKK3 (BD Biosciences), and anti-I $\kappa$ B $\alpha$  antibody (Santa Cruz Biotechnology). As a loading control,  $\beta$ -actin was detected by immunoblotting analysis in the same samples using an anti- $\beta$ -actin antibody (Sigma-Aldrich, St. Louis, MO) at a 1/5000 dilution. Peroxidase-conjugated anti-rabbit immunoglobulin G was purchased from Sigma-Aldrich. Immunoreactive proteins were detected with a chemiluminescent system (Pierce, Rockford, IL).

#### *FACS analysis*

Lentivirus-siRNA-transduced cells were analysed and sorted by FACS Aerial. Briefly,  $1 \times 10^5$  cells were sorted at 4° in DMEM containing 0.5% fetal bovine serum. GFP-positive cells were then analysed using FACSCALIBUR and CELLQUEST software (BD Biosciences). Sorted cells were cultured for 3–4 days more and then used for experiments.

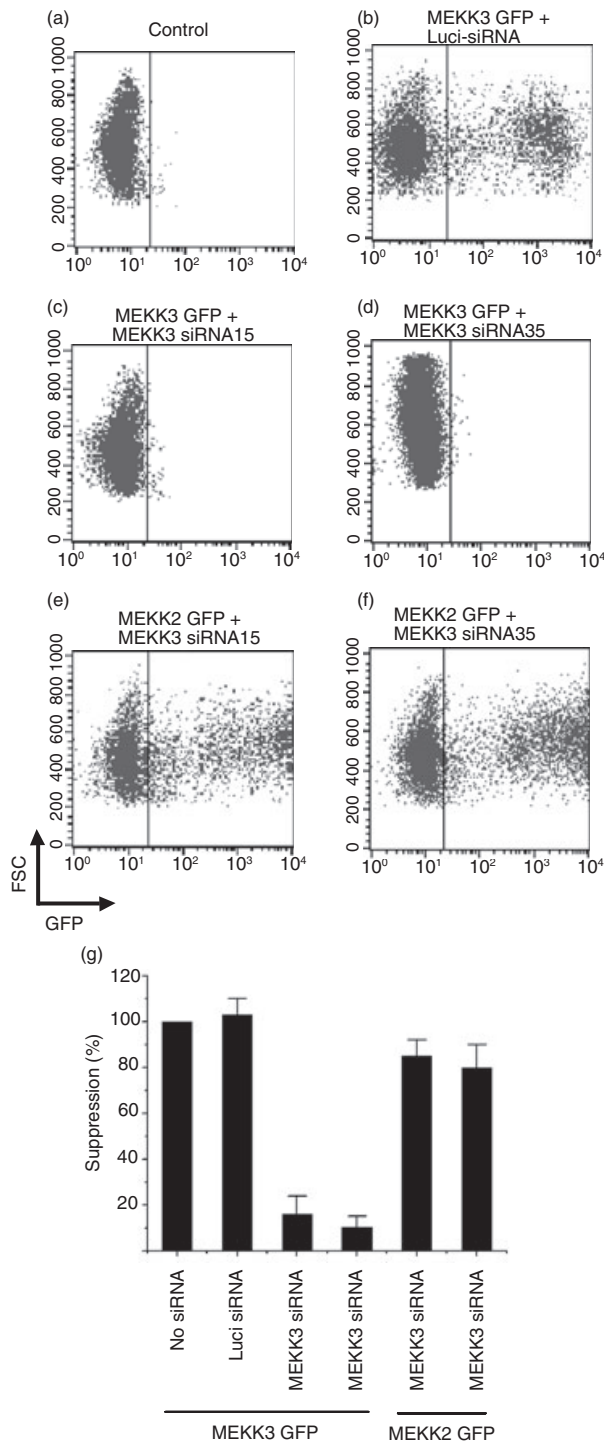
## Results

### Construction of *Mekk3*-siRNA expression cassettes

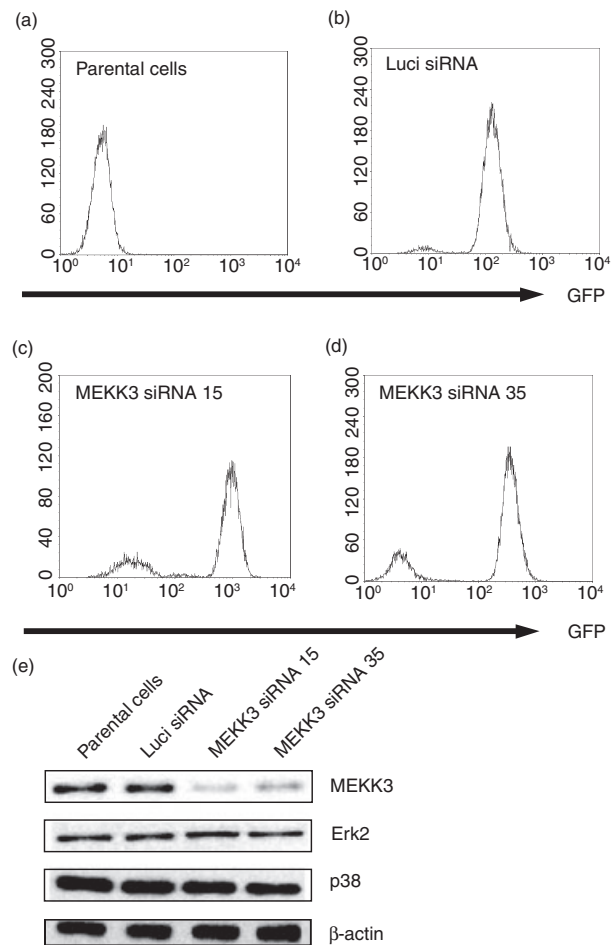
We designed four *Mekk3* hairpin siRNA expression cassettes by subcloning the annealed oligonucleotide sequences into the *BbsI* and *XhoI* sites in the pBS-SKII-huU6 expression vector, as described in Materials and methods. We tested the specificity of these siRNA cassettes by cotransfecting the siRNA expression vectors with a MEKK3-GFP fusion protein expression vector.<sup>26</sup> As shown in Fig. 1, while the control non-specific luciferase-siRNA (*Luci*-siRNA) had no effect on MEKK3-GFP expression (Fig. 1b), two *Mekk3*-siRNA expression plasmids, *Mekk3*-siRNA15 and *Mekk3*-siRNA35 (Fig. 1c,d), were found to specifically suppress MEKK3-GFP expression. To confirm that the knockdown of MEKK3-GFP expression by the *Mekk3*-siRNAs was specific, we examined their effect on a similar control expression vector for MEKK2-GFP expression and showed that they had no effect on MEKK2-GFP expression (Fig. 1e,f). Expression of the *Mekk3*-siRNAs was capable of inhibiting more than 90% of the expression of the cotransfected MEKK3-GFP as compared with the expression of the cotransfected *Luci*-siRNA, which had no effect on MEKK3-GFP expression (Fig. 1g). These results thus demonstrated that the expression of either *Mekk3*-siRNA15 or *Mekk3*-siRNA35 specifically knocked down *Mekk3* gene expression.

### Knockdown of MEKK3 expression in Raw264.7 cells by *Mekk3*-siRNA lentivirus

We next subcloned the *Mekk3*-siRNA expression cassettes into the lentiviral vector FG12 that had been originally designed for germ-line gene transduction.<sup>28</sup> This vector also expresses a human UbiC-driven GFP gene that serves as a marker and enables transduced cells to be tracked.<sup>28</sup> To understand the function of MEKK3 in macrophages, we used the lentiviruses that express either *Luci*-siRNA or one of the two *Mekk3*-siRNAs to transduce Raw264.7 cells. We enriched the numbers of transduced Raw264.7 cells by performing FACS sorting of the GFP-positive cells. As shown in Fig. 2(a), mock-transduced Raw264.7 cells expressed no GFP. However, more than 95% of the cells transduced by *Luci*-siRNA or by *Mekk3*-siRNA



**Figure 1.** MEKK3-GFP expression knocked down by *Mekk3*-siRNAs. (a–f) 293T cells were transfected with an empty vector (a) or with siRNA expression vectors against luciferase (*Luci*-siRNA) (b) or against *Mekk3* (c, d) alone (a) or together with expression vectors for MEKK3-GFP (b–d) or MEKK2-GFP (e, f). Transfected cells were analysed 36 hr later by FACS. (g) Quantification of knock down of MEKK3-GFP and MEKK2-GFP expression by FACS. Data shown are the percentage of GFP-positive cells transfected with different siRNAs compared with GFP-positive cells that were transfected with an empty vector. One representative FACS plot is shown for each condition.



**Figure 2.** Knocked down MEKK3 expression in the macrophage line Raw264.7. (a–d) Raw264.7 cells (a) and lentivirus-infected Raw264.7 cells that express *Luci*-siRNA (b), *Mekk3*-siRNA15 (c), or *Mekk3*-siRNA35 (d) after sorting for GFP-positive cells followed by FACS analysis. (e) Equal amounts of cell lysates prepared from the cells described above in (a–d) were analysed by immunoblotting using antibodies against MEKK3, ERK2, p38 and β-actin, as indicated. One representative experiment of three is shown.

(*Mekk3*-siRNA15 or *Mekk3*-siRNA35) were GFP-expressing cells (i.e. positively transduced cells) (Fig. 2c,d).

Next, the levels of the MEKK3 protein and several control proteins in Raw264.7 cells transduced with different siRNAs were measured by performing an immunoblot analysis. As shown in Fig. 2(e), while the endogenous levels of the control proteins ERK2, p38 and β-actin were constant, the endogenous MEKK3 protein level decreased dramatically in cells transduced with either *Mekk3*-siRNA15 or *Mekk3*-siRNA35 but not in control cells transduced with *Luci*-siRNA. These results demonstrated that expressing either the *Mekk3*-siRNA15 or *Mekk3*-siRNA35 by means of a lentiviral vector effectively and specifically suppressed endogenous MEKK3 expression in the macrophage line Raw264.7.

### Knockdown MEKK3 expression impairs LPS-induced IL-6 and GM-CSF but not TNF- $\alpha$ and IL-1 $\beta$ production in Raw264.7 cells

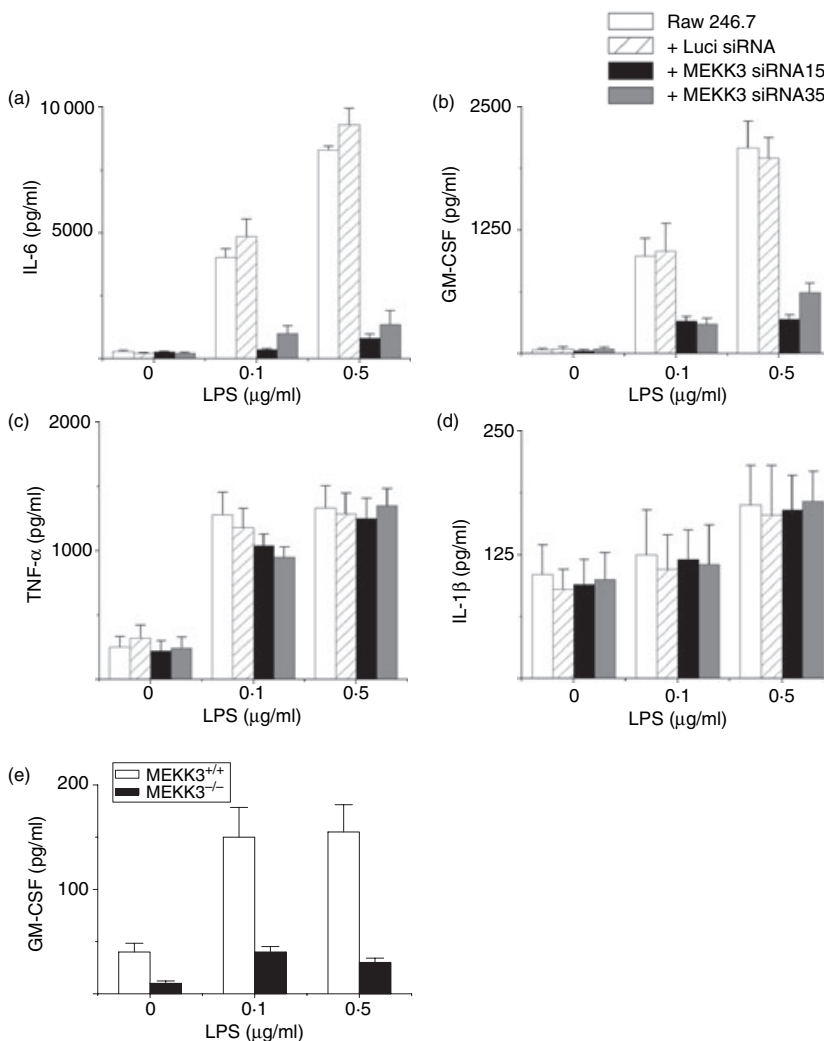
To determine whether MEKK3 must be present for macrophages to produce proinflammatory cytokines in response to LPS, we determined the effect of knocked-down MEKK3 expression by *Mekk3*-siRNA-expressing lentiviral vectors on the production of various cytokines before and after LPS stimulation in Raw264.7 cells. While the parental and *Luci*-siRNA-transduced Raw264.7 cells produced high levels of IL-6, the *Mekk3*-siRNA15- and *Mekk3*-siRNA35-transduced cells produced significantly reduced levels of IL-6 (Fig. 3a). In addition, the production of GM-CSF was severely impaired in *Mekk3*-siRNA-transduced cells but not in parental or *Luci*-siRNA-transduced cells (Fig. 3b). Surprisingly, the LPS-induced TNF- $\alpha$  (Fig. 3c) and IL-1 $\beta$  (Fig. 3d) production were not affected in *Mekk3*-siRNA-transduced cells. These results suggest that MEKK3 plays a pivotal role in LPS-stimulated IL-6

and GM-CSF production in macrophage Raw264.7 cells. In contrast, TNF- $\alpha$  and IL-1 $\beta$  production may not require MEKK3. Alternatively, the loss of MEKK3 function may be compensated for by different pathways.

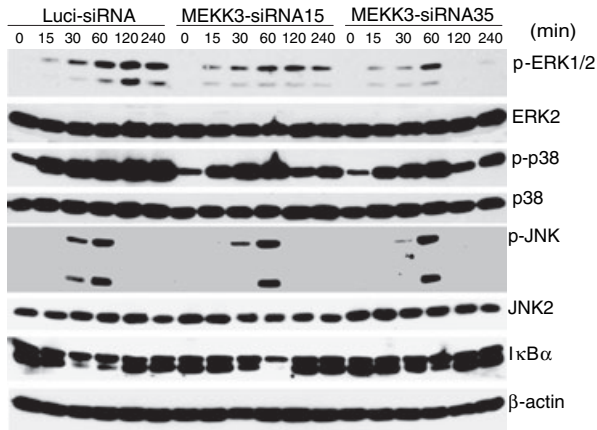
To confirm the critical role of MEKK3 in GM-CSF production, we stimulated wild-type and MEKK3<sup>-/-</sup> MEFs and then determined their GM-CSF production. The MEKK3<sup>-/-</sup> MEFs produced significantly less GM-CSF than did the wild-type cells, consistent with the results obtained in the MEKK3 knockdown Raw264.7 cells (Fig. 3E). Together, these results demonstrated that MEKK3 is critical for both IL-6 and GM-CSF production induced by LPS.

### Effects of MEKK3 knockdown on JNK, p38 and ERK activation and I $\kappa$ B $\alpha$ degradation in Raw264.7 cells

The JNK, ERK and p38 MAPKs are crucial downstream molecules for TLR signalling. To determine whether MEKK3 is required for the LPS-mediated activation of



**Figure 3.** MEKK3 is required for LPS-induced IL-6 and GM-CSF production but not TNF- $\alpha$  and IL-1 $\beta$  production. (a–d) Parental Raw264.7 cells or lentiviral siRNA-transduced cells were stimulated with the indicated amounts of LPS for 24 hr. The culture supernatants were collected for determination of IL-6, GM-CSF, TNF- $\alpha$  and IL-1 $\beta$  production by ELISA. (e) Wild-type and MEKK3<sup>-/-</sup> MEFs were stimulated with LPS for 24 hr, and analysed for GM-CSF production by ELISA. One representative experiment of three is shown.



**Figure 4.** Effect of MEKK3 knockdown on NF- $\kappa$ B and MAPK pathway activation. Raw264.7 cells transduced with lentiviruses expressing *Luci*-siRNA, *Mekk3*-siRNA15, or *Mekk3*-siRNA35 were stimulated with LPS (0.5  $\mu$ g/ml) for 0, 15, 30, 60, 120 and 240 min, as indicated. Cell lysates were prepared and analysed by immunoblotting using anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, anti-p38, anti-phospho-JNK, anti-JNK2, anti-I $\kappa$ B $\alpha$  and anti- $\beta$ -actin antibodies, as indicated.

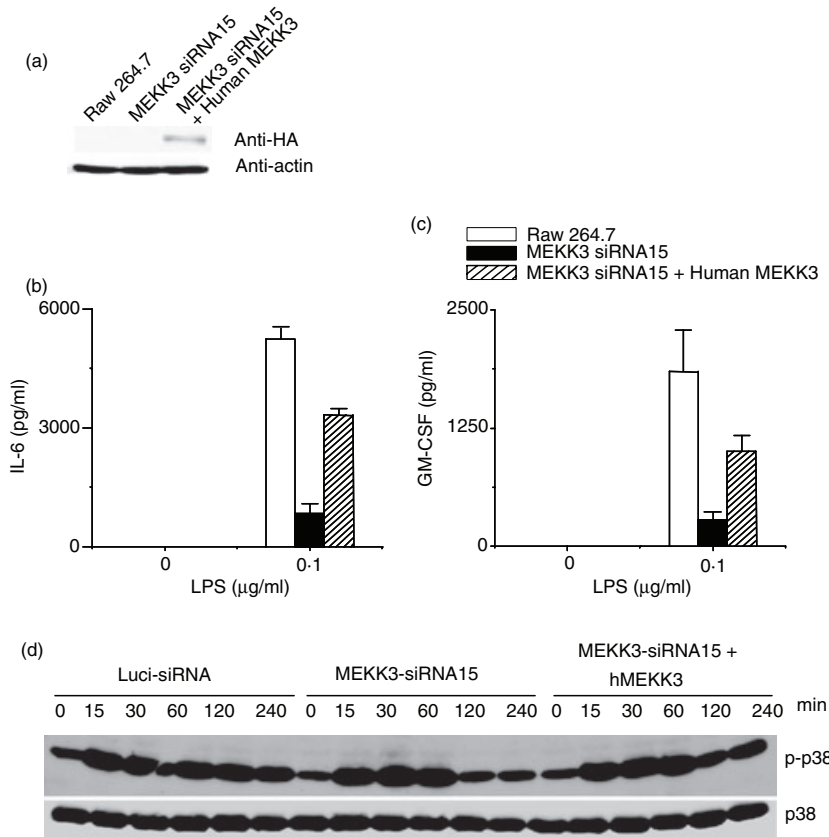
the JNK, ERK and p38 pathways in macrophages, we stimulated the control and *Mekk3*-siRNA-transduced Raw264.7 cells with LPS for different time-points. Cell extracts were prepared for the determination of ERK1/2, p38 and JNK MAPK activation by immunoblotting analyses. Interestingly, although the ERK1/2, p38 and JNK MAPKs were still induced in the *Mekk3* knockdown cells, their levels were consistently lower than those in the control cells with similar kinetics at early time-points (at 15, 30 and 60 min stimulation) (Fig. 4). However, at later time-points (120 and 240 min post-stimulation), we found that both ERK1/2 and p38 activation were significantly reduced. These results suggest that knockdown MEKK3 may be crucial for the ERK1/2 and p38 MAPK activation at later time-points. We also determined the NF- $\kappa$ B activation in MEKK3 knockdown cells and found a partial inhibition of I $\kappa$ B $\alpha$  degradation compared to that in wild-type cells. Thus, these results indicate that the blockade of MAPK activation at later time-points, together with a partial inhibition of the NF- $\kappa$ B pathway caused the inhibition of cytokine gene expression. Interestingly, in our previous study, we did not observe any defect in ERK activation by LPS in fibroblasts. In the *Mekk3* knockdown macrophages, ERK1/2 activation was markedly inhibited at later time-points, especially in the *Mekk3*-siRNA35-transduced cells. These results suggest that MEKK3 participates in the ERK1/2 activation in macrophages but not in fibroblasts. These results also indicate that the MAPK pathways still function in the MEKK3 knockdown Raw264.7 cells and that initial TLR4 signalling to MAPKs is not completely blocked.

### Restoration of LPS-induced IL-6 and GM-CSF expression in MEKK3 knockdown macrophages with human *Mekk3* cDNA

Because siRNA-mediated gene knockdown sometimes introduces artefacts that are not associated with the targeted genes, we decided to confirm our results by examining the restoration of MEKK3 expression in the knockdown cells. Human and mouse MEKK3 is 99% identical at the protein level but only 85% identical at the DNA level. Because three nucleotides in *Mekk3*-siRNA15 differ from those in the corresponding human *Mekk3* DNA sequence, we expected that it would not target the human *Mekk3* cDNA. Therefore, we transfected the *Mekk3*-siRNA15-transduced Raw264.7 cells with an expression vector for a haemagglutinin-tagged human MEKK3 (HA-hMEKK3). Expression of the HA-hMEKK3 was determined by immunoblotting with an anti-HA-antibody (Fig. 5a). To determine if the expression of human MEKK3 in the knockdown Raw264.7 cells restored LPS-induced IL-6 and GM-CSF production, we stimulated the parental, *Mekk3*-siRNA15-transduced or *Mekk3*-siRNA15-transduced Raw264.7 cells restored with human MEKK3 with LPS for 24 hr and then measured IL-6 and GM-CSF production in the culture supernatants by ELISA. As shown in Fig. 5(b,c), expression of the human *Mekk3* cDNA restored almost 50–60% of IL-6 and GM-CSF production in the *Mekk3*-siRNA15-transduced cells. To determine whether the expression of human MEKK3 in the *Mekk3*-siRNA15-transduced Raw264.7 cells restored LPS-induced signalling cascade, we stimulated the parental (*Luciferase*-siRNA), *Mekk3*-siRNA15-transduced, or *Mekk3*-siRNA15-Raw264.7 cells that were restored with human MEKK3 with LPS. As shown in Fig. 5(d), expression of the human *Mekk3* cDNA restored about 40–60% of p38 activation in the hMEKK3-transfected *Mekk3*-siRNA15-transduced cells. This confirmed that the defect in IL-6 and GM-CSF production in Raw264.7 cells transduced with *Mekk3*-specific siRNAs was indeed the result of the knockdown of MEKK3 expression.

### Discussion

In this study, we observed that MEKK3 plays a pivotal role in LPS-induced IL-6 and GM-CSF production but not in the production of two other cytokines, TNF- $\alpha$  and IL-1 $\beta$ . Thus, we conclude that MEKK3 is a crucial and specific regulator of the proinflammatory cytokines IL-6 and GM-CSF in macrophages. We found that the ERK1/2 and p38 MAPK induction by LPS was also inhibited in the MEKK3 knockdown cells. The JNK activation and the I $\kappa$ B $\alpha$  degradation appear to be moderately affected. This study also provided an alternative method for investigating MEKK3 function in other immune cells.



**Figure 5.** Restoration of LPS-induced IL-6 and GM-CSF expression in MEKK3 knockdown macrophages by a human *Mekk3* cDNA (hMEKK3). Raw264.7 cells infected with lentiviral vector for *Mekk3*-siRNA15 were transfected with an empty vector or HA-hMEKK3-expressing vector. HA-hMEKK3 expression in the transfected cells was determined by immunoblotting using an anti-HA antibody (a). (b) Control or (c) HA-hMEKK3-transfected cells were stimulated with LPS for 24 hr. The culture supernatants were collected and analysed for IL-6 and GM-CSF production by ELISA as described in Fig. 3. One representative experiment of three is shown. (d) Raw264.7 cells transduced with lentiviruses expressing *Luci*-siRNA, *Mekk3*-siRNA15 or HA-hMEKK3-transfected siRNA15 were stimulated with LPS (0.5 µg/ml) for 0, 15, 30, 60, 120 and 240 min, as indicated. Cell lysates were prepared and analysed by immunoblotting using anti-phospho-p38 and total p38 as indicated.

In our previous study, we demonstrated that MEKK3 plays a critical role in IL-1- and LPS-induced IL-6 production in embryonic fibroblasts.<sup>26</sup> However, because of the early embryonic lethality of the MEKK3 germ-line knockout in mice, we have been prevented from investigating the role of MEKK3 in proinflammatory cytokine production in immune cells such as macrophages and dendritic cells, which are the key types of cells controlling the innate immune responses and inflammation. To overcome this problem, we developed a strategy to knock down *Mekk3* gene expression in the macrophage line Raw264.7 through the use of a lentiviral vector-mediated delivery of siRNA expression vectors. This strategy inhibited the expression of the cotransfected *Mekk3* gene and also the endogenous *Mekk3* gene by up to 80%. We showed in this study that MEKK3 knockdown in macrophages caused a severe impairment in IL-6 and GM-CSF expression.

Only two of the four siRNAs, *Mekk3*-siRNA15 and *Mekk3*-siRNA35 that we tested against the mouse *Mekk3* gene worked in this study. However, these two siRNAs were specific because they had no effect on the expression of *Mekk2*, a close homologue of *Mekk3*. We also tested these two siRNAs on another unrelated gene, *Mip1* and again there was no effect (data not shown). In addition, we designed an anti-human *Mekk3*-siRNA, h*Mekk3*-siRNA9, with only one nucleotide change from the mouse

sequence. While this siRNA appeared able to knock down human *Mekk3* expression, it had no effect on mouse *Mekk3* expression (data not shown). These results strongly suggest that *Mekk3*-siRNA15 and *Mekk3*-siRNA35 were specific to the *Mekk3* gene and the impairment in cytokine expression was probably the result of the knock down of MEKK3.

To confirm that the observed impairment in cytokine production was indeed the result of the defective MEKK3 expression, we restored the cytokine expression by reconstitution of MEKK3 expression in the knockdown cells using a human *Mekk3* cDNA that could not be targeted by the mouse *Mekk3* siRNAs. Although this strategy did not completely restore IL-6 and GM-CSF production, the production of both cytokines was significantly increased compared with production in cells transfected with an empty vector. This incomplete restoration may have been partially the result of transfection inefficiency because not all cells were transfected.

Although the activation of the ERK1/2, p38 and JNK MAPKs in MEKK3 knockdown cells was only moderately affected at the early time-points, we found strong inhibition of the ERK1/2 and p38 activation later, at 2–4 hr after stimulation. We also observed a partial inhibition of IκBα degradation in MEKK3 knockdown cells suggesting that the NF-κB pathway may also be affected in the MEKK3 knockdown cells. These results suggest that the

defect in cytokine gene expression in MEKK3 knockdown cells was the result of the inhibition of MAPK at later time-points, and may be partially the result of NF- $\kappa$ B inhibition. Whether other pathways besides the ERK1/2, JNK and p38, or NF- $\kappa$ B pathways, are also impaired in MEKK3 knockdown macrophages is currently unknown. The ERK5/BMPK activation is defective in MEKK2-deficient cells.<sup>31</sup> Interestingly, while we found that ERK1/2 activation was defective in MEKK3 knockdown macrophages, we did not find such a defect in our previous study involving embryonic fibroblasts.<sup>26</sup> This finding in the present study suggests that MEKK3 signalling may be cell-type-specific.

In addition to the defect in IL-6 production in the MEKK3 knockdown cells, this study also revealed that MEKK3 is required for the expression of another cytokine, GM-CSF, which was confirmed in MEKK3-deficient fibroblasts (Fig. 4e). Interestingly, MEKK3 does not seem to be required for TNF- $\alpha$  and IL-1 $\beta$  production because both cytokines were expressed at a similar level in both the knockdown and control cells. Together, these results suggest that distinct cytokine genes may specifically use the MEKK3 signalling pathway.

In summary, we used siRNAs to knock down MEKK3 expression in a macrophage line and provided evidence that MEKK3 plays an important and specific role in mediating proinflammatory cytokine gene expression in immune cells. While other experimental systems such as conditional MEKK3 knockout mice are important and may be central to investigating the function and regulation of MEKK3 in different immune cells, the siRNA strategy developed in this study promises to serve as a valuable alternative system in the study of other immune cells, especially in the light of the potential improvement in the delivery efficiency of lentiviruses expected in the future. Finally, this strategy is crucial for the study of human immune cells, because mouse genetic manipulation does not work for studies of the human system.

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