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ORIGINAL ARTICLE

TRAF6-mediated ubiquitination of MST1/STK4 attenuates the TLR4-NF-κB signaling pathway in macrophages

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Abstract

Pattern-recognition receptors including Toll-like receptors (TLRs) recognize invading pathogens and trigger an immune response in mammals. Here we show that mammalian ste20-like kinase 1/serine/threonine kinase 4 (MST1/STK4) functions as a negative regulator of lipopolysaccharide (LPS)-induced activation of the TLR4-NF-κB signaling pathway associated with inflammation. Myeloid-specific genetic ablation of MST1/STK4 increased the susceptibility of mice to LPS-induced septic shock. Ablation of MST1/STK4 also enhanced NF-κB activation triggered by LPS in bone marrow-derived macrophages (BMDMs), leading to increased production of proinflammatory cytokines by these cells. Furthermore, MST1/STK4 inhibited TRAF6 autoubiquitination as well as TRAF6-mediated downstream signaling induced by LPS. In addition, we found that TRAF6 mediates the LPS-induced activation of MST1/STK4 by catalyzing its ubiquitination, resulting in negative feedback regulation by MST1/STK4 of the LPS-induced pathway leading to cytokine production in macrophages. Together, our findings suggest that MST1/STK4 functions as a negative modulator of the LPS-induced NF-κB signaling pathway during macrophage activation.

Keywords Lipopolysaccharides · MST1/STK4 · NF-κB · TRAF6

Introduction

Macrophages play a critical role in the innate immune response of mammals to bacterial infection [1–3]. Lipopolysaccharide (LPS) derived from the cell wall of Gram-negative bacteria induces macrophage activation after its recognition by Toll-like receptor 4 (TLR4) expressed on the cell surface [4–7]. The activated cells produce proinflammatory cytokines that contribute to the immune response to infection [8]. An excessive immune response, however, can lead to septic shock or even death of the host [9].

The binding of LPS to TLR4 induces the recruitment of adaptor proteins including myeloid differentiation primary response gene-88 (MyD88), interleukin (IL)-1 receptor-associated kinases (IRAKs), and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) [10–12]. TRAF6, an E3 ubiquitin ligase that catalyzes the formation of Lys^{63}-linked polyubiquitin chains on itself and other substrates, mediates the activation of transforming growth factor-β-activated kinase 1 (TAK1) and IκB kinase (IKK) [13, 14]. IKK is an enzyme complex that consists of two catalytic subunits, IKKα and IKKβ, as well as the

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regulatory subunit IKKγ (also known as NEMO). The IKK complex phosphorylates inhibitor of nuclear factor (NF)-κB (IκB), which results in IκB ubiquitination and consequent proteasomal degradation. The degradation of IκB releases NF-κB from an inactive complex with its inhibitor in the cytosol and promotes nuclear translocation of NF-κB, which is essential for the expression of the proinflammatory cytokines [15, 16].

Mammalian Ste20-like kinase 1/serine/threonine kinase 4 (MST1/STK4) belongs to the family of germinal center kinases [17–19] and plays a role in a variety of biological events including morphogenesis, cell proliferation, stress response, and apoptosis [20, 21]. Emerging evidence also suggests that MST1/STK4 modulates the immune response and inflammation [22–27]. In particular, MST1/STK4 was shown to regulate various inflammation-related events by targeting several components of the NF-κB signaling pathway including IRAK1 and IκB [24, 25]. To provide insight into the mechanism by which MST1/STK4 modulates inflammation, we have now examined its possible role in the TLR4-dependent activation of NF-κB signaling in macrophages. We found that genetic ablation of MST1/STK4 enhanced LPS-induced lethal sepsis in mice in association with increased production of proinflammatory cytokines. Furthermore, exposure of macrophages to LPS triggered the TRAF6-mediated polyubiquitination and stimulation of MST1/STK4. MST1/STK4, in turn, inhibited LPS-induced NF-κB signaling events through suppression of TRAF6 activity. Our findings thus uncover a previously unrecognized mechanism by which MST1/STK4 negatively regulates LPS-induced cytokine production in macrophages.

Materials and methods

Animals

MST1/STK4$^{-/-}$ [28] and *p62/sequestosome 1 (SQSTM1)*$^{-/-}$ C57BL/6 mice [29] were described previously. Myeloid cell-specific MST1/STK4-deficient mice were generated by crossing mice harboring floxed alleles of the MST1/STK4 gene (*MST1*$^{fl/fl}$ mice) with *LysM-Cre* mice, which harbor a Cre transgene under the control of the LysM (lysozyme M, also known as lysozyme 2) gene promoter (The Jackson Laboratory, Bar Harbor, ME). *MST1*$^{fl/fl}$ mice were kindly provided by T. Kinashi (Kansai Medical University) [30] and were used as controls in experiments with *MST1*$^{fl/fl}$*;LysM-Cre* mice. All mice were maintained at the Korea University Laboratory Animal Center, and all animal procedures were approved by the Institutional Animal Care and Use Committee of Korea University.

LPS shock model

Eight- to twelve-week-old *MST1*$^{fl/fl}$ and *MST1*$^{fl/fl}$*; LysM-Cre* mice were injected intraperitoneally with LPS (50 mg/kg) or saline vehicle, and then monitored for survival. Serum and lung tissue were collected at 12 h after LPS injection for measurement of cytokine levels and for histological analysis, respectively. For histological analysis, the lung tissue specimens were fixed in 3.7% neutral-buffered formalin, dehydrated in increasing concentrations of isopropyl alcohol, embedded in paraffin, sectioned as a thickness of 3 μm, and stained with Hematoxylin–Eosin.

Flow cytometric analysis

Single-cell suspensions isolated from lymph nodes and the spleen of mice were incubated for 15 min at room temperature with phycoerythrin-conjugated antibodies to CD11b, fluorescein isothiocyanate-conjugated antibodies to CD11c, or allophycocyanin-conjugated antibodies to F4/80 in phosphate-buffered saline containing 0.5% FBS and were then analyzed with a BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell culture and transfection

Mouse embryonic fibroblasts (MEFs) were prepared from WT and *p62/SQSTM1*$^{-/-}$ mice as described previously [29]. MEFs and HEK293 cells were cultured under a humidified atmosphere of 5% CO_2 at 37 °C in DMEM supplemented with 10% FBS. Bone marrow-derived macrophages (BMDMs) were isolated from the hind leg bones of 8- to 12-week old C57BL/6 mice, plated in 100-mm culture dishes (5×10^6 cells per dish), and maintained for 6 days in DMEM supplemented with recombinant murine M-CSF (Peprotech, Seoul, Korea) at 25 ng/ml and 10% FBS, after which nonadherent cells were removed and adherent cells were harvested for experiments. For DNA transfection, BMDMs were plated in 6-well plates (1×10^6 cells per well) and then exposed to expression vectors in the presence of jet-PEI reagent (Polyplus-transfection, Illkirch, France). HEK293 cells were transfected with indicated plasmid vectors with the use of polyethylenimine (Sigma, St. Louis, MO, USA).

Plasmids

A pME18S vector for Flag-tagged mouse MST1/STK4 was kindly provided by S. Yonehara (Kyoto University, Japan). A pET-23b/hexahistidine (His_6)-MST1(K59R) vector was described previously [31], and a pcDNA3.1-HisA/

Xpress-Ub vector and a pRK5-HA-ubiquitin(WT) vector were provided by S. Kang (Korea University, Korea). For generation of a plasmid encoding Myc epitope-tagged TRAF6, the mouse TRAF6 cDNA was amplified by PCR from a pRK5 vector (kindly provided by D. Goeddel of Tularik Inc.) and subcloned into the HinIII and EcoRI sites of the pcDNA3.1/Myc-His A vector. To generation of a vector for MBP-tagged TRAF6, the mouse TRAF6 cDNA was amplified by PCR from a pCMV5 vector (Addgene) and subcloned into the SspI and BamHI sites of the pET His6 MBP TEV LIC cloning vector (pET/MBP, Addgene). For generation of expression vectors for HA-tagged p62/SQSTM1, the human p62/SQSTM1 cDNA was amplified by PCR from a pOTB7 vector and subcloned into the EcoRI and XhoI sites of pcDNA3. The pME28S/Flag-MST1(K59R), pME28S/Flag-MST1(K221R), pET-23b/His-MST1(K59R/K221R), Myc-TRAF6(C70A), and pRK5-HA-Ub (K63R) vectors for mutant constructs were prepared with a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

Antibodies and reagents

Rabbit polyclonal antibodies to MST1/STK4 and to TAK1 as well as rabbit monoclonal antibodies to phospho-MST1/STK4(Thr^{183}), phospho-IKKα/β (Ser$^{176/180}$), to phospho-TAK1 (Thr$^{184/187}$), to phospho-p65 (Ser^{536}), to phospho-IκBα Ser$^{32/36}$, to phospho-IRAK4 (Thr^{345}/Ser^{346}), to IRAK4, to IRAK1, to IKKβ, and to TRAF6 were obtained from Cell Signaling Technology (Boston, MA). Rabbit monoclonal antibodies to p62/SQSTM1 and to TRAF6 as well as a mouse monoclonal antibody to p62/SQSTM1 were from Abcam (Cambridge, UK). A mouse monoclonal antibody to the Flag epitope and rabbit polyclonal antibody to phospho-IRAK1 (Thr^{209}) were from Sigma. A mouse monoclonal antibody to ubiquitin was from Millipore (Billerica, MA, USA). Rabbit polyclonal antibody to IκBα and mouse monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody to Xpress was purchased from Invitrogen (Carlsbad, CA, USA). Human antibody to K63-linked polyubiquitin chains was kindly provided by V. M. Dixit (Genentech, South San Francisco, CA, USA). A phycoerythrin-conjugated rat monoclonal antibody to CD11b, allophycocyanin-conjugated rat monoclonal antibody to F4/80 were obtained from eBioscience (San Diego, CA, USA), and fluorescein isothiocyanate-conjugated Armenian hamster monoclonal antibody to CD11c was obtained from BD Biosciences. Rabbit polyclonal antibody to p62/SQSTM1 was generated with a synthetic peptide (NH_2-SSLDPSQEGPTGLK-OH) as immunogen. Rabbit polyclonal antibody to MST1/STK4 was generated with a recombinant glutathione S-transferase (GST) fusion protein containing amino acids 276 to 487 of the human protein as immunogen. LPS of *Escherichia coli* O55:B5 was obtained from Sigma.

Cytokine measurement

Culture supernatants or mouse serum were assayed for IL-6 and TNF-α with the use of ELISA kits (BioLegend, San Diego, CA, USA).

RT and real-time PCR analysis

Total RNA was extracted from BMDMs with the use of Trizol reagent (Invitrogen). The RNA (1 μg) was subjected to RT with an oligo(dT) primer, and the resulting cDNA was subjected to real-time PCR analysis with primers (each at 250 nM) and SYBR Green Supermix (Thermo Fisher Scientific, Waltham, MA, USA) in an iQ5 thermocycler (Bio-Rad, Hercules, CA, USA). Each reaction was performed in triplicate, and data were analyzed with iQ5 optical system software (Bio-Rad). The amount of each target mRNA was normalized by that of GAPDH mRNA as an internal control. The sequences of the primers (forward and reverse, respectively) are as follows: IL-6, 5′-TCTAATTCATATCTTCAACCAAGAGG-3′ and 5′- TGGTCCTTAGCCACTCCTTC-3′; TNFα, 5′-TCTTCTCATTCCTGCTTGTGG-3′ and 5′-GGTCTGGGCCATAGAACTGA-3′; GAPDH, 5′-CGTGCGCCTGGAGAAACC-3′ and 5′-TGGAAGAGTGGGAGTTGCTGTTG-3′.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot analysis were performed as previously described [32].

Immune complex kinase assay

Immune complex kinase assays were performed, as described previously [33, 34], with the use of myelin basic protein (Millipore) or other indicated proteins as substrate.

Ubiquitination assay

For cell-based assay of ubiquitination in BMDMs, the cells were lysed with buffer A [20 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 12 mM *β*-glycerophosphate, 5 mM EGTA, 10 mM NaF, 5 mM N-ethylmaleimide]. The lysates were incubated for 10 min at 50 °C and then subjected to immunoprecipitation with antibodies to TRAF6 or to MST1/STK4. The resulting precipitates were examined by immunoblot analysis with indicated antibodies to ubiquitin.

For in vitro assay of TRAF6-mediated K63-linked polyubiquitin chain formation, recombinant MBP-TRAF6 (3 μg)

was incubated for 1 h at 37 °C in 20 µl of reaction solution A [5 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM ATP, 50 mM Tris–HCl (pH 7.6)] containing 4 µg of ubiquitin (Sigma), 50 ng of E1 (Boston Biochem, Cambridge, MA, USA), 200 ng of GST-Ubc13, and 200 ng of GST-Uev1a. The reaction mixtures were then subjected to SDS-PAGE and immunoblot analysis with antibody to K63-linked polyubiquitin chains.

For in vitro assay of TRAF6-mediated MST1/STK4 ubiquitination, Myc-TRAF6 immunoprecipitates were prepared with anti-Myc antibody from HEK293 cells overexpressing Myc-TRAF6. Bacterially expressed and purified His_6-tagged MST1/STK4 proteins [MST1(K59R) and MST1(K59R/K221R)] (5 µg) were incubated for 1 h at 37 °C with Myc-TRAF6 immunoprecipitates in 30 µl of reaction solution A containing 2 µg of ubiquitin (Sigma), 50 ng of E1 (Boston Biochem), 200 ng of GST-Ubc13, and 200 ng of GST-Uev1a. The reaction mixtures were centrifuged at 12,000 × g for 1 min, the resulting supernatants were immunoprecipitated with antibody to ubiquitin, and the resulting precipitates were examined by immunoblot analysis with antibody to MST1/STK4.

RNA interference

The siRNA oligonucleotides for green fluorescent protein (control) as well as p62/SQSTM1 and TRAF6 were synthesized by Invitrogen. The control siRNA was targeted to the sequences 5′-ATG ACT GTC AGG ATG TTG C-3′, whereas the p62/SQSTM1, and TRAF6 siRNAs were targeted to the sequences 5′-GCT GAA ACA TGG ACA CTT TGG-3′ and 5′-CCC AGG CTG TTC ATA ATG TTA-3′, respectively. BMDMs were transfected with the siRNA duplexes with the use of the Lipofectamine RNAiMAX (Invitrogen).

Statistical analysis

Quantitative data are presented as means ± SD and were analyzed with Student's *t* test as performed with Excel 2010 software (Microsoft, Redmond, WA, USA). Survival data were analyzed by the log-rank test with the use of GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). A p value of < 0.05 was considered statistically significant.

Result

Myeloid-specific genetic ablation of MST1/STK4 enhances the susceptibility of mice to LPS-induced sepsis

To investigate the role of MST1/STK4 in the macrophage-mediated immune response to LPS, we established myeloid cell-specific MST1/STK4-knockout (*MST1*$^{fl/fl}$*;LysM-Cre*, or *MST1/STK4* cKO) mice by crossing *MST1*$^{fl/fl}$ mice with *LysM-Cre* transgenic mice. We confirmed MST1/STK4 deficiency in BMDMs isolated from *MST1/STK4* cKO mice (Fig. S1A). *MST1/STK4* cKO mice were born in a ratio consistent with Mendelian inheritance and appeared normal. They showed no significant differences in the numbers of F4/80$^+$CD11b$^+$ macrophages and CD11b$^+$CD11c$^+$ dendritic cells in the spleen or lymph nodes relative to their *MST1*$^{fl/fl}$ control littermates (Fig. S1B). *MST1/STK4* cKO mice and control littermates were injected intraperitoneally (i.p.) with LPS (50 mg/kg) and was monitored for mortality. The survival rate of the *MST1/STK4* cKO mice was significantly lower than that of the control littermates (Fig. 1a). Furthermore, the *MST1/STK4* cKO mice showed higher concentrations of the proinflammatory cytokines IL-6 and TNF-α in serum (Fig. 1b) as well as an increased extent of inflammatory damage (Fig. 1c) and increased expression of intercellular adhesion molecule-1 (ICAM-1) (Fig. 1d) in lung tissue compared with control littermates. These results thus suggested that myeloid-specific ablation of MST1/STK4 enhanced the susceptibility of mice to LPS-induced septic shock.

MST1/STK4 negatively regulates LPS-induced NF-κB signaling in BMDMs

Given that the TLR4-NF-κB signaling pathway plays a pivotal role in the LPS-induced immune response [6, 9], we next examined whether MST1/STK4 might modulate the LPS-induced activation of this pathway in BMDMs. The extent of the LPS-induced expression of NF-κB target genes including those for IL-6 and TNF-α was greater in BMDMs from MST1/STK4 knockout (*MST1/STK4*$^{-/-}$) mice than in those from wild-type (WT) mice (Fig. 2a). Consistent with this finding, the extent of LPS-induced secretion of IL-6 and TNF-α from *MST1/STK4*$^{-/-}$ BMDMs was greater than that from WT cells (Fig. 2b). Furthermore, the effects of LPS on IκB degradation and on the accumulation of the Ser^{536}-phosphorylated form of the p65 subunit of NF-κB were more pronounced in *MST1/STK4*$^{-/-}$ BMDMs than in WT cells (Fig. 2c). The extents of the LPS-induced autoubiquitination of TRAF6 as well

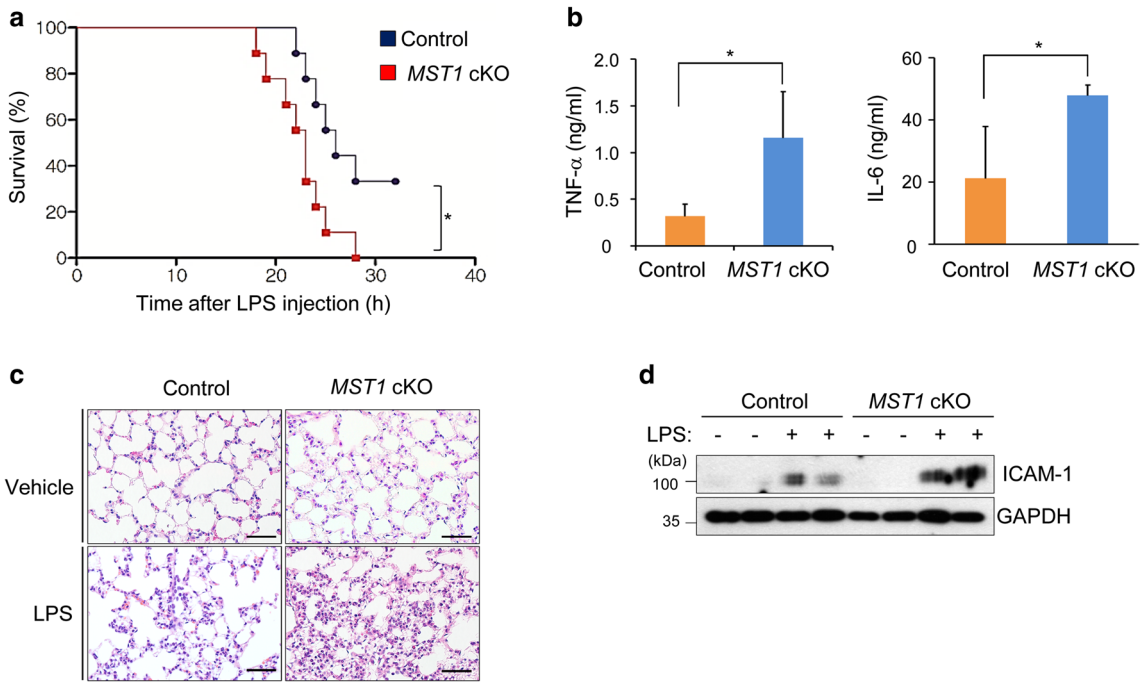


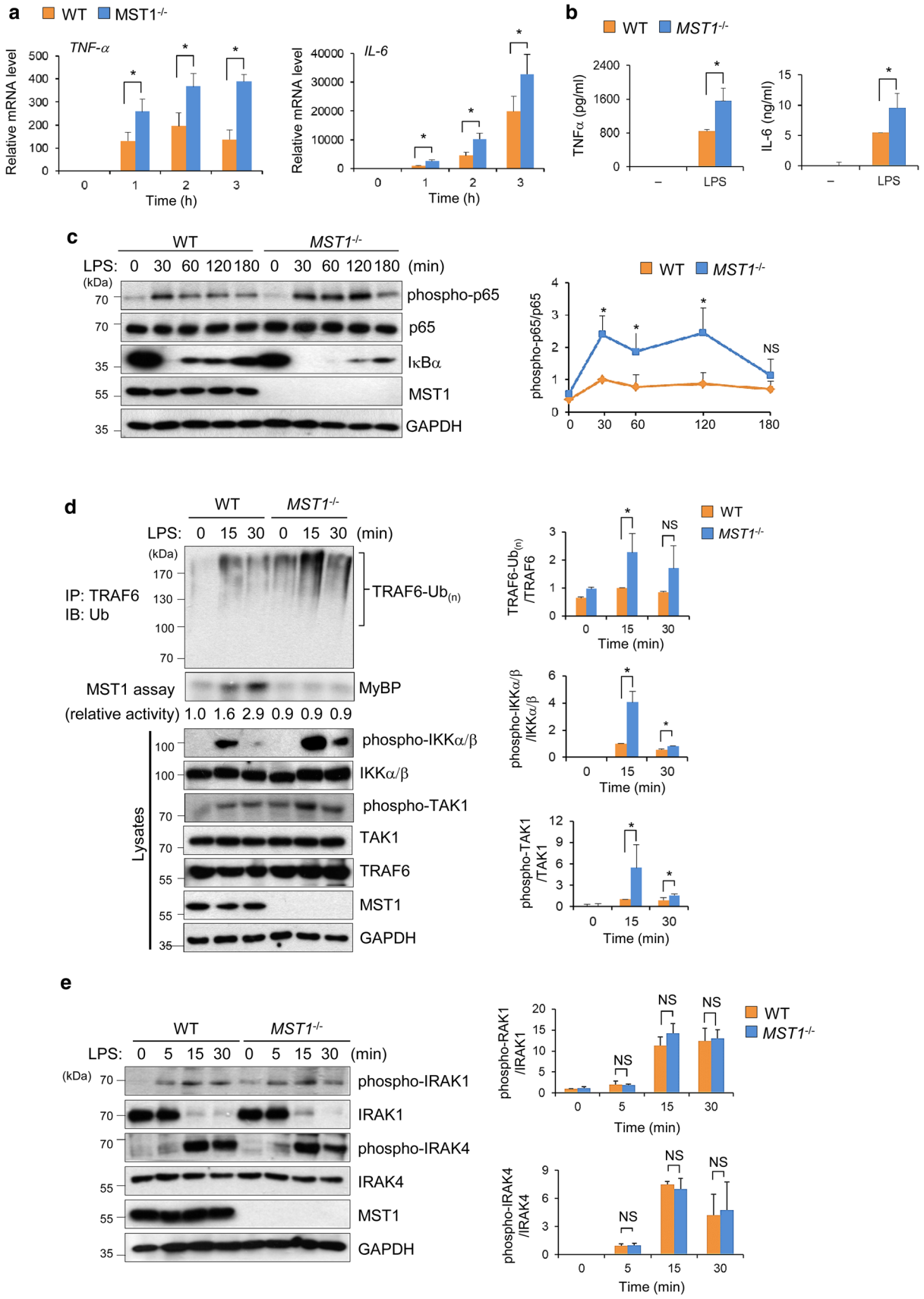
Fig. 1 Myeloid-specific genetic ablation of MST1/STK4 in mice increases susceptibility to LPS-induced sepsis. **a** *MST1/STK4* cKO mice and control (*MST1/STK4*$^{fl/fl}$) littermates ($n=9$ per group) were injected with LPS (50 mg/kg, i.p.) and then monitored every hour for survival. *$p<0.05$. **b** Serum concentrations of TNF-α and IL-6 in *MST1/STK4* cKO mice and control littermates at 12 h after LPS injection. Data are mean ± SD ($n=3$ per group). *$p<0.05$. **c** Representative hematoxylin–eosin staining of lung sections from *MST1/STK4* cKO mice and control littermates ($n=4$ per group) at 12 h after LPS injection. Scale bar, 50 μm. **d** Lung tissue isolated from *MST1/STK4* cKO mice and control littermates at 12 h after LPS injection was subjected to immunoblot analysis with antibodies to ICAM-1 and to GAPDH

as phosphorylation (activation) of TAK1 and IKKα/β were also greater in *MST1/STK4*$^{-/-}$ BMDMs than in WT cells (Fig. 2d). Together, these results suggested that MST1/STK4 negatively regulates the LPS-induced autoubiquitination of TRAF6 and downstream signaling events in the NF-κB pathway in BMDMs. In contrast, the LPS-induced phosphorylation (activation) of either IRAK1 or IRAK4 was not affected by ablation of MST1/STK4 in BMDMs (Fig. 2e).

Given that MST1/STK4 deficiency increased LPS-induced TRAF6 ubiquitination in BMDMs (Fig. 2d), we examined whether MST1/STK4 might directly inhibit the E3 ligase activity of TRAF6. An in vitro ubiquitination assay revealed that a constitutively active MST1/STK4 [MST1-CA, or MST1(1–326)] did not affect the ability of TRAF6 to catalyze formation of a K63-linked ubiquitin chain (Fig. S2A). In addition, in vitro phosphorylation analysis showed that MST1/STK4 immunoprecipitates prepared from LPS-treated BMDMs did not mediate the phosphorylation of TRAF6, whereas they did catalyze that of MOB1, a known substrate of MST1/STK4 (Fig. S2B), indicating that TRAF6 is not a substrate of MST1/STK4.

p62/SQSTM1 promotes TRAF6-mediated NF-κB signaling in BMDMs

Our finding that TRAF6 does not appear to be a direct target of MST1/STK4 (Fig. S2) prompted us to investigate further the mechanism by which MST1/STK4 attenuates the LPS-induced up-regulation of the E3 ligase activity of TRAF6 in BMDMs. The E3 ligase activity of TRAF6, which is critical for the LPS-induced activation of NF-κB signaling [13, 14, 35], has been shown to be modulated positively or negatively by various proteins [36]. In particular, the adaptor protein p62/SQSTM1 promotes the oligomerization and increases the E3 activity of TRAF6 [37, 38]. Indeed, the LPS-induced increase in K63-linked autoubiquitination of TRAF6 as well as that in IKK phosphorylation (activation) were both markedly attenuated in BMDMs derived from p62/SQSTM1 knockout (*p62/SQSTM1*$^{-/-}$) mice compared with those derived from WT mice (Fig. 3a). Furthermore, genetic ablation of p62/SQSTM1 abolished the LPS-induced association of TRAF6 with TAK1 and IKKβ in BMDMs (Fig. 3b), while it downregulates the LPS-induced expression of IL-6 and TNFα mRNA (Fig. 3c). Similar results were


a
WT
MST1-/-
TNF-α
Relative mRNA level
Time (h)
IL-6
b
WT
MST1-/-
TNFα (pg/ml)
IL-6 (ng/ml)
LPS
c
WT
MST1-/-
LPS: 0 30 60 120 180 0 30 60 120 180 (min)
(kDa)
phospho-p65
p65
IκBα
MST1
GAPDH
phospho-p65/p65
NS
d
LPS: 0 15 30 0 15 30 (min)
IP: TRAF6
IB: Ub
TRAF6-Ub(n)
MST1 assay
MyBP
(relative activity) 1.0 1.6 2.9 0.9 0.9 0.9
Lysates
phospho-IKKα/β
IKKα/β
phospho-TAK1
TAK1
TRAF6
MST1
GAPDH
TRAF6-Ub(n) /TRAF6
phospho-IKKα/β /IKKα/β
phospho-TAK1 /TAK1
Time (min)
e
LPS: 0 5 15 30 0 5 15 30 (min)
phospho-IRAK1
IRAK1
phospho-IRAK4
IRAK4
MST1
GAPDH
phospho-RAK1 /IRAK1
phospho-IRAK4 /IRAK4

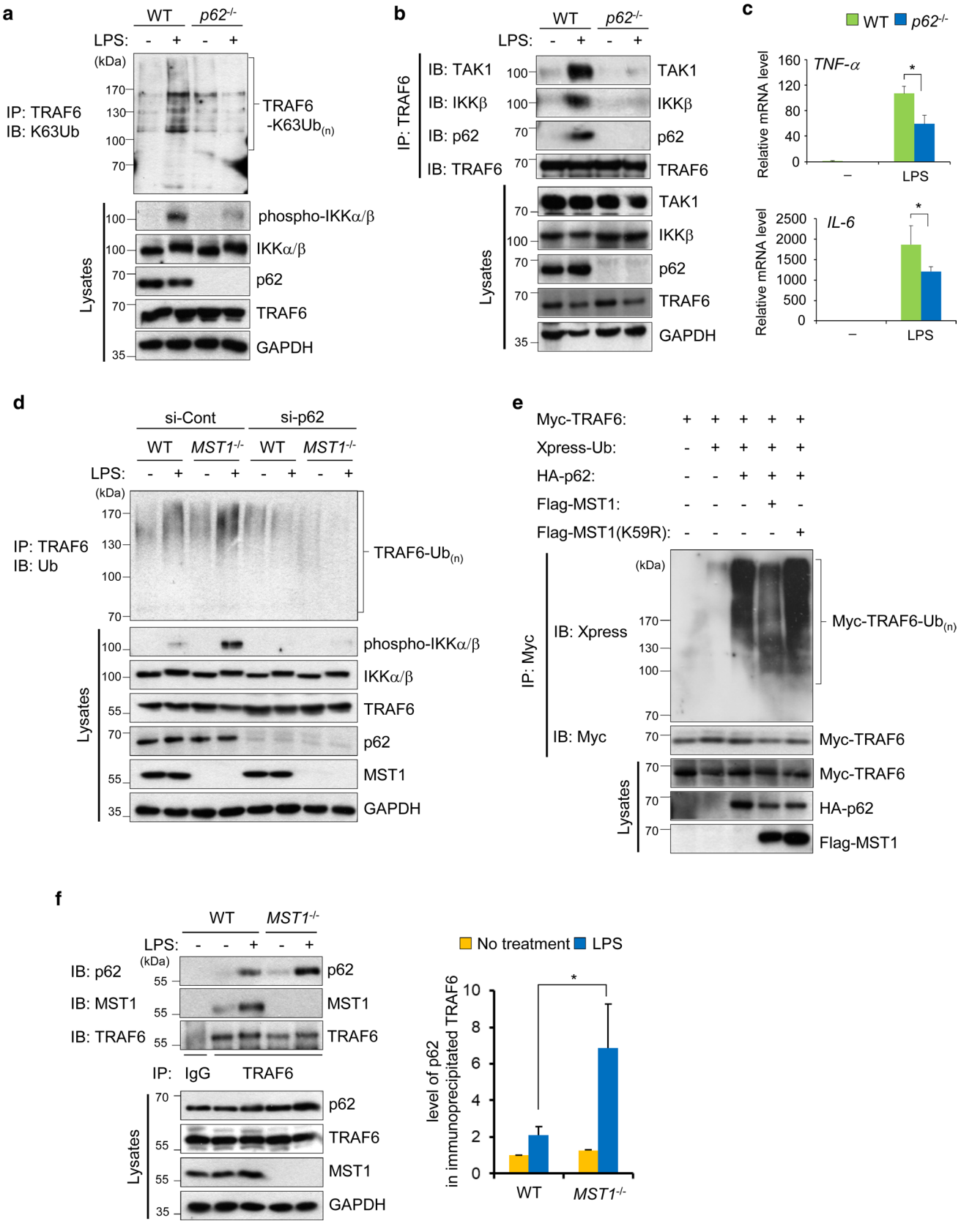
◂**Fig. 2** MST1/STK4 negatively regulates LPS-induced NF-κB signaling in BMDMs. **a** BMDMs isolated from WT and *MST1/STK4*$^{-/-}$ mice were exposed to LPS (1 μg/ml) for the indicated times in culture and then assayed for IL-6 and TNF-α mRNA by quantitative RT-PCR analysis. Data are representative of three independent experiments (means ± SD of triplicate samples). *$p < 0.05$. **b** BMDMs were incubated in the absence or presence of LPS (1 μg/ml) for 12 h, after which IL-6 and TNF-α released into culture media were quantified by ELISA. Data are means ± SD from three independent experiments. *$p < 0.05$. **c** BMDMs exposed to LPS (1 μg/ml) for the indicated times were subjected to immunoblot analysis with antibodies to phospho-p65 (Ser^{536}), to p65, to IκBα, to MST1/STK4, or to GAPDH. The intensity of bands corresponding to phospho-p65 was quantified by densitometry, and normalized by that of those for p65. The relative values of the intensity from five independent experiments were shown below. *$p < 0.05$. *NS* not significant. **d** BMDMs exposed to LPS (1 μg/ml) for the indicated times were immunoprecipitated with antibodies to TRAF6 or to MST1/STK4. The TRAF6 precipitates were examined by immunoblot analysis (IB) with anti-ubiquitin antibody, whereas the MST1/STK4 precipitates were subjected to an immune complex kinase assay with myelin basic protein (MyBP) as substrate. The relative values for MST1/STK4 activity are shown. Cell lysates were also immunoblotted directly with antibodies to phospho-IKKα/β (Ser$^{176/180}$), to IKKα/β, to phospho-TAK1 (Thr$^{184/187}$), to TAK1, to TRAF6, to MST1/STK4, or to GAPDH. The intensity of the bands for ubiquitinated TRAF6 [TRAF6-Ub$_{(n)}$], phospho-IKKα/β, or phospho-TAK1 normalized by that of those for the corresponding total proteins was quantified by densitometry, and the relative values of the intensity were shown. Quantitative data are mean ± SD from three independent experiments. *$p < 0.05$. *NS* not significant. **e** BMDMs exposed to LPS (1 μg/ml) for the indicated times were immunoblotted with antibodies to phospho-IRAK1 (Thr^{209}), to IRAK1, to phospho-IRAK4 (Thr^{345}/Ser^{346}), to IRAK4, to MST1/STK4, or to GAPDH. The intensity of the bands for phospho-IRAK1 or phospho-IRAK4 normalized by that of those for the corresponding total proteins was quantified by densitometry, and the relative values of the intensity were shown. Quantitative data are mean ± SD from three independent experiments. *NS* not significant

obtained with p62/SQSTM1$^{-/-}$ MEFs, with p62/SQSTM1 deficiency suppressing TRAF6 auto-ubiquitination (Fig. S3A), IKKα/β phosphorylation, and IκBα phosphorylation induced by LPS (Fig. S3B). These results thus confirmed that p62/SQSTM1 promotes the LPS-induced stimulation of TRAF6 E3 ligase activity and thereby facilitates TRAF6-mediated NF-κB signaling.

Given that MST1/STK4 negatively regulates the LPS-induced activation of TRAF6 and that p62/SQSTM1 promotes LPS-TRAF6-NF-κB signaling, we examined whether MST1/STK4 might affect the p62/SQSTM1-mediated stimulation of TRAF6 activity in BMDMs exposed to LPS. The enhancement of the LPS-induced ubiquitination of TRAF6 and phosphorylation (activation) of IKK apparent in *MST1/STK4*$^{-/-}$ BMDMs was abolished by siRNA-mediated depletion of p62/SQSTM1 (Fig. 3d), suggesting that MST1/STK4 inhibits the p62/SQSTM1-dependent promotion of the TRAF6 activity induced by LPS. Transfection study using HEK293 cells also revealed that p62/SQSTM1 enhanced the autoubiquitination of TRAF6 and that this enhancement was reduced by wild-type MST1/STK4, but not by a kinase-dead mutant of MST1/STK4, MST1(K59R) (Fig. 3e). Given that the interaction of p62/SQSTM1 with TRAF6 facilitates the activation of TRAF6 [37, 39], we examined the possible effect of MST1/STK4 on the LPS-induced formation of the TRAF6-p62/SQSTM1 complex in BMDMs. The LPS-induced binding of p62/SQSTM1 to TRAF6 was more pronounced in *MST1/STK4*$^{-/-}$ BMDMs than in WT cells (Fig. 3f). Together, these results suggested that inhibition of p62/SQSTM1-mediated TRAF6 activation contributes to the attenuation of LPS-induced TRAF6 activation by MST1/STK4 in BMDMs.

TRAF6 mediates the LPS-induced K63-polyubiquitination and activation of MST1/STK4 in BMDMs

We next investigated the mechanism by which LPS induces the activation of MST1/STK4 in BMDMs. Given that LPS triggered the interaction of MST1/STK4 with TRAF6 in BMDMs (Fig. 3e) and that forcefully expressed TRAF6 increased the kinase activity of recombinant MST1/STK4 in co-transfected HEK293 cells (Fig. 4a), we examined the possible role of TRAF6 in the LPS-induced activation of MST1/STK4. We found that LPS triggered not only the activation but also the ubiquitination of MST1/STK4 in BMDMs, and that these effects of LPS were abrogated by siRNA-mediated depletion of TRAF6 (Fig. 4b), suggesting that TRAF6 mediates both the activation and ubiquitination of MST1/STK4 in response to LPS stimulation. In addition, forced expression of TRAF6 promoted the ubiquitination of MST1/STK4 in HEK293 cells coexpressing WT ubiquitin, but not in those coexpressing a K63R mutant of ubiquitin (Fig. 4c). In a separate cotransfection experiment using HEK293 cells, the ubiquitination of MST1/STK4 was mediated by WT TRAF6, but not by TRAF6(C70A), which lacks its E3 ubiquitin ligase activity (Fig. 4d). Moreover, an in vitro ubiquitination assay demonstrated that TRAF6 catalyzed the polyubiquitination of a His$_6$-tagged kinase-dead mutant (K59R) of MST1/STK4 (Fig. 5a), indicating that MST1/STK4 is a substrate for the E3 ligase activity of TRAF6. A kinase-dead mutant of MST1/STK4 was tested as the substrate in this assay because a sufficient amount of the WT protein could not be prepared by bacterial expression. Amino acid residues 220–229 (GKPPYADIHP) of mouse MST1/STK4 were found to conform to a consensus site for TRAF6-mediated polyubiquitination [-(hydrophobic)-K-(hydrophobic)-X-X-(hydrophobic)-(polar)-(hydrophobic)-(polar)-(hydrophobic)-], where K is the ubiquitinated lysine residue and X is any amino acid [40]. Indeed, TRAF6 failed to ubiquitinate a K221R mutant of His$_6$-MST1(K59R) in vitro (Fig. 5a). Furthermore, forcefully expressed TRAF6 did not mediate the ubiquitination of MST1(K221R) in transfected HEK293 cells (Fig. 5b).


a
WT
p62-/-
LPS: - + - +
(kDa)
IP: TRAF6
IB: K63Ub
TRAF6
-K63Ub(n)
Lysates
phospho-IKKα/β
IKKα/β
p62
TRAF6
GAPDH
b
IP: TRAF6
IB: TAK1
IB: IKKβ
IB: p62
IB: TRAF6
TAK1
IKKβ
p62
TRAF6
Lysates
c
WT p62-/-
TNF-α
IL-6
Relative mRNA level
LPS
d
si-Cont
si-p62
MST1-/-
IP: TRAF6
IB: Ub
TRAF6-Ub(n)
phospho-IKKα/β
IKKα/β
TRAF6
p62
MST1
GAPDH
e
Myc-TRAF6:
Xpress-Ub:
HA-p62:
Flag-MST1:
Flag-MST1(K59R):
IP: Myc
IB: Xpress
IB: Myc
Myc-TRAF6-Ub(n)
Myc-TRAF6
HA-p62
Flag-MST1
f
IB: p62
IB: MST1
IB: TRAF6
IP: IgG TRAF6
No treatment LPS
level of p62
in immunoprecipitated TRAF6

◂**Fig. 3** Genetic ablation of MST1/STK4 enhances the p62/SQSTM1-dependent activation of TRAF6 induced by LPS in BMDMs. **a**, **b** WT or *p62/SQSTM1*$^{-/-}$ BMDMs were left untreated or treated with 1 μg/ml LPS for 15 min, after which cell lysates were immunoprecipitated with antibody to TRAF6 and the resulting precipitates were examined by immunoblot analysis with antibody to K63-linked polyubiquitin chains (**a**) or to TAK1, to IKKβ, to p62/SQSTM1, or to TRAF6 (**b**). Cell lysates were also immunoblotted with indicated antibodies. **c** Quantitative RT-PCR analysis of TNF-α and IL6 mRNAs in WT and *p62/SQSTM1*$^{-/-}$ BMDMs after LPS treatment (1 μg/ml, 1 h). Data are representative of two independent experiments (means ± SD of triplicate samples). *$p < 0.05$. **d** WT or *MST1/STK4*$^{-/-}$ BMDMs were transfected with p62/SQSTM1 (si-p62/SQSTM1) or control (si-Cont) siRNA for 24 h, incubated in the absence or presence of LPS (1 μg/ml) for 15 min, and then immunoprecipitated with antibody to TRAF6. The resulting precipitates were immunoblotted with antibody to ubiquitin. The cell lysates were also subjected directly to immunoblot analysis with antibodies to indicated antibodies. **e** HEK293 cells were transfected for 48 h with plasmid vectors for the indicated combinations of proteins, lysed, and subjected to immunoprecipitation with anti-Myc antibody. The resulting precipitates were examined by immunoblot analysis with antibodies to Xpress or to Myc. The lysates were also examined directly by immunoblot analysis with antibodies to Myc, to HA, or to Flag. **f** WT or *MST1/STK4*$^{-/-}$ BMDMs were left untreated or treated with 1 μg/ml LPS for 15 min, after which cell lysates were immunoprecipitated with anti-TRAF6 antibody (or with rabbit preimmune IgG) and the resulting precipitates were immunoblotted with antibodies to p62/SQSTM1, to MST1/STK4, or to TRAF6. The intensity of the bands for p62/SQSTM1 present in the TRAF6 immunoprecipitates was quantified by densitometry, and normalized by the intensity of the bands for the corresponding immunoprecipitated TRAF6. Quantitative data are mean ± SD from three independent experiments. *$p < 0.05$

Together, these results suggested that Lys^{221} of MST1/STK4 is a site of TRAF6-mediated ubiquitination.

We next examined whether LPS might induce the K63-linked polyubiquitination of MST1/STK4 at Lys^{221} in BMDMs with using *MST1/STK4*$^{-/-}$ cells reconstituted with Flag-tagged WT or K221R mutant forms of MST1/STK4. LPS induced the K63-linked polyubiquitination of Flag-MST1/STK4(WT), but not that of Flag-MST1(K221R), in the reconstituted cells (Fig. 5c). LPS also increased the Thr^{183}-phosphorylation and the kinase activity of MST1/STK4 in the cells reconstituted with WT MST1/STK4 but not in those reconstituted with MST1(K221R). Phosphorylation at Thr^{183} within the activation loop of MST1/STK4 has been shown to be required for the activation of the kinase [41, 42]. Of note, MST1(K221R) was stimulated in cellular response to H_2O_2 (Fig. S4), suggesting that its catalytic activity is intact. Collectively, these results suggested that the TRAF6-mediated ubiquitination of MST1/STK4 at Lys^{221} is critical for LPS-induced MST1/STK4 activation in BMDMs.

TRAF6-mediated ubiquitination of MST1/STK4 at Lys^{221} attenuates LPS-induced cytokine production in BMDMs

Finally, we examined the effect of TRAF6-mediated ubiquitination of MST1/STK4 at Lys^{221} on LPS-induced cytokine production in BMDMs. BMDMs from *MST1/STK4*$^{-/-}$ mice were reconstituted with WT or K221R mutant forms of MST1/STK4 and then examined for LPS-induced production of proinflammatory cytokines TNF-α and IL-6. LPS stimulation increased the production of TNF-α (Fig. 6a) and IL-6 (Fig. 6b) in *MST1/STK4*$^{-/-}$ cells transfected with an empty vector, and these effects of LPS were attenuated in the cells reconstituted with wild-type MST1/STK4, but not in the cells reconstituted with MST1(K221R) (Fig. 6a, b). Together, these results suggested that Lys^{221} is a critical residue for MST1/STK4 to attenuate the LPS-induced production of the cytokines TNF-α and IL-6 in BMDMs.

Discussion

We have here established that MST1/STK4 functions as a negative modulator of the TLR4-TRAF6-NF-κB signaling pathway associated with inflammation in macrophages. Myeloid-specific ablation of MST1/STK4 increased the susceptibility of mice to LPS-induced septic toxicity as well as enhanced NF-κB signaling-dependent processes including the production of proinflammatory cytokines. Genetic ablation of MST1/STK4 also enhanced the LPS-induced auto-ubiquitination of TRAF6 as well as downstream signaling events in BMDMs, suggesting that MST1/STK4 negatively regulates the LPS-induced activation of TRAF6 in these cells. However, we found that MST1/STK4 did not either phosphorylate TRAF6 or directly inhibit TRAF6 auto-ubiquitination, indicating that TRAF6 is not a direct target of MST1/STK4. The negative effect of MST1/STK4 on LPS-induced TRAF6 autoubiquitination was apparent in BMDMs only in the presence of p62/SQSTM1, which functions as a signaling adaptor in the NF-κB signaling pathway [37, 43–47]. We found that MST1/STK4 reduces the LPS-induced association between TRAF6 and p62/SQSTM1 in BMDMs. Moreover, MST1/STK4 inhibited the p62/SQSTM1-dependent activation of TRAF6 and downstream signaling events in the NF-κB pathway. We therefore propose that the inhibition of p62/SQSTM1-dependent TRAF6 activation may be an integral part of the mechanism by which MST1/STK4 negatively regulates the LPS-TLR4-NF-κB signaling pathway.

The p62/SQSTM1 adapter was initially discovered as a protein that binds to the Src homology 2 (SH2) domain of the kinase p56^{lck} in a phosphotyrosine-independent manner [48, 49]. It was subsequently shown to function as an adapter

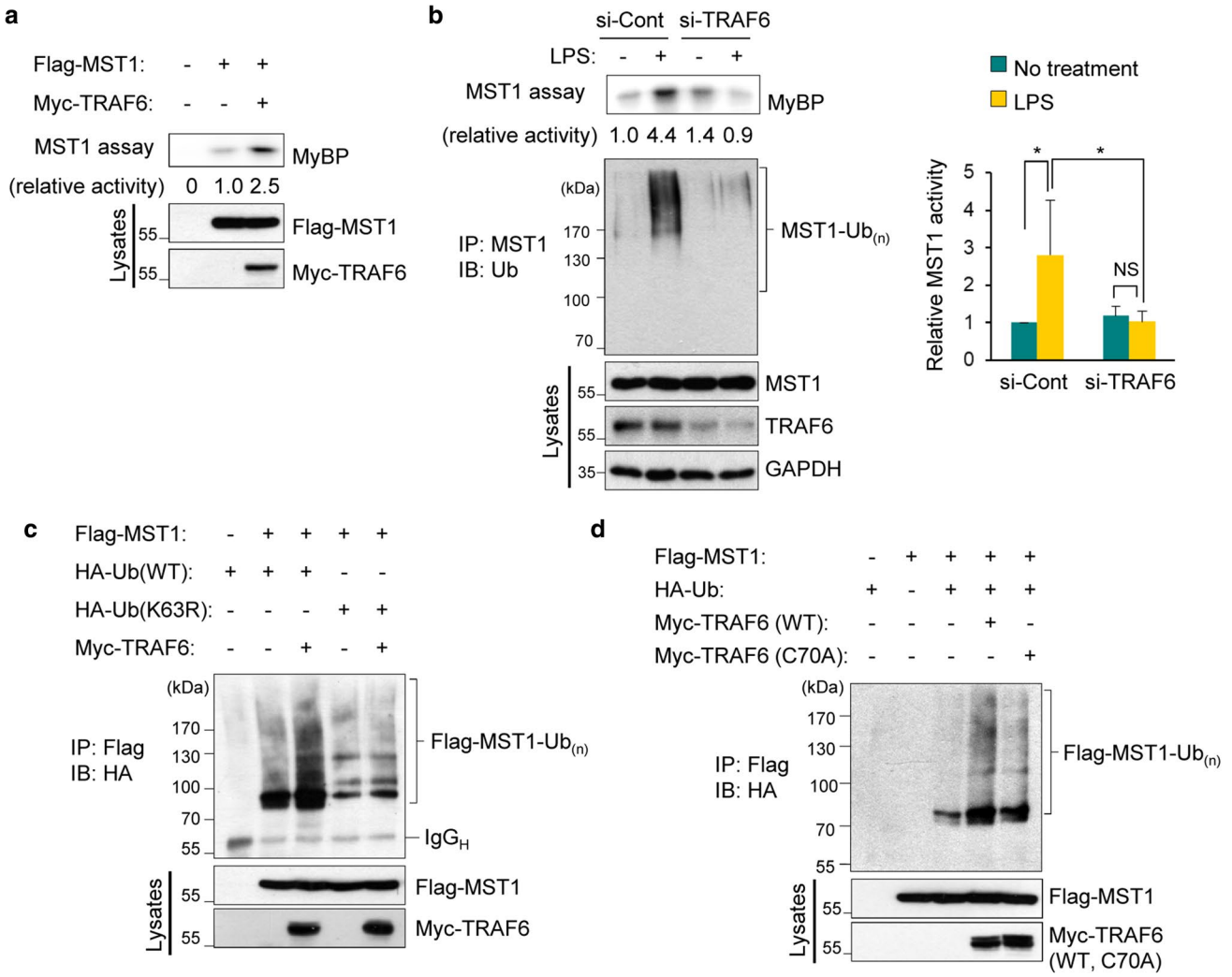


Fig. 4 TRAF6 mediates the LPS-induced activation of MST1/STK4. **a** HEK293 cells were transfected for 24 h with expression vectors for the indicated combinations of proteins, lysed, and subjected to immunoprecipitation with anti-Flag antibody. The resulting precipitates were examined by an immune complex kinase assay with myelin basic protein (MyBP) as substrate and the relative values for MST1/STK4 activity are shown. The cell lysates were examined also directly by immunoblot analysis with antibodies to Flag or to Myc. **b** BMDMs were transfected for 24 h with control or TRAF6 siRNA, left untreated or treated with 1 μg/ml LPS for 30 min, and subjected to immunoprecipitation with anti-MST1/STK4 antibody. The resulting precipitates were examined for MST1/STK4 activity with an immune complex kinase assay as well as for MST1/STK4 ubiquitination by immunoblot analysis with anti-ubiquitin antibody. Quantitation of relative MST1/STK4 activity from four independent experiments is also shown. $*p < 0.05$. *NS* not significant. **c**, **d** HEK293 cells were transfected for 48 h with expression vectors for the indicated combinations of proteins, lysed, and subjected to immunoprecipitation with anti-Flag antibody. The resulting precipitates were examined by immunoblot analysis with antibody to HA. IgG_H heavy chain of immunoglobulin G. The lysates were also examined directly by immunoblot analysis with antibodies to Flag or Myc

protein in the NF-κB signaling pathway and thereby to enhance transcription-stimulating activity of NF-κB [44–46, 50]. In particular, p62/SQSTM1 was shown to interact with TRAF6, with formation of the TRAF6-p62/SQSTM1 complex being important for downstream signaling events of the NF-κB pathway [37, 38, 45, 47, 50, 51]. Although the mechanism underlying regulation of the TRAF6-p62/SQSTM1 interaction has been unclear, p62/SQSTM1 appears to interact with polyubiquitinated TRAF6 through its TRAF6 binding site and carboxy-terminal ubiquitin associated domain [37, 39, 44, 45]. Our additional experiments are currently underway for clarification of a molecular mechanism how MST1/STK4 suppresses the TRAF6-p62/SQSTM1 interaction.

We have also shown that TRAF6 is a key mediator of LPS-induced MST1/STK4 activation in BMDMs. TRAF6 was thus found to mediate the K63-linked polyubiquitination of MST1/STK4 at Lys^{221} as well as MST1/STK4 activation in cells exposed to LPS. Furthermore, LPS failed to induce the ubiquitination and activation of a K221R

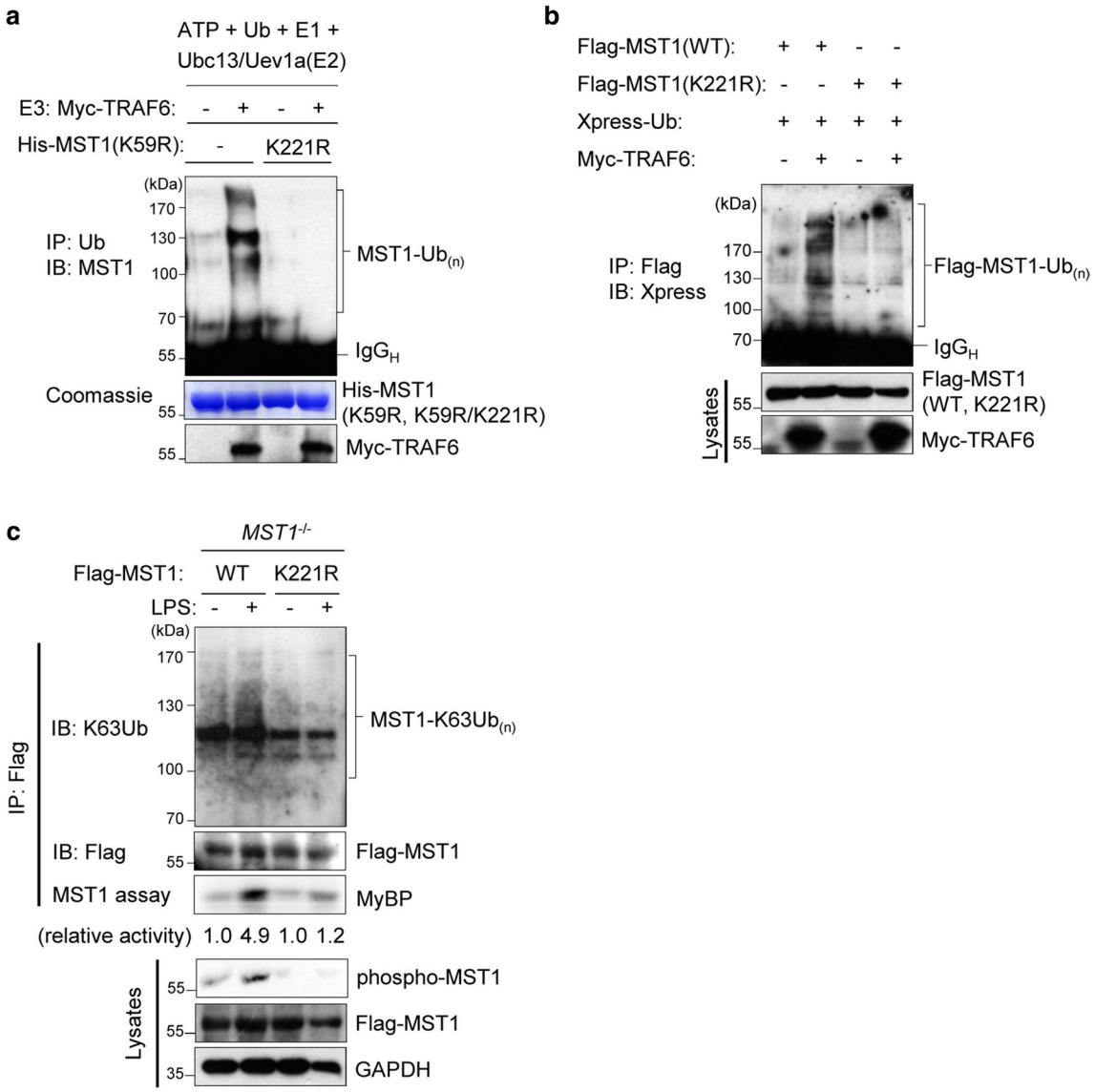


Fig. 5 TRAF6-mediated ubiquitination of MST1/STK4 at Lys^{221} is important for LPS-induced MST1/STK4 activation. **a** In vitro assay for TRAF6-catalyzed MST1/STK4 ubiquitination. His_6-MST1/STK4 variants (K59R and K59R/K221R) were subjected to an in vitro ubiquitination reaction in the absence or presence of Myc-TRAF6, which was obtained by immunoprecipitation of transfected HEK293 cells with anti-Myc antibody. The reaction mixtures were then centrifuged, the resulting supernatants were immunoprecipitated with anti-ubiquitin antibody, and the precipitates were examined by immunoblotting with anti-MST1/STK4 antibody. The input of the His_6-MST1/STK4 proteins to the assay was visualized by Coomassie staining, and that of Myc-TRAF6 was examined by immunoblot analysis with anti-Myc antibody. IgG_H heavy chain of immunoglobulin G. **b** HEK293 cells were transfected for 48 h with expression vectors for the indicated combinations of proteins, lysed, and subjected to immunoprecipitation with anti-Flag antibody. The resulting precipitates were examined by immunoblot analysis with antibody to Xpress. The lysates were also examined directly by immunoblot analysis with antibodies to Flag or Myc. **c** *MST1/STK4*$^{-/-}$ BMDMs were transfected for 24 h with vectors for Flag-tagged MST1/STK4 variants (WT and K221R), incubated in the absence or presence of LPS (1 μg/ml) for 30 min, lysed, and immunoprecipitated with anti-Flag antibody. The resulting precipitates were examined for MST1/STK4 ubiquitination by immunoblot analysis with antibodies to K63-linked polyubiquitin chains or to Flag. The Flag precipitates were also examined for MST1/STK4 activity with an immune complex kinase assay, and the cell lysates were examined directly by immunoblot analysis with antibodies to phospho-MST1/STK4(Thr^{183}), or to Flag, or to GAPDH

mutant of MST1/STK4, suggesting that TRAF6-mediated ubiquitination is a key step for LPS-induced activation of MST1/STK4. The mechanism by which the ubiquitination of MST1/STK4 at Lys^{221} increases the enzymatic activity of MST1/STK4 remains unclear, however. Given that homodimer formation contributes to MST1/STK4 activation [52], it will be of interest to investigate whether the TRAF6-medated ubiquitination of MST1/STK4 promotes its homodimerization in intact cells.

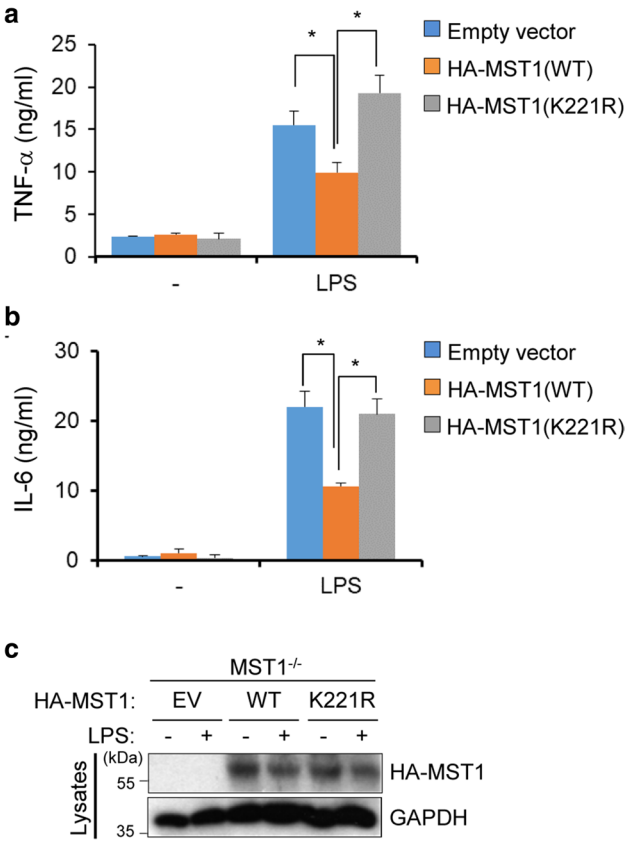


Fig. 6 TRAF6-mediated ubiquitination of MST1/STK4 at Lys^{221} negatively regulates LPS-induced cytokine production. BMDMs isolated from *MST1/STK4*$^{-/-}$ mice were transfected for 24 h with an empty vector (EV) or vectors encoding HA-MST1/STK4 variants (WT or K221R). Then, the cells were left untreated or treated with LPS (1 μg/ml) for 12 h before assay of TNF-α (**a**) or IL-6 (**b**) secreted into the culture medium by ELISA. The cells were also lysed and examined by immunoblot analysis with antibodies to MST1/STK4 or to GAPDH (**c**). Data are representative of two independent experiments (means ± SD of triplicate samples). $*p < 0.05$

The NF-κB signaling pathway plays a pivotal role in inflammation and the immune response [53], and it must therefore be subject to strict regulation to prevent its excessive activation and consequent uncontrolled inflammation and immunopathology [53, 54]. Accordingly, the initial activation of the NF-κB pathway should be attenuated by negative feedback regulation that prevents overactivation of the pathway and facilitates the proper resolution of inflammation [55]. We have now uncovered a previously unrecognized role of MST1/STK4 in the TRAF6-mediated activation of the NF-κB signaling pathway induced by LPS. Furthermore, given that TRAF6 also mediates the NF-κB pathway induced by other stimuli including IL-1, CD40, RANK, and NGF [10, 56–59], it is intriguing to investigate a possibility that MST1/STK4 may function as a common key regulator of the diverse NF-κB signaling events. Thus, our findings in this study warrants further study for its potential to serve as a basis for development of novel therapeutics for inflammatory disorders.

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Author contributions KHR, YL, IYL, DL, EK, EP, and JHY conducted the experiments. KHR, YL, EP, TSK, HKS, JHY and EJC designed the experiments. DSL and JS provided materials. KHR and EJC wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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