Cellular & Molecular Immunology (2012) 9, 27–33

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RESEARCH ARTICLE

MLK4 has negative effect on TLR4 signaling

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The stimulation of Toll-like receptors (TLRs) on macrophages triggers production of proinflammatory cytokines such as tumor-necrosis factor-α (TNF-α). The TNF production is mediated by a series of signaling events and subsequent transcriptional and post-transcriptional activation of the *TNF* gene. Termination of TLR-mediated cellular signaling is also important for a proper immunoresponse, since sustained cytokine expression can result in immune disorders. Here we identified that mixed-lineage kinase (MLK) 4 is a TLR4-interacting protein. Unlike previously characterized MLK group members, MLK4 cannot act as a mitogen-activated protein kinase kinase kinase (MAP3K) to mediate c-Jun N-terminal kinase (JNK), p38 or extracellular signal-regulated kinase (ERK) activation. Rather, MLK4 appears to be able to inhibit lipopolysaccharide (LPS)-induced activation of the JNK or ERK pathways, but does not have effect on LPS-induced p38 or NF-κB activation. The LPS-induced TNF production in MLK4 knockdown and overexpression cells were also increased and reduced, respectively. These data demonstrate that MLK4 is a negative regulator of TLR4 signaling.

Cellular & Molecular Immunology (2012) **9,** 27–33; doi:10.1038/cmi.2011.15; published online 23 May 2011

Keywords: Kinase; MLK4; TLR4

INTRODUCTION

Toll-like receptors (TLRs) are innate immune sensors, each responding to specific molecules of microbial origin.[1,2] Binding of pathogen-associated pattern molecules, such as lipopolysaccharides (LPS), to cell surface TLRs results in recruitment of signaling adaptors, such as myeloid differentiation primary response gene 88 and TIR domain-containing adapter-inducing interferon-β.[3–6] This recruitment allows for the activation of members of the interleukin-1 receptor-associated kinase family of kinases and further recruitment of the adaptor molecule tumor-necrosis factor (TNF) receptor-associated factor 6 (TRAF6).[2] The signaling pathways of the transcription factor NF-κB[7] and mitogen-activated protein kinases (MAPKs), which include c-Jun N-terminal kinase (JNK),[8] extracellular signal-regulated kinase (ERK)[9] and p38,[10, 11] are subsequently activated after receptor engagement with microbial pattern molecules.[2,6] The activation of these signaling pathways is believed to be essential for the production of proinflammatory cytokines, such as TNF, IL-6, IL-1 and IL-12, as well as chemokines.[12] Although the production of proinflammatory cytokines serves an important role in host defense against the invading microbes, overexpression of cytokines can lead to inflammatory disorders, therefore necessitating intrinsic pathways that shut down cytokine production.[12] However, the mechanisms that limit cytokine production have not been extensively studied.

Mixed-lineage kinases (MLKs) belong to the superfamily of MAP kinase kinase kinases (MAP3Ks).[13] The MLK family members are characterized by the presence of signature sequences of Ser/Thr and Tyr kinases within their catalytic domain. The MLK1–4 contains an N-terminal Src homology (SH3) domain, followed by the kinase domain, a leucine-zipper region, and a Cdc42/Rac-interactive binding (CRIB) motif.[13,14] The overall sequence identity of these domains among MLK1–4 is about 65%. The carboxyl terminal regions of these proteins diverge, suggesting that these proteins may have different functions.[13] It was shown that some of the MLK family members, such as MLK3, can activate the JNK and p38 MAPKs.[10,13–22] The MAPK kinases that lie between MLK3 and JNK in the kinase cascades are MKK4 and 7.[13,14,18] Some data implicate that MKK3 and 6 are involved in MLK3-triggered p38 activation.[13,14,18] An interplay between MLK3 and other MAP3Ks was reported, as AKT (also named protein kinase B) can inhibit MLK3 by direct phosphorylation at a specific residue on the MLK3 C-terminal regulatory domain.[23] It was reported that MLK3 behaves as a pro-apoptotic kinase, leading to cell death upon trophic factor withdrawal or in response to neurotoxic assault in an animal model of Parkinson's disease.[14,21,22,24,25] To date, MLK4 has not been biochemically characterized. The detailed function of MLK group members, including MLK3, is still largely unknown.

In searching for intracellular interacting proteins of TLR4, we used a yeast two-hybrid screen to identify proteins that interacted with the intracellular domain of TLR4 and found that MLK4 can interact with TLR4. We show here that MLK4 can suppress TLR4-mediated JNK and ERK activation, but not p38 or NF-κB activation. Knockdown of MLK4 increases LPS-induced TNF production, and overexpression of

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Received 9 March 2011; revised 18 April 2011; accepted 21 April 2011

MLK4 reduces LPS-induced TNF production. Therefore, MLK4 appears to be a negative regulator of TLR4 signaling.

MATERIALS AND METHODS

Reagents

LPS were from *Escherichia coli* O111:B4 (List Biological Laboratories, Campbell, CA, USA), Anti-Flag (F3165; Sigma, St Louis, MO, USA), anti-Myc (A-14), anti-V5 (Invitrogen, San Diego, CA, USA) and anti-IkBa (C-21; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and antibodies to phosphorylated p38 (9211), Jnk (9251) and Erk (9010; Cell Signaling, Beverly, MA, USA).

Plasmids

Mammalian expression vectors for human TLR4 and its deletion mutants were described previously.[26] Mammalian expression vectors of MLK4α and MLK4β were constructed by subcloning full-length cDNAs into pcDNA3 vector and fused with V5 or Flag tag at its N-terminal. Expression plasmids of different MAPKs were described previously.[27] The deletion of MLK4β was made by using polymerase chain reaction. Small interfering RNA (siRNA)-expressing vectors were made by cloning double stranded synthetic oligos into pH1-neo vector. The sequences of the shRNA are available upon request.

Co-immunoprecipitation assay

HEK293T (293T) cells (1×10^6) grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum were transfected with 5 μg of mammalian expression vectors using lipofectamine 2000 (Invitrogen). After 24 h, cells were chilled to 4 °C and lysed by incubating for 15 min in lysis buffer (25 mM HEPES, pH 7.6, 137 mM NaCl, 3 mM EDTA, 3 mM β-glycerophosphate, 1% Triton X-100, 0.1 mM Na_3VO_4, 1 mM phenylmethsulfonyl fluoride). Nuclei (cell pellet) were removed by centrifugation at 130 000*g* for 10 min. Flag- or V5-tagged proteins were purified from the cell lysate by immunoprecipitation. The immunoprecipitates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting analysis with different antibodies.

Measurement of MAPK activity

293T cells were transfected with expression vectors of MLK4, TAK1, MKK7, MEK1, p38, JNK1 or ERK2 in different combinations using lipofectamine 2000. After 24 h, the cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation and western blotting analysis. The regulatory phosphorylation levels of p38, JNK1 or ERK2 were detected by anti-phospho-p38, anti-phospho-JNK or anti-phospho-ERK antibodies. The levels of the phosphorylation were used to evaluate the activity of these MAPKs.

Generation of stable cell lines

RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. siRNA-expressing vectors or MLK4α expression vectors were transfected in RAW264.7 cells using electroporation method. The stable lines were established by culturing the cells in G418 (1 mg/ml) for 2–3 weeks. The pool of survived clones expressing gene of interest were collected and used.

Measurement of TNF

Supernatants of macrophage cultures were collected and concentrations of TNF were measured by enzyme-linked immunosorbent assay according to the kit manufacturer's protocol (eBioscience, San Diego, CA, USA).

Yeast two-hybrid screen

Two-hybrid screening was done as described with a mixed human fetal brain and spleen cDNA library.[28] A portion of human TLR4 spanning the sequence encoding the intracellular protein domain (amino acids 653–839) was subcloned into pAS2 vector and was used as "bait"; 5×10^6 transformants were screened.

RESULTS

MLK4 interacts with TLR4

We used a yeast two-hybrid screen to identify proteins that interact with the intracellular domain of TLR4 (amino acids 653–839).[26] One of the positive clones encoded MLK4β, myeloid differentiation primary response gene 88 and 4-1BBL were isolated in the same screen, suggesting that the two-hybrid screen accurately identified proteins that were functionally associated with TLR4. We determined by the yeast two-hybrid interaction assay that MLK4β, but not negative control proteins, including the p38, the tumor suppressor p53, and lamin, interacted with the TLR4 intracellular domain (Figure 1a). Yeast containing the TLR4 intracellular domain in a "bait" vector and MLK4β in a "prey" vector grew in media lacking histidine (because of the interaction-mediated expression of a *HIS3* reporter gene). To confirm the interaction in mammalian cells, we co-expressed Flag-TLR4 with V5-tagged MLK4β in human embryonic kidney 293T cells. Flag interleukin-3 receptor (IL-3R) was used as a control for TLR4. We found that MLK4β can be coprecipitated with Flag-TLR4, but not with Flag-IL-3R, when the cell lysates were immunoprecipitated with anti-flag antibodies (Figure 1b). Thus, MLK4β can interact with TLR4 in a yeast two-hybrid system and in mammalian cells.

Domains that are required for MLK4–TLR4 interaction

MLK4 expressed in two splicing variants, MLK4α and MLK4β (Figure 2a). The difference of these two isoforms is located in their C-terminal portion. We asked whether MLK4α can interact with TLR4 by using co-immunoprecipitation assay and found that MLK4α can also be pull down by co-expressed TLR4 (Figure 2b). Thus, both the short and long variants of MLK4 can interact with TLR4. We then mapped the region of MLK4 required for its interaction with TLR4. MLK4 contains a number of domain structures (Figure 2a).[13,14] We generated a number of deletion mutants of MLK4 as indicated in

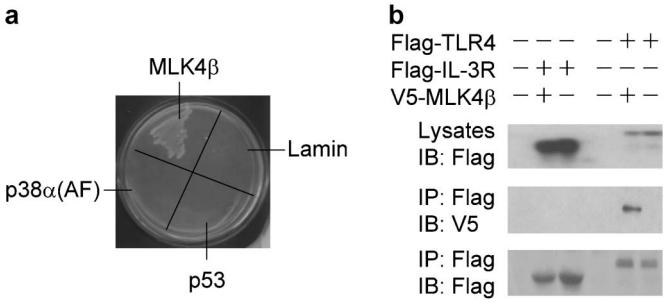

Figure 1 MLK4β can interact with TLR4. (**a**) Growth of yeast cells transfected with expression plasmids encoding amino acids 653–839 of TLR4 and amino acids 12–1036 of MLK4β, dominant negative p38α(AF), p53 or lamin, on medium lacking histidine. (**b**) Co-immunoprecipitation assay of 293T cells transfected with plasmids expressing V5-tagged MLK4β (V5-MLK4β) and Flag-tagged TLR4 or IL-3R as indicated. '−' indicates that empty vector was included. Cells were lysed 24 h after transfection; two-thirds of the lysates were immunoprecipitated (IP) with anti-Flag. The cell lysates and immunoprecipitates were analyzed by immunoblotting (IB) with antibodies as indicated. MLK, mixed-lineage kinase; TLR, Toll-like receptor.

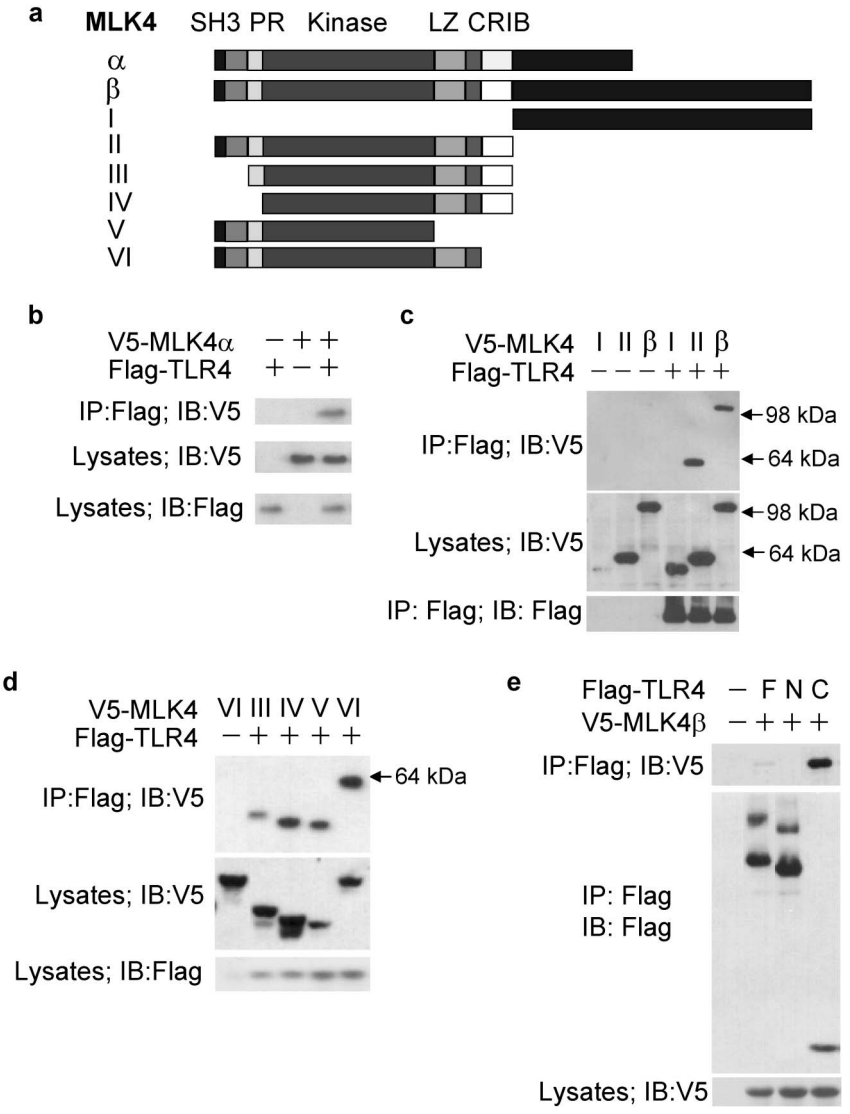

Figure 2 Intracellular domain of TLR4 and kinase domain of MLK4 are required for TLR4–MLK4 interaction. (**a**) Diagram of MLK4α, MLK4β and MLK4 deletion mutants. (**b**) Co-immunoprecipitation assay of 293T cells transfected with plasmids expressing Flag-tagged TLR4 and V5-tagged MLK4α. Cell lysates were immunoprecipitated with anti-Flag. The cell lysates and immunoprecipitates were analyzed by immunoblotting with antibodies as indicated. (**c**) Co-immunoprecipitation assay of 293T cells transfected with plasmids expressing Flag-tagged TLR4 or empty vector and V5-tagged MLK4β, MLK4 mutant I and II. Cell lysates were immunoprecipitated with anti-Flag. The cell lysates and immunoprecipitates were analyzed by immunoblotting with antibodies as indicated. (**d**) Co-immunoprecipitation assay of 293T cells transfected with plasmids expressing Flag-tagged TLR4 or empty vector and V5-tagged MLK4 mutant III, IV, V and VI. Cell lysates were immunoprecipitated with anti-Flag. The cell lysates and immunoprecipitates were analyzed by immunoblotting with antibodies as indicated. (**e**) Co-immunoprecipitation assay of 293T cells transfected with plasmids expressing V5-tagged MLK4β and Flag-tagged TLR4 (F), TLR4 lacking the cytosolic domain (N) or TLR4 lacking the extracellular domain (C). Cell lysates were immunoprecipitated with anti-Flag. The cell lysates and immunoprecipitates were analyzed by immunoblotting with antibodies as indicated. Data are representative of two to three independent experiments. CRIB, Cdc42/Rac-interactive binding; IB, immunoblotting; IP, immunoprecipitation; MLK, mixed-lineage kinase; TLR, Toll-like receptor.

Figure 2a. We first examined the interaction of TLR4 with MLK4 mutants that contain the N-terminal region, which contains all domains except the C-terminal region, or the C-terminal region of MLK4β alone (termed II and I, respectively in the figure). As shown in Figure 2c, the N-terminal region, but not the C-terminal region, can interact with TLR4 in co-immunoprecipitation studies. We then tested the sequential deletion of the SH3 domain, SH3+proline-rich motif, leucine zipper and CRIB domain of MLK, and found that deletion of any of these domains did not affect the binding of MLK4 to TLR4 (Figure 2d), indicating that none of these domains is required for MLK4 to interact with TLR4. Based on the data shown in Figure 2c and d, we concluded that the kinase domain of MLK4 is essential for its interaction with TLR4.

Since our two-hybrid screen was performed using TLR4 intracellular domain, we further asked whether other part TLR4 can interact with MLK4. As shown in Figure 2e, the full-length TLR4 and intracellular domain (termed C in the figure), but not the TLR4 with the intracellular domain deletion (termed N in the figure), can be co-immunoprecipitated with MLK4β. A higher amount of intracellular domain (C) was coprecipitated with MLK4β than that of the full-length TLR4 (Figure 2e), which is most likely due to the fact that the non-membrane-associated TLR4 intracellular domain has more of a

chance to encounter expressed MLK4 in the cytosol. The double bands of Flag-TLR4 and TLR4(N) in the immunoprecipitation of Flag proteins likely resulted from formation of homodimers of TLR4 and TLR4(N). Collectively, we concluded that the MLK4–TLR4 interaction requires the cytoplasmic domain of TLR4.

MLK4β does not interact with Cdc42, TRAF2 or TRAF6

MLK3 was shown to interact with active Cdc42 and can be activated by co-expressed active Cdc42.[29,30] The CRIB domain has been demonstrated to be a Cdc42/Rac-interacting motif in MLK3.[29–31] Since MLK4β contains a CRIB domain, we determined whether MLK4β can interact with Cdc42. We co-expressed dominant active Cdc42 with MLK4β in 293T cells and examined their interaction by co-immunoprecipitation. As shown in Figure 3a, we did not detect any interaction between Cdc42 and MLK4β, while Cdc42 can be pull down by MLK3. A major difference between MLK4 and MLK3 is the C-terminal domain. To test whether the C-terminal domain in MLK4β interferes with the interaction between MLK4 and Cdc42, we included a C-terminal deletion mutant of MLK4β in the experiment, and found that the deletion mutant (II) still cannot interact with Cdc42. Thus, we concluded that MLK4 cannot interact with Cdc42.

TRAF6 is an effector protein recruited to the TLR4 receptor complex after cells are stimulated with LPS.[32,33] Because MLK4 interacts with TLR4, we tested whether MLK4β interacts with TRAF6 using a co-immunoprecipitation assay. TRAF2 was also included in the experiment. We also included deletion mutants I and II of MLK4β, which contain either the C-terminal domain or the N-terminal domain, respectively. The interaction of Flag-TAB1 and Myc-TRAF6 was included in a parallel experiment as positive control (data not shown). As shown in Figure 3b, MLK4β and its mutants did not interact with either TRAF6 or TRAF2.

Overexpression of MLK4β cannot activate p38

Previous studies have shown that co-expression of MLK3 can lead to activation of JNK and p38.[13,29,30] We performed similar experiments to examine whether MLK4β can play a role of MAP3K in the JNK and p38 pathways. Phosphorylation levels of MAPKs were used to measure the activation of these kinases. Co-expression of MLK4β with JNK1 did not increased phosphorylation levels of JNK1 in 293 cells (Figure 4a). Similarly, co-expression of MLK4β with ERK2 did not increase phosphorylation of ERK2 (Figure 4b). In contrast, co-expression of MKK7 or MEK1 increased phosphorylation of JNK1 and ERK2, respectively (Figure 2a and b). We further analyzed the effect of MLK4β overexpression on p38 activity in details. MLK4β co-expression had no effect on p38 activity, whereas co-expressed MAP3K TAK1 significantly increased phosphorylation levels of p38 (Figure 4c). Because the SH3 domain of MLK3 has been shown to auto-inhibit its kinase activity, to exclude the possibility that some domain structures in MLK4 prevented MLK4 kinase domain to activate p38 in our co-expression assay, we co-expressed p38 with a series of deletion mutants of MLK4β, in which the SH3 domain or other domains have been deleted or truncated. None of these MLK4 deletion mutants is capable of activating p38 (Figure 4c). Thus, unlike MLK3, MLK4 seems unable to act as a MAP3K for the MAPK pathways.

MLK4β has negative effect on TLR4 signaling

To determine if there is any role of MLK4 in TLR4-mediated cellular responses, we next examined whether reducing or increasing MLK4 in RAW264.7 macrophage cells affects LPS-induced activation of NF-κB and MAPKs pathways, as well as TNF production.

We designed a number of MLK4-targeting siRNAs and identified by co-expression assay that two siRNAs that can effectively knock down MLK4. We named these two siRNAs Si-MLK4-1 and Si-MLK4-2. siRNA that targets GFP (siRNA-targeting GFP (Si-GFP)) was used as control. We stably expressed Si-MLK4-1, Si-MLK4-2 or Si-GFP in RAW264.7 cells and measured LPS-induced activation of NF-κB and MAPKs pathways. It is known that IκB degradation is a step in releasing NF-κB to the nucleus for transcription of NF-kB-dependent genes, and therefore, we used IκB protein levels to measure the activity of NF-κB. Since the regulatory phosphorylation of MAPKs represents their activation, we used phosphorylation levels of ERK, JNK and p38 to measure the activities of these MAPKs. As shown in Figure 5a, knockdown of MLK4 did not affect LPS-induced IκB degradation, and did not affect LPS-induced p38 phosphorylation. However, LPS-induced phosphorylation of JNK1/2 and ERK1/2 were significantly enhanced in MLK4 knockdown cells, suggesting an inhibitory

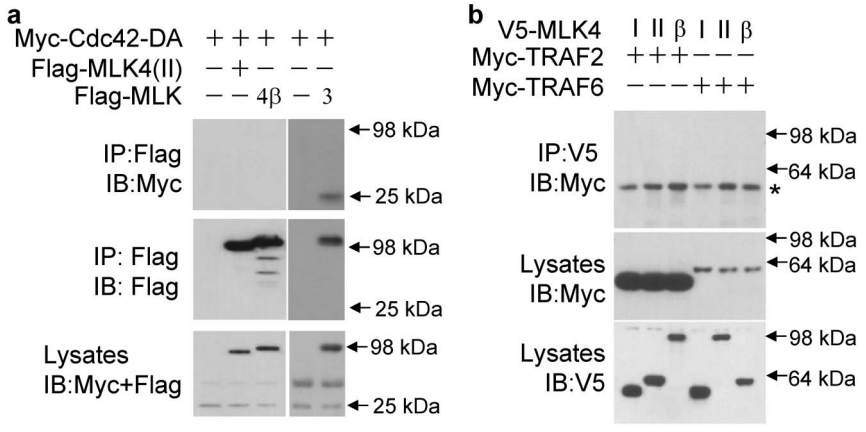

Figure 3 MLK4 does not interact with Cdc42, TRAF6 and TRAF2. (**a**) Co-immunoprecipitation assay of 293T cells transfected with plasmids expressing Flag-tagged MLK4β, Flag-MLK4 mutant II or Myc-Cdc42 dominant active mutant (Myc-Cdc42-DA) in different combinations as indicated. Flag-MLK3 was included as positive control. Cell lysates were immunoprecipitated with anti-Flag. The cell lysates and immunoprecipitates were analyzed by immunoblotting with antibodies as indicated. (**b**) Co-immunoprecipitation assay of 293T cells transfected with plasmids expressing V5-tagged MLK4β, V5-MLK4 mutant I or II, Myc-TRAF6 or TRAF2 as indicated. Cell lysates were immunoprecipitated with anti-V5. The cell lysates and immunoprecipitates were analyzed by immunoblotting with antibodies as indicated. Data are representative of two to three independent experiments. IB, immunoblotting; IP, immunoprecipitation; MLK, mixed-lineage kinase; TRAF, TNF receptor-associated factor 6.

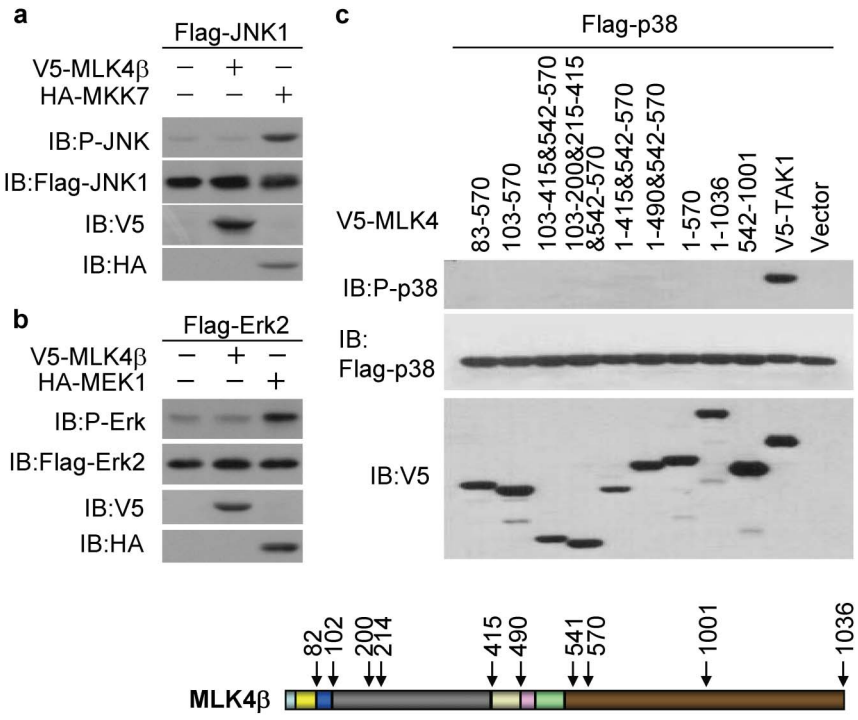

Figure 4 MLK4 cannot function as a MAP3K for ERK, JNK and p38. (**a**) 293T cells were transfected with an expressing vector of Flag-JNK1 with or without an expression vector of V5-MLK4β or HA-MKK7 for 24 h. The cell lysates were immunoblotted with antibodies as indicated. (**b**) 293T cells were transfected with an expressing vector of Flag-ERK2 with or without an expression vector of V5-MLK4β or HA-MEK1 for 24 h. The cell lysates were immunoblotted with antibodies as indicated. (**c**) 293T cells were transfected with an expressing vector of Flag-p38 with an expression vector of V5-MLK4β or its deletion mutants as indicated. V5-TAK1 was included as a positive control. The cell lysates were immunoblotted with antibodies as indicated. Data are representative of two independent experiments. ERK, extracellular signal-regulated kinase; IB, immunoblotting; IP, immunoprecipitation; JNK, c-Jun N-terminal kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MLK, mixed-lineage kinase.

role of MLK4 in JNK and ERK activation. To confirm the negative regulation of JNK and ERK by MLK4, we overexpressed MLK4α in RAW264.7 cells and examined JNK and ERK phosphorylation. As shown in Figure 5b, MLK4 overexpression significantly reduced the level of ERK phosphorylation in LPS-treated cells, and slightly affected the level of LPS-induced phosphorylation of p38 and JNK. LPS-induced degradation of IκB was not influenced by MLK4 overexpression. Collectively, these data suggest an inhibitory role of MLK4 in LPS/TLR4-mediated signaling.

To further evaluate the negative effect of MLK4 on TLR4 signaling in LPS-induced cellular activation, we measured LPS-induced TNF production in cells with reduced or increased MLK4 expression. LPS induced more TNF production in MLK4 knockdown cells in comparison with Si-GFP or Si-RNAscra (containing a scramble sequence) expressing RAW264.7 cells (Figure 6a). Consistently, LPS induced less TNF production in MLK4-overexpressing RAW264.7 cells in comparison with cells transfected with an empty vector. These data support the notion that MLK4 negatively regulates TLR4 signaling.

DISCUSSION

Of the MLK group kinases, MLK2 (also named MKN28-derived kinase) and MLK3 (also named SH3 domain-containing proline-rich kinase) were previously shown to be able to activate JNK and p38.[16,18,21,22,29,34] We show here that MLK4 does not activate p38 and JNK, demonstrating the distinct functions of various MLK group kinases. The finding of interaction between MLK4 and TLR4 suggests a role of MLK4 in inflammatory responses. The effect of knockdown or overexpression of MLK4 on LPS-induced TNF production indicates the negative role of MLK4 in TLR4 signaling.

The negative regulation of LPS-induced TNF production by MLK4 is at least in part due to the inhibitory effect of MLK4 on TLR4-mediated ERK and JNK activation. Knockdown of MLK4 enhanced LPS-induced activation of JNK and ERK (Figure 5a), while overexpression only reduced LPS-induced ERK activation (Figure 5b), suggesting that the effects of MLK4 on JNK and ERK activation are not all the same. Similarly, knockdown of MLK4 has no effect of p38 activation, while MLK4 overexpression reduced LPS-induced p38 phosphorylation (Figure 5a and b), suggesting that the effect of MLK4 level on p38 phosphorylation is not in a linear fashion. Since MLK3 can activate JNK, the inhibition of JNK by MLK4 could be due to the effect of competition among MLKs. Since MLK4 interacts with TLR4, the inhibitory effect of MLK4 on TLR4 signaling could result from directly modification of TLR4 by phosphorylation or other means. It is also possible that MLK4's effect on LPS-induced TNF production is an indirect effect resulting from the cellular changes caused by MLK knockdown and overexpression. Since MLK4 cannot activate the JNK and p38 pathways, its function should include more than affecting the activity of MAPKs.

The sequence difference between MLK3 and MLK4 is primarily located in the C-terminal domain. However, deletion of the C-terminal domain did not enable MLK4 to activate p38 (Figure 4c), suggesting that the other structural feature(s) is required for MLK to function as MAP3K. Although MLK4 and MLK3 share 75% sequence identity in their kinase domain, it is still possible that the 25%

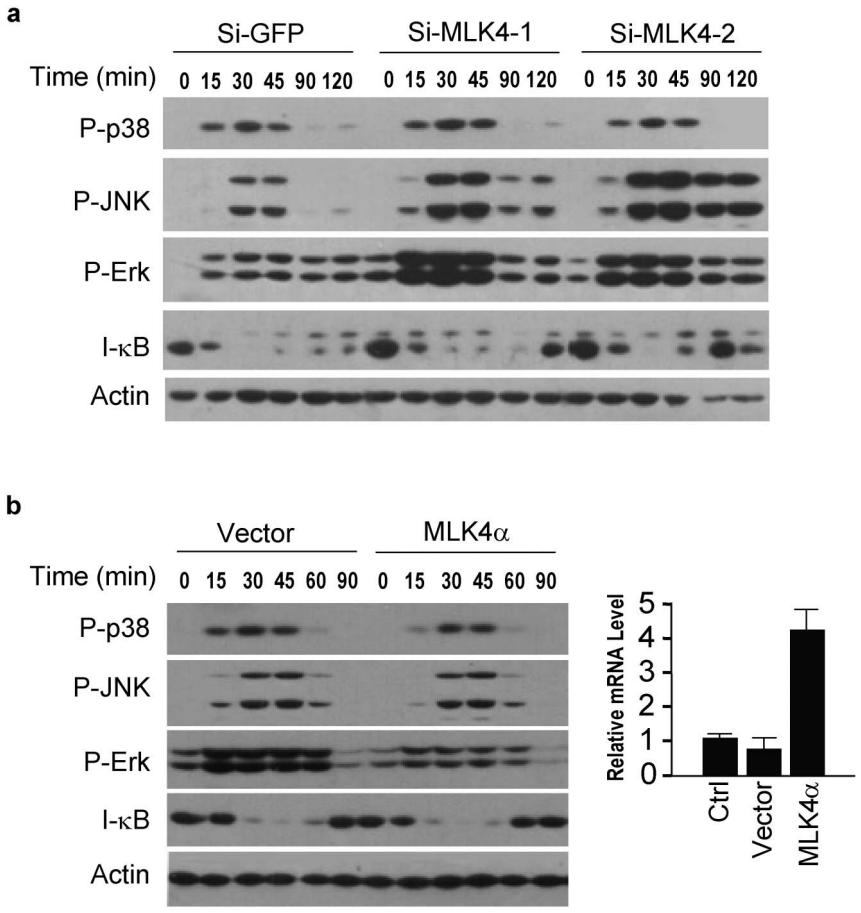

Figure 5 MLK4 negatively regulates LPS-induced JNK activation. (**a**) The RAW264.7 lines stably expressing siRNA-targeting GFP (Si-GFP) or MLK4 (Si-MLK4-1 or Si-MLK4-2) were treated with LPS (100 ng/ml) for different times as indicated. The cell lysates were immunoblotted with antibodies as indicated. (**b**) The RAW264.7 lines stably transfected with empty vector or MLK4α-expressing vector were treated with LPS (100 ng/ml) for different times, as indicated. The cell lysates were immunoblotted with antibodies as indicated. Data are representative of two independent experiments. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MLK, mixed-lineage kinase; siRNA, small interfering RNA.

difference prevented MLK4 from activating p38. It seems that the CRIB domain alone is insufficient to mediate an interaction between MLK and Cdc42, suggesting that another region or domain in MLK3 is involved in the interaction with Cdc42. The difference between MLK3 and MLK4 in interacting with Cdc42 provides more evidence of the distinct regulation between these MLKs.

The engagement of LPS with the TLR4 receptor complex leads to activation of a number of intracellular signaling pathways and results

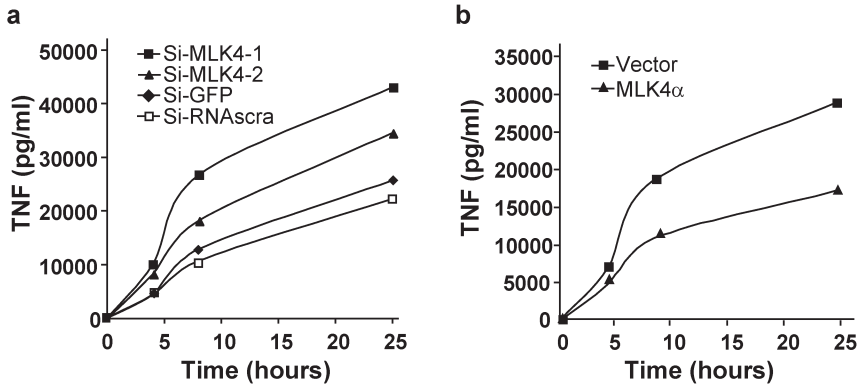

Figure 6 MLK4 negatively regulates LPS-induced TNF production. (**a**) The RAW264.7 lines stably expressing scramble siRNA, siRNA-targeting GFP (Si-GFP) or MLK4 (Si-MLK4-1 or Si-MLK4-2) were treated with LPS (100 ng/ml) for different times as indicated. TNF concentrations in the medium were measured. (**b**) The RAW264.7 lines stably transfected with empty vector or MLK4α-expressing vector were treated with LPS (100 ng/ml) for different times as indicated. TNF concentrations in the medium were measured. The expression levels of MLK4 were measured by real-time PCR and shown as right panel. LPS, lipopolysaccharide; MLK, mixed-lineage kinase; PCR, polymerase chain reaction; siRNA, small interfering RNA; TNF, tumor-necrosis factor.

in inflammatory cytokine production and other cellular responses. Inactivation of these intracellular pathways is essential for termination of inflammation. The negative effect of MLK4 on LPS-induced ERK and JNK activation could be very important in controlling the level of cytokine expression, and thus maintaining the balance of inflammatory responses.

ACKNOWLEDGEMENTS

This work was supported by grants from NSF China (30830092, 30921005, 91029304 and 81061160512), 973 Program (2009CB522200) and National Institutes of Health (AI41637 and AI68896).

1 Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002; **20**: 197–216.
2 Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; **4**: 499–511.
3 Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H *et al*. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003; **301**: 640–643.
4 Biswas SK, Bist P, Dhillon MK, Kajiji T, del Fresno C, Yamamoto M *et al*. Role for MyD88-independent, TRIF pathway in lipid A/TLR4-induced endotoxin tolerance. *J Immunol* 2007; **179**: 4083–4092.
5 Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO *et al*. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 2003; **424**: 743–748.
6 Beutler B. Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* 2004; **430**: 257–263.
7 Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signaling. *Immunol Rev* 2006; **210**: 171–186.
8 Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr Opin Cell Biol* 2007; **19**: 142–149.
9 Cobb MH, Hepler JE, Cheng M, Robbins D. The mitogen-activated protein kinases, ERK1 and ERK2. *Semin Cancer Biol* 1994; **5**: 261–268.
10 Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res* 2005; **15**: 11–18.
11 Cook R, Wu CC, Kang YJ, Han J. The role of the p38 pathway in adaptive immunity. *Cell Mol Immunol* 2007; **4**: 253–259.
12 Han J, Ulevitch RJ. Limiting inflammatory responses during activation of innate immunity. *Nat Immunol* 2005; **6**: 1198–1205.
13 Gallo KA, Johnson GL. Mixed-lineage kinase control of JNK and p38 MAPK pathways. *Nat Rev Mol Cell Biol* 2002; **3**: 663–672.
14 Wang LH, Besirli CG, Johnson EM Jr. Mixed-lineage kinases: a target for the prevention of neurodegeneration. *Annu Rev Pharmacol Toxicol* 2004; **44**: 451–474.
15 Sathyanarayana P, Barthwal MK, Kundu CN, Lane ME, Bergmann A, Tzivion G *et al*. Activation of the *Drosophila* MLK by ceramide reveals TNF-alpha and ceramide as agonists of mammalian MLK3. *Mol Cell* 2002; **10**: 1527–1533.
16 Rana A, Gallo K, Godowski P, Hirai S, Ohno S, Zon L *et al*. The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator, SEK-1. *J Biol Chem* 1996; **271**: 19025–19028.
17 Brancho D, Ventura JJ, Jaeschke A, Doran B, Flavell RA, Davis RJ. Role of MLK3 in the regulation of mitogen-activated protein kinase signaling cascades. *Mol Cell Biol* 2005; **25**: 3670–3681.
18 Tibbles LA, Ing YL, Kiefer F, Chan J, Iscove N, Woodgett JR *et al*. MLK-3 activates the SAPK/JNK and p38/RK pathways *via* SEK1 and MKK3/6. *EMBO J* 1996; **15**: 7026–7035.
19 Kim KY, Kim BC, Xu Z, Kim SJ. Mixed lineage kinase 3 (MLK3)-activated p38 MAP kinase mediates transforming growth factor-beta-induced apoptosis in hepatoma cells. *J Biol Chem* 2004; **279**: 29478–29484.
20 Vacratsis PO, Gallo KA. Zipper-mediated oligomerization of the mixed lineage kinase SPRK/MLK-3 is not required for its activation by the GTPase CDC 42 but is necessary for its activation of the JNK pathway. Monomeric SPRK L410P does not catalyze the activating phosphorylation of Thr258 of murine mitogen-activated protein kinase kinase 4. *J Biol Chem* 2000; **275**: 27893–27900.
21 Xu Z, Maroney AC, Dobrzanski P, Kukekov NV, Greene LA. The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. *Mol Cell Biol* 2001; **21**: 4713–4724.
22 Mota M, Reeder M, Chernoff J, Bazenet CE. Evidence for a role of mixed lineage kinases in neuronal apoptosis. *J Neurosci* 2001; **21**: 4949–4957.
23 Rangasamy V, Mishra R, Mehrotra S, Sondarva G, Ray RS, Rao A *et al*. Estrogen suppresses MLK3-mediated apoptosis sensitivity in ER^+ breast cancer cells. *Cancer Res* 2010; **70**: 1731–1740.
24 Saporito MS, Brown EM, Miller MS, Carswell S. CEP-1347/KT-7515, an inhibitor of c-jun N-terminal kinase activation, attenuates the 1-methyl-4-phenyl tetrahydropyridine-mediated loss of nigrostriatal dopaminergic neurons *in vivo*. *J Pharmacol Exp Ther* 1999; **288**: 421–427.
25 Mishra R, Barthwal MK, Sondarva G, Rana B, Wong L, Chatterjee M *et al*. Glycogen synthase kinase-3beta induces neuronal cell death *via* direct phosphorylation of mixed lineage kinase 3. *J Biol Chem* 2007; **282**: 30393–30405.
26 Kang YJ, Kim SO, Shimada S, Otsuka M, Seit-Nebi A, Kwon BS *et al*. Cell surface 4-1BBL mediates sequential signaling pathways 'downstream' of TLR and is required for sustained TNF production in macrophages. *Nat Immunol* 2007; **8**: 601–609.
27 Han J, Lee JD, Jiang Y, Li Z, Feng L, Ulevitch RJ. Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J Biol Chem* 1996; **271**: 2886–2891.
28 Han J, Jiang Y, Li Z, Kravchenko VV, Ulevitch RJ. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature* 1997; **386**: 296–299.
29 Teramoto H, Coso OA, Miyata H, Igishi T, Miki T, Gutkind JS. Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. *J Biol Chem* 1996; **271**: 27225–27228.
30 Bock BC, Vacratsis PO, Qamirani E, Gallo KA. Cdc42-induced activation of the mixed-lineage kinase SPRK *in vivo*. Requirement of the Cdc42/Rac interactive binding motif and changes in phosphorylation. *J Biol Chem* 2000; **275**: 14231–14241.
31 Burbelo PD, Drechsel D, Hall A. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J Biol Chem* 1995; **270**: 29071–29074.
32 Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A *et al*. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* 1999; **13**: 1015–1024.
33 Deng L, Wang C, Spencer E, Yang L, Braun A, You J *et al*. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 2000; **103**: 351–361.
34 Nagata K, Puls A, Futter C, Aspenstrom P, Schaefer E, Nakata T *et al*. The MAP kinase kinase kinase MLK2 co-localizes with activated JNK along microtubules and associates with kinesin superfamily motor KIF3. *Embo J* 1998; **17**: 149–158.