MicroRNA-92a Negatively Regulates Toll-like Receptor (TLR)-triggered Inflammatory Response in Macrophages by Targeting MKK4 Kinase[*]

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Background: microRNAs (miRNAs) participate in innate immune responses.
Results: miR-92a decreases rapidly in macrophages once stimulated with TLR ligands, and miR-92a controls inflammatory response by targeting MKK4/JNK/c-Jun pathway.
Conclusion: TLR-mediated miR-92a reduction feedback enhances TLR-triggered inflammatory response.
Significance: Our findings reveal a novel positive feedback loop in which TLR-reduced miR-92a expression functions to regulate the innate inflammatory responses.

Toll-like receptors (TLRs) play a critical role in the initiation of immune responses against invading pathogens. MicroRNAs have been shown to be important regulators of TLR signaling. In this study, we have found that the stimulation of multiple TLRs rapidly reduced the levels of microRNA-92a (miRNA-92a) and some other members of the miRNA-92a family in macrophages. miR-92a mimics significantly decreased, whereas miR-92a knockdown increased, the activation of the JNK/c-Jun pathway and the production of inflammatory cytokines in macrophages when stimulated with ligands for TLR4. Furthermore, mitogen-activated protein kinase kinase 4 (MKK4), a kinase that activates JNK/stress-activated protein kinase, was found to be directly targeted by miR-92a. Similar to the effects of the miR-92a mimics, knockdown of MKK4 inhibited the activation of JNK/c-Jun signaling and the production of TNF-α and IL-6. In conclusion, we have demonstrated that TLR-mediated miR-92a reduction feedback enhances TLR-triggered production of inflammatory cytokines in macrophages, thus outlining new mechanisms for fine-tuning the TLR-triggered inflammatory response.

Recognition of microbial pathogens is an essential element for the initiation of innate immune responses. Toll-like receptors (TLRs)[3] are germline-encoded pattern recognition recep-

tors that play significant roles in the recognition of and responses to microbial pathogens (1, 2). TLRs trigger a signaling cascade through the MyD88-dependent and/or MyD88-independent signaling pathways, which in turn transmit a series of signaling cascades that leads to the activation of MAPK and NF-κB, resulting in the production of proinflammatory cytokines (*e.g.* TNF-α, IL-6) (3). There are three major subfamilies of MAPKs involved in innate immunity: the extracellular signal-regulated protein kinases (ERK), the p38 MAP kinases, and the c-Jun NH$_2$-terminal kinases (JNK) (4). All three MAPKs are activated by phosphorylation of the threonine and tyrosine residues that is mediated by a conserved protein kinase cascade. ERK is activated by the MAP kinase kinases (MKKs), MKK1 and MKK2; the p38 MAP kinases are activated by MKK3 and MKK6; and the JNK pathway is activated by MKK4 and MKK7 (5). These MKKs are further phosphorylated and activated by MAP kinase kinase kinases (MKKKs) such as TAK1, TPL-2, and ASK1, which are activated in TLR stimulation (6). Members of the MAPK family phosphorylate and activate AP-1, a transcription factor, found in the promoters of proinflammatory cytokines and many other genes that are up-regulated in response to TLR ligation.

The mitogen-activated protein kinase kinase 4 (*Map2k4*, *MKK4*) was first identified in a cDNA library from *Xenopus laevis* embryos (7). MKK4 is activated by the majority of MKKKs, such as MEKK, ASK, mixed-lineage kinase (MLK), and TAK (8). The D domain type docking site in the N-terminal region of MKK4 is for binding of the JNK substrates. It was proved that MKK4 preferentially phosphorylates the tyrosine residue of JNKs, which is to be required for optimal JNK activation (9). MKK4 is involved in various physiological and pathophysiological processes. It is an important gene required for viability and embryonic development in mice (10). Studies on the function of MKK4 in the immune system have been mostly carried out on chimeric *Sek1*$^{-/-}$ *Rag2*$^{-/-}$ mice and have controversial results. One study showed that these mice had a reduction in thymus size due to a decrease in the population of

[3] The abbreviations used are: TLR, Toll-like receptor; AP-1, activator protein 1; miRNA, microRNA; miR, microRNA; MKK, MAP kinase kinase; MKKK, MKK kinase; qRT-PCR, quantitative RT-PCR; ASK, apoptosis signal-regulating kinase; TAK, TGF-β-activated protein kinase; TRIF, TIR domain-containing adapter-inducing interferon-β; TIR, Toll/interleukin-1 receptor; p, phospho.

peripheral T cells (11), whereas another study showed that MKK4 was required for maintenance of a normal peripheral lymphoid compartment, but is not required for primary lymphocyte development (12). In innate immunity, it has been investigated that endogenous macrophage MKK4 is able to transphosphorylate both p46 and p54 JNK isoforms and that both isoforms are phosphorylated in response to stimulation with TNF-α in macrophages (13). MKK4 is also involved in CCR7-mediated c-Jun N-terminal kinase activation to regulate cell migration in mature dendritic cells (14).

MicroRNAs (miRNAs) are highly conserved, small noncoding RNAs that have recently been shown to act as important regulators of gene expression at the posttranscriptional level. They induce gene degradation or suppress translation mainly by binding to the 3'-untranslated region (UTR) of target mRNAs (15). Currently, miRNAs have been found to play key roles in many biological processes, such as cellular development and tumorigenesis (16, 17). An initial study in 2004 indicated that miRNAs such as miR-142a, miR-181a, and miR-223 can control hematopoiesis and regulate the immune responses (18). From then on, more and more miRNAs have been shown to participate in both innate (19, 20) and adaptive immune responses (21, 22). Following TLR activation, miR-155 (23, 24), miR-146 (25), miR-21 (26), miR-147 (27), and miR-101a (28) are up-regulated to inhibit or enhance the TLR-triggered inflammatory response in monocytic cell lines or mouse macrophages. The down-regulation of miR-98 and let-7 may be relevant to the regulation of TLR-mediated epithelial innate immune response and the endotoxin tolerance in macrophages (29, 30).

The miR-17–92 cluster has shown to be activated in cancer cells (31), solid tumors (32–34), and neuroblastomas (35) and is also essential for B cell development (36). The miRNAs of this cluster can regulate different cellular targets for tumorigenesis or cell development, either in certain combinations with additive effects or individually. Among these, miR-92a has been reported to promote the migration and invasion of esophageal squamous cell carcinoma cells, partially via suppression of its target gene (*CDH1*) expression (37). miR-92a has also been identified to be an endogenous repressor of the angiogenic process by targeting mRNAs corresponding to several proangiogenic proteins, including the integrin subunit $\alpha 5$ in endothelial cells (38). Additionally, miR-92a increases cell proliferation by negative regulation of an isoform of the cell cycle regulator p63 (39). In addition, miR-25, miR-92b, miR-32, miR-363, and miR-367 are members of miR-92a family that have similar seed sequences that may target the same mRNA UTR. These miRNAs have been implied to participate in the development of different cancers (40–43).

However, whether miR-92a or its family members take part in TLR-induced innate immune response is still unclear. In this study, we first analyzed the expression profile of miR-92a family members in mouse macrophages after TLR activation and demonstrated its possible function in the TLR-triggered immune response. We found that the expression level of miR-92a is the highest among its family members in the macrophages and is down-regulated quickly in macrophages after activation through multiple TLR ligations. miR-92a negatively regulates TLR-triggered inflammatory cytokine production through targeting MKK4 involved in the JNK/c-Jun pathway. We thus for the first time have identified a new positive feedback loop in which TLR-mediated miR-92a reduction functions to regulate the innate inflammatory responses.
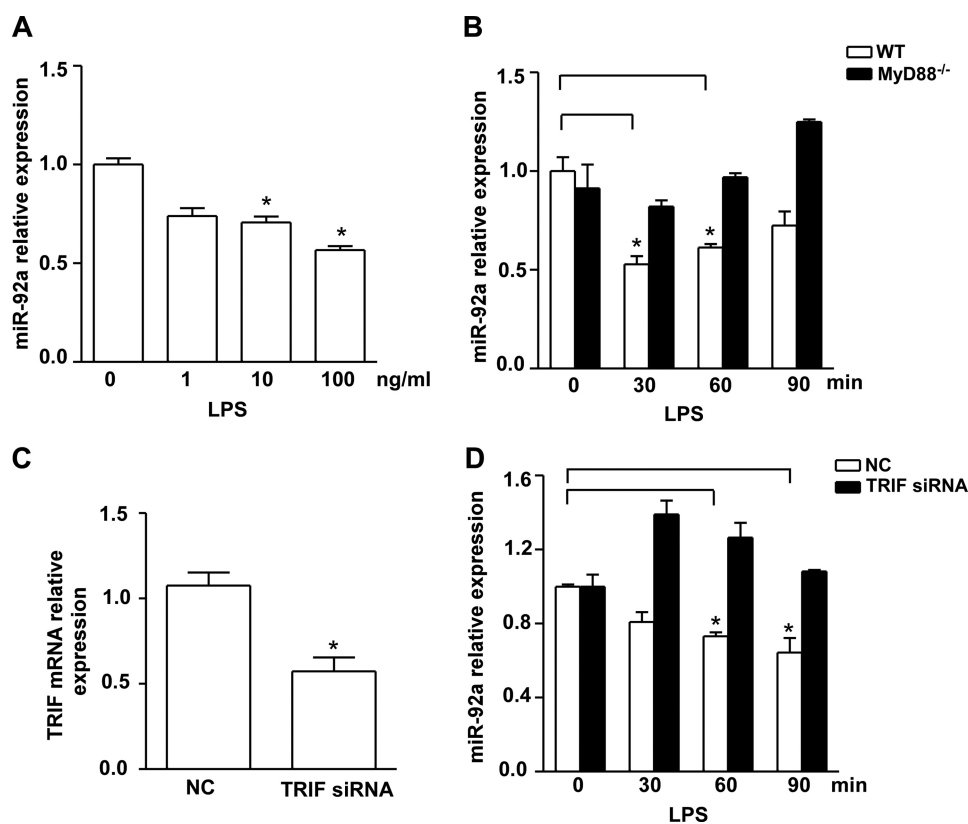
EXPERIMENTAL PROCEDURES

Mice and Reagents—C57BL/6 mice (5–6 weeks old) were purchased from SIPPR-BK Experimental Animal Ltd. Co. (Shanghai, China). *MyD88* knock-out mice were provided by Prof. S. Akira (Osaka University, Osaka, Japan). Experiments and animal care were performed according to protocols approved by the Zhejiang University Institutional Animal Care and Use Committee. Lipopolysaccharide (LPS), polyinosinic-polycytidylic acid (poly(I:C)), and peptidoglycan were purchased from Sigma. Antibodies specific to MKK4, IκBα, and β-actin and HRP-coupled secondary antibodies were obtained from Santa Cruz Biotechnology. Antibodies specific to p-JNK, p-c-Jun, JNK, p-ERK1/2, p-p38, and p-p65 were obtained from Cell Signaling Technology.

MiRNA Mimics, Inhibitors, and Small Interfering RNA—miR-92a dsRNA mimics and miR-92a ssRNA inhibitors from GenePharma (Shanghai, China) were used for the overexpression and inhibition of miR-92a activity in cells, respectively. The cells mentioned above were transfected with RNAs at a final concentration of 20 nM. Negative control mimics or inhibitors were transfected as matched controls. The MKK4-specific siRNA (mixture) sequences were 5'-GGACUUGAAAGAC-CUUGGATT-3' (sense), 5'-UCCAAGGUCUUUCAAGUCCT-C-3' (antisense) and 5'-GCCGUAUAUGGCACCUGAATT-3' (sense), 5'-UUCAGGUGCCAUAUACGGCTT-3' (antisense). The TRIF-specific siRNA sequences were 5'-GGAAAGCAGUG-GCCUAUUA-3' (sense), 5'-UAAUAGGCCACUGCUUUCC-3' (antisense).

Cell Culture and Transfection—HEK293 and RAW 264.7 cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM containing 10% FCS. A total of 1×10^4 HEK-293 cells were seeded onto 96-well plates and incubated overnight. jetSI-ENDO transfection reagents (Polyplus-transfection, Illkirch, France) were used for the co-transfection of plasmids and RNAs as we described previously (44). Thioglycollate-elicited mouse peritoneal macrophages were prepared and cultured as described previously (45). A total of 0.5 ml of 2×10^5 cells were seeded onto each well of 24-well plates and incubated overnight, and then transfected with RNAs using INTERFERin (Polyplus-transfection), according to the manufacturer's instructions. All of the cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

3'-UTR Luciferase Reporter Assays—The wild-type mouse MKK4 3'-UTR luciferase reporter vectors were constructed by amplifying the mouse MKK4 mRNA 3'-UTR containing two potential binding sites and cloning it into the PGL3-promoter vector (Promega). The MKK4 UTR mutant 1 was generated from the MKK4 3'-UTR by mutating the first one of two potential miR-92a binding sites. The MKK4 UTR mutant 2 was generated from the MKK4 3'-UTR by mutating the second one of two potential miR-92a binding sites. Also, both miR-92a potential binding sites were mutated in the MKK4 UTR mutant vector. HEK-293 cells were co-transfected with luciferase reporter

FIGURE 1. **Reduction of the miR-92a family by the stimulation of TLR ligands.** *A*, expression of miR-92a, miR-92b, miR-25, miR-32, miR-363, and miR-367 in mouse primary peritoneal macrophage cells was measured by qRT-PCR and normalized to the expression of U6 in each sample. *B* and *C*, mouse primary peritoneal macrophages cells were stimulated with LPS (100 ng/ml) for different time periods as indicated. Expressions of miR-155 (*B*) and miR-92a, miR-25, miR-92b, and miR-32 (*C*) were measured by qRT-PCR. *D* and *E*, mouse primary peritoneal macrophages cells were stimulated with peptidoglycan (*PGN*) (10 μg/ml) or poly(I:C) (20 μg/ml). Expressions of miR-92a, miR-25, miR-92b, and miR-32 were measured by qRT-PCR. Data are expressed as the mean $\pm$ S.D. (*n* = 4). All of the data shown above are representative of three independent experiments. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$.

plasmid, thymidine kinase promoter-*Renilla* luciferase reporter plasmid, and the indicated miRNA mimics, inhibitors, or controls (final concentration, 20 nM). After 24 h, the cells were collected for application in the Dual-Luciferase reporter system (Promega, Madison, WI) following the manufacturer's instructions. All of the Dual-Luciferase reporter assays were done in triplicate within each experiment, and three independent experiments were conducted.

RNA Isolation and Real-time Quantitative PCR—For reverse transcription PCR (RT-PCR), total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instruc-

tions. Real-time quantitative PCR, using SYBR Green detection chemistry (Takara), was performed on a 7500 real-time PCR system (Applied Biosystems) as we described previously (29). For miRNA analysis, the RT primer sequences used were: miR-92a, 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTT-GAGTCAGGCCG-3′; miR-92b, 5′-CTCAACTGGTGTCGTG-GAGTCGGCAATTCAGTTGAGTGGAGGCC-3′; miR-25, 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGT-TCAGAC-3′; miR-32, 5′-CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGTTGCAAC-3′; miR-363, 5′-CTCAACT-GGTGTCGTGGAGTCGGCAATTCAGTTGAGTTACAGA-

FIGURE 2. **Reduction of the miR-92a was both MyD88 and TRIF signaling-dependent.** *A*, mouse primary peritoneal macrophages cells were stimulated with LPS at different dose as indicated for 60 min. Expression of miR-92a was measured by qRT-PCR. *B*, primary peritoneal macrophages cells derived from wild-type or *MyD88*$^{-/-}$ mouse were stimulated with LPS (100 ng/ml) for 60 min. Expression of miR-92a was measured by qRT-PCR. *C*, mouse primary peritoneal macrophages cells were transfected with control or TRIF siRNA at a final concentration of 40 nM. After 24 h, cells were stimulated with LPS (100 ng/ml) for 60 min. *D*, expression of miR-92a was measured by qRT-PCR. Similar results were obtained in three independent experiments. *, $p < 0.05$. *NC*, negative control.

3′; miR-367, 5′-CTCAACTGGTGTCGTGGAGTCGGCAATT-CAGTTGAGTTCACCA-3′. The quantitative PCR primer sequences used were: miR-92a, 5′-GCTGAGTATTGCACTTGTC-CCG-3′ (forward); miR-92b, 5′-TTGGTATTGCACTCGTCCC-3′ (forward); miR-25, 5′-TGGCATTGCACTTGTCTCG-3′ (forward); miR-32, 5′-TGGGGCTATTGCACATTACTAA-3′ (forward); miR-363, 5′-TGGAATTGCACGGTATCCA-3′ (forward); miR-367, 5′-TGGGAATTGCACTTTAGCAA-3′ (forward), and the universal reverse primer, 5′-GTGTCGTG-GAGTCGGCAA-3′. The U6 small nuclear RNA sequence was: 5′-CTCGCTTCGGCAGCACA-3′ (forward) and 5′-AACGC-TTCACGAATTTGCGT-3′ (reverse). The relative expression level of miRNA was normalized to that of the internal control U6. The following primers were used: murine β-actin, 5′-AGT-GTGACGTTGACATCCGT-3′ (forward) and 5′-GCAGCTC-AGTAACAGTCCGC-3′ (reverse); murine TNF-α, 5′-CAAA-ATTCGAGTGACAAGCCTG-3′ (forward) and 5′-GAGATC-CATGCCGTTGGC-3′ (reverse); murine IL-6, 5′-GTTGCCT-TCTTGGGACTGATG-3′ (forward) and 5′-GGAGTGGGTAT-CCTCTGTGAAGTCT-3′ (reverse); TRIF, 5′-GGTTCACGA-TCCTGCTCCTGAC-3′ (forward) and 5′-GCTGGGCCTGA-GAACACTCAAG-3′ (reverse); and MKK4, 5′-GA CTGAGA-ACACACAGCATTGAG-3′ (forward) and 5′-cgtccaatttctcca-aggtctt-3′ (reverse). Data were normalized to the level of β-actin expression in each sample.

ELISA Assay—After stimulation with LPS at the indicated time points, the cell supernatants were collected and analyzed using ELISA kits (eBioscience) according to the manufacturer's protocols.

Immunoblotting—The cells were washed twice with cold PBS and lysed with cell lysis buffer (Cell Signaling Technology) supplemented with PMSF (Beyotime Institute of Biotechnology). The lysates were loaded onto SDS-PAGE gels and transferred onto nitrocellulose membranes as described previously (46). Detection of proteins was conducted using ECL reagent (Thermo Fisher).

Statistical Analysis—All experiments were repeated at least three times, and the data were expressed as mean ± S.D. Statistical significance was determined by Student's *t* test and analysis of variance using SPSS (version 10.0), with values of $p < 0.05$ considered to be statistically significant.

RESULTS

TLR Ligand Stimulation Reduces the Expression of Most miR-92a Family Members in Macrophages—We first detected the expression levels of all the miR-92a family members in the primary peritoneal macrophages and found that miR-92a was significantly enriched in macrophages and much more enriched than the other miRNAs in its family. miR-363 and miR-367 were barely detectable in macrophages (Fig. 1*A*). Then, to investigate the kinetics of miR-92a family induction, primary peritoneal macrophages were stimulated with different TLR ligands, and the expression levels of miR-92a family were evaluated by qRT-PCR. Treatment of primary peritoneal macro-
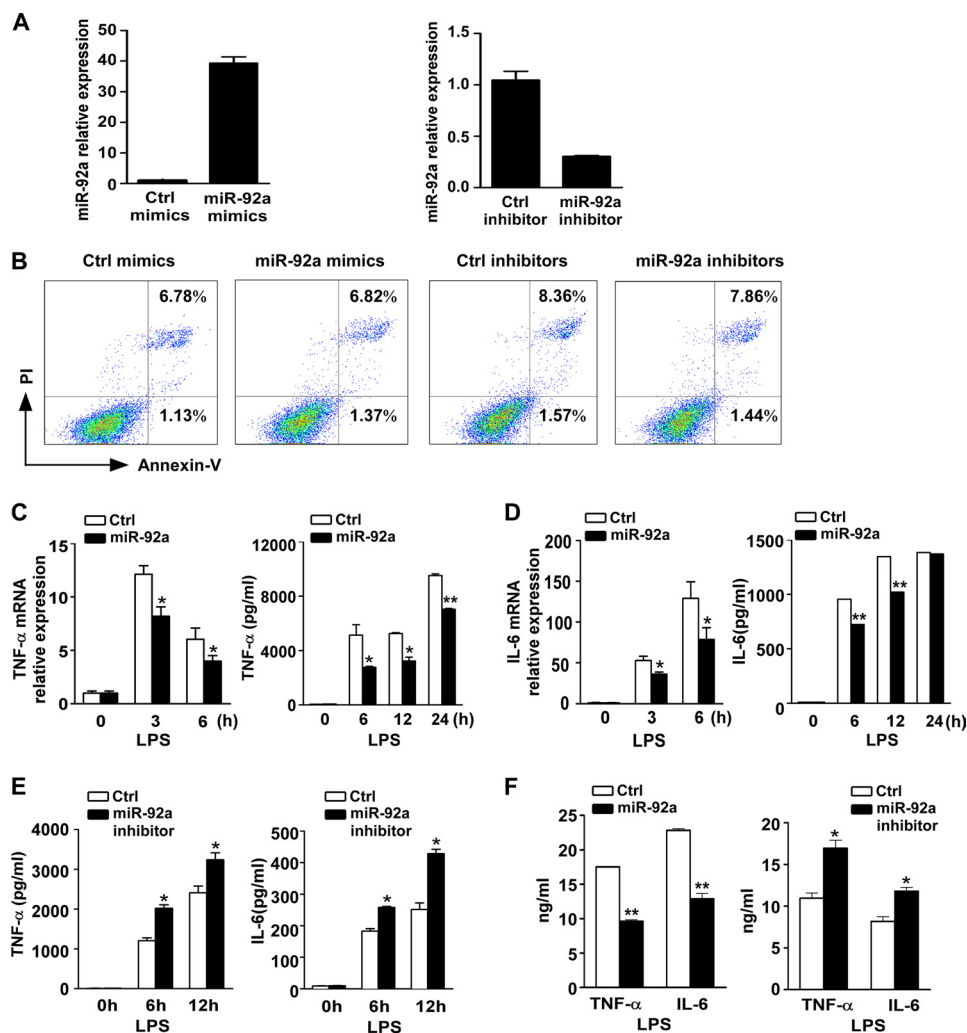
FIGURE 3. **miR-92a is a negative regulator of TLR-triggered inflammatory responses in macrophages.** *A*, RAW264.7 cells were transfected with control (*Ctrl*) or miR-92a mimics (*left*) or control inhibitor or miR-92a inhibitor (*right*), as indicated at a final concentration of 20 nm. After 24 h, miR-92a expression was measured by qRT-PCR and normalized to the expression of U6. *B*, cells were transfected with miR-92a mimics or inhibitors as indicated. After 24 h, apoptosis of the cells was detected by annexin V-FITC/propidium iodide double staining with FACS analysis. *C* and *D*, cells were transfected with control or miR-92a mimics as indicated. At 24 h after transfection, the cells were cultured with or without 100 ng/ml LPS. The mRNA levels of TNF-α (*C*) and IL-6 (*D*) were evaluated by qRT-PCR and normalized to β-actin, and the protein levels of TNF-α (*C*) and IL-6 (*D*) in the supernatants were determined by ELISA. *E*, cells were transfected with control or miR-92a inhibitor as indicated. At 24 h, the cells were cultured with or without 100 ng/ml LPS. The production of TNF-α and IL-6 was measured by ELISA. *F*, mouse primary peritoneal macrophages cells were transfected as described above. At 24 h, the cells were cultured with or without 100 ng/ml LPS for 12h. The production of TNF-α and IL-6 was measured by ELISA. Data are the mean $\pm$ S.D. of one representative experiment. Similar results were obtained in three independent experiments.*, $p < 0.05$ and **, $p < 0.01$.

phages with TLR4 ligand LPS rapidly induced the up-regulation of miR-155 expression, indicating that the peritoneal macrophages were activated properly (Fig. 1*B*).

As shown in Fig. 1*C*, LPS reduced the expression of miR-92a at time points from 30 min and also slightly decreased the levels of miR-25 and miR-92b at 60 min, whereas the expression of miR-32 remained almost unchanged. Stimulation with peptidoglycan, a TLR2 ligand, also reduced the expression of miR-92a and miR-92b in macrophages, but had no effect on expression of miR-25 and even induced the expression of miR-32 (Fig. 1*D*). Poly(I:C), a TLR3 ligand, also reduced the expressions of the miR-92a family members (Fig. 1*E*). The expression of miR-92a was also decreased with lower concentrations of LPS (Fig. 2*A*). These data demonstrated that the expression of miR-92a family members including miR-92a, miR-25, and miR-92b decreased after the activation by TLR agonists in macrophages,

and among these family members, miR-92a is abundantly expressed in macrophages, suggesting that miR-92a may be involved in the regulation of TLR-triggered response in macrophages.

We next investigated the underlying mechanism through which miR-92a was reduced. We used the *MyD88*-deficient macrophages from *MyD88*$^{-/-}$ mice and also used TRIF-siRNA to decrease expression of TRIF in macrophages (Fig. 2, *B–D*). This result suggested that LPS-reduced miR-92a expression was dependent on both MyD88 and TRIF signaling and may be mainly TRIF-dependent as the expression of miR-92a significantly increased after LPS stimulation in TRIF-silenced macrophages.

miR-92a Negatively Regulates TLR-triggered Production of Proinflammatory Cytokines in Macrophages—To assess whether miR-92a may be involved in the regulation of innate immune responses to microbial infection, we transfected RAW264.7

cells with miR-92a mimics, inhibitors or their controls, and analyzed the production of proinflammatory cytokines. As shown in Fig. 3*A*, transfection with miR-92a mimics increased miR-92a expression, whereas the miR-92a inhibitor decreased its expression level. To exclude the possibility that miR-92a could influence cell survival, apoptosis of the cells was detected by propidium iodide/annexin V staining with FACS analysis. Neither overexpression nor inhibition of miR-92a had any effect on cell viability (Fig. 3*B*).

Interestingly, miR-92a overexpression significantly inhibited LPS-stimulated TNF-α and IL-6 production at both the mRNA and the protein levels (Fig. 3, *C* and *D*). Accordingly, inhibition of miR-92a significantly increased LPS-induced TNF-α and IL-6 production (Fig. 3*E*). Similarly, overexpression of miR-92a also inhibited the production of the above cytokines in LPS-stimulated primary peritoneal macrophages, whereas inhibition of miR-92a increased the production of these cytokines (Fig. 3*F*). Taken together, these data indicated that miR-92a may act as a negative regulator of TLR-triggered innate inflammatory response in macrophages by suppressing the production of proinflammatory cytokines such as IL-6 and TNF-α.

miR-92a Inhibits LPS-induced Activation of JNK Pathway—
After ligand binding, TLRs activate a series of signaling cascades involved in the activation of MAPKs and NF-κB, resulting in the production of proinflammatory cytokines (*e.g.* TNF-α, IL-6) (3). Three major subfamilies of MAPKs are involved in this process, including the ERK1/2, p38 MAP kinases, and JNK. To further investigate the mechanisms by which miR-92a regulates proinflammatory cytokine production, we assessed the effect of miR-92a mimics on the activation of several components of the NF-κB and MAPK pathway in RAW264.7 cells. Cells were transfected with miR-92a mimics and then stimulated with LPS for different time periods. Activation of NF-κB and MAPK pathways including ERKs, JNK/stress-activated protein kinase, and p38 MAPKs were measured by Western blot analysis. As shown in Fig. 4*A*, after LPS treatment, there was no difference in NF-κB, ERK1/2, and p38 activation in cells transfected with miR-92a mimics as compared with control cells because the levels of p-p65, p-IκBα, p-ERK, and p-p38 were comparable.

However, transfection with the miR-92a mimics significantly attenuated the phosphorylation of JNK and c-Jun after LPS stimulation (Fig. 4*B*). By contrast, treatment of cells with the miR-92a inhibitors markedly increased the phosphorylation of JNK and c-Jun (Fig. 4*C*), thus suggesting that LPS-induced miR-92a exhibits its anti-inflammatory phenotype mainly through suppression of the JNK/Jun pathway.

miR-92a Directly Targets Mouse MKK4—We next investigated possible targets by which miR-92a may act through to modulate the LPS-triggered JNK/Jun pathway in regulating the inflammatory response. Computational prediction via TargetScan revealed that miR-92a is one of the broadly conserved miRNAs that putatively targets two conserved sites of the murine MKK4 3′-UTR (Fig. 5*A*). Previous studies have shown that MKK4, which is known to be a member of the MKK family, directly phosphorylates and activates JNK in response to cellular stresses and proinflammatory cytokines (47, 48). In turn, active JNK phosphorylates and activates AP-1, Jun, and activat-
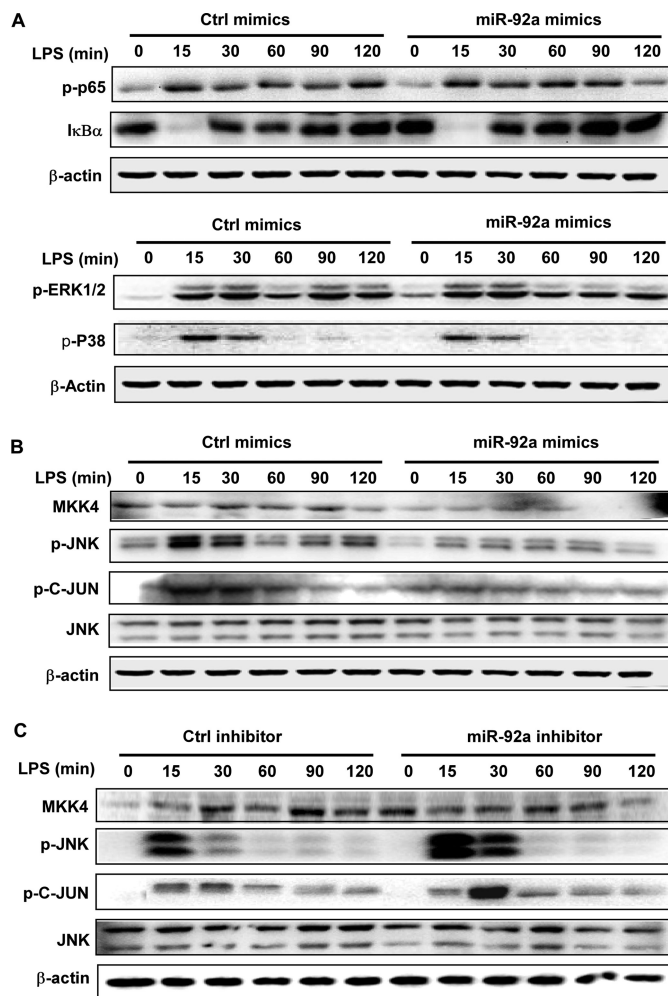

FIGURE 4. **miR-92a regulates the LPS-induced activation of JNK.** *A* and *B*, RAW264.7 cells were transfected with control (*Ctrl*) or miR-92a mimics and then stimulated with LPS for different time periods as indicated. Cell lysates were analyzed by Western blot analysis using anti-IκBα, p-ERK1/2, p-p38, and p-p65 (*A*) and p-JNK, p-c-Jun, and JNK (*B*). β-Actin was used as the loading control. *C*, cells were transfected with control or miR-92a inhibitors and then stimulated with LPS for different time periods as indicated. p-JNK, p-c-Jun, and JNK were measured as in *B*. Data shown are representative of one experiment out of three.

ing transcription factor 2 (ATF2), thereby altering gene transcription. c-Jun has been implicated in having a central role in inflammatory responses in TLR signaling (49).

To obtain direct evidence that MKK4 is a direct target of miR-92a, luciferase reporter constructs were generated by cloning either the wild-type 3′-UTR or a mutated portion of the 3′-UTR of MKK4 into the pGL3 vector (Fig. 5*B*). By co-transfection of the reporter plasmids and miR-92a mimics or inhibitors into HEK293 cells, we found that miR-92a mimics markedly inhibited, whereas miR-92a inhibitors enhanced, the luciferase activity of cells transfected with wild-type MKK4 3′-UTR vector as compared with the control dsRNA. However, they had no effect on the luciferase activity of cells transfected with MKK4 UTR mutant or PGL3 empty vector (Fig. 5*C*). In addition, the effect of miR-92a mimics or inhibitors was partially diminished when either one of the 3′-UTR binding sites was mutated, suggesting that both sites contributed to the binding with miR-92a.
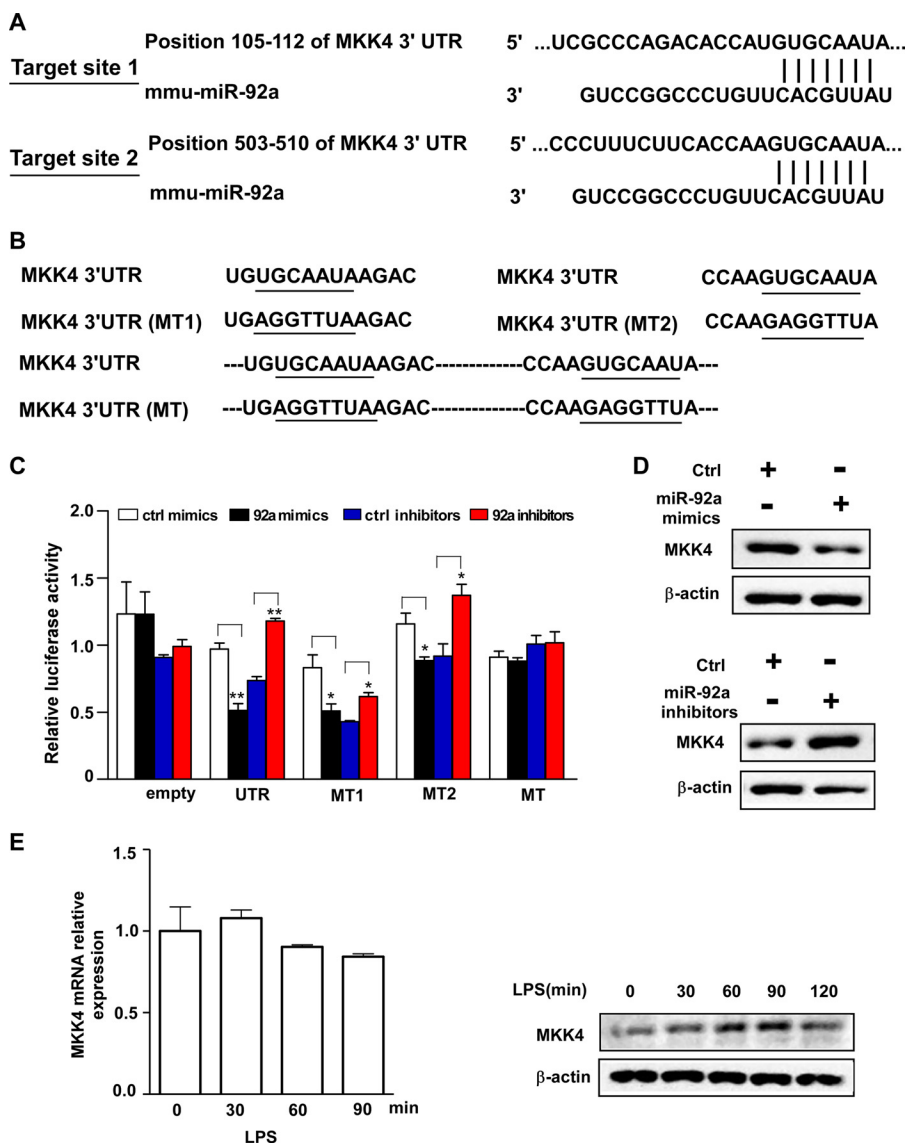
FIGURE 5. **miR-92a directly targets mouse MKK4.** *A*, mouse MKK4 might be the target of miR-92a. Shown is the alignment of miR-92a and its target sites in the 3′-UTR of MKK4, which was downloaded from TargetScan. *mmu, Mus musculus. B*, sequence of the MKK4 3′-UTR seed mutant for luciferase reporter assays. *WT*, wild type; *MT*, mutant. *C*, HEK293 cells were co-transfected with pGL3-Luc vector carrying the wild-type, empty, or different mutant portion of the 3′-UTR of MKK4 and pTK-*Renilla* luciferase plasmids, together with control (*ctrl*) or miR-92a mimics (*left*) or control or miR-92a inhibitors (*right*) (final concentration: 20 nM) as indicated. After 24 h, firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. *D*, cells were transfected with control or miR-92a mimics (*left*) or control or miR-92a inhibitors (*right*) at a final concentration of 20 nM. The protein level of MKK4 was determined by immunoblotting. *β*-Actin served as a loading control. *E*, cells were stimulated with 100 ng/ml LPS for the indicated time points. The mRNA (*left*) and protein (*right*) levels of MKK4 were detected by qRT-PCR and Western blotting. Data are the mean ± S.D. of one representative experiment. Similar results were obtained in at least three independent experiments. *, $p < 0.05$ and **, $p < 0.01$.

Furthermore, transfection with miR-92a mimics markedly decreased the protein expression of MKK4 as compared with that in cells transfected with control RNAs. In contrast, transfection with miR-92a inhibitors significantly enhanced the protein level of MKK4 (Fig. 5*D*).

We further observed the mRNA and protein expression levels of MKK4 in macrophages after LPS stimulation at different time points. As shown in Fig. 5*E*, the mRNA level of MKK4 seemed unchanged, whereas the protein level of MKK4 significantly increased in a short time period upon LPS treatment. This result suggested that an interaction may exist between miR-92a and MKK4 in macrophages during TLR4-triggered inflammatory response. Taken together, the results showed that MKK4 is a new target of miR-92a and that endogenous

MKK4 can be directly regulated by miR-92a in macrophages during pathogen-associated molecular pattern stimulation.

Knockdown of MKK4 Inhibits LPS-induced Inflammatory Responses and Activation of JNK—To further confirm the mechanisms by which miR-92a regulates LPS-triggered proinflammatory cytokine production, cells were transfected with MKK4 siRNA to knock down the expression of MKK4. A significant inhibition at both the mRNA and the protein levels of MKK4 was observed (Fig. 6*A*). Knockdown of MKK4 also markedly reduced LPS-triggered TNF-α and IL-6 production in macrophages, at both the mRNA and the protein levels (Fig. 6, *B* and *C*), suggesting that MKK4 plays an essential role in regulating the effect of LPS on the production of proinflammatory cytokines.
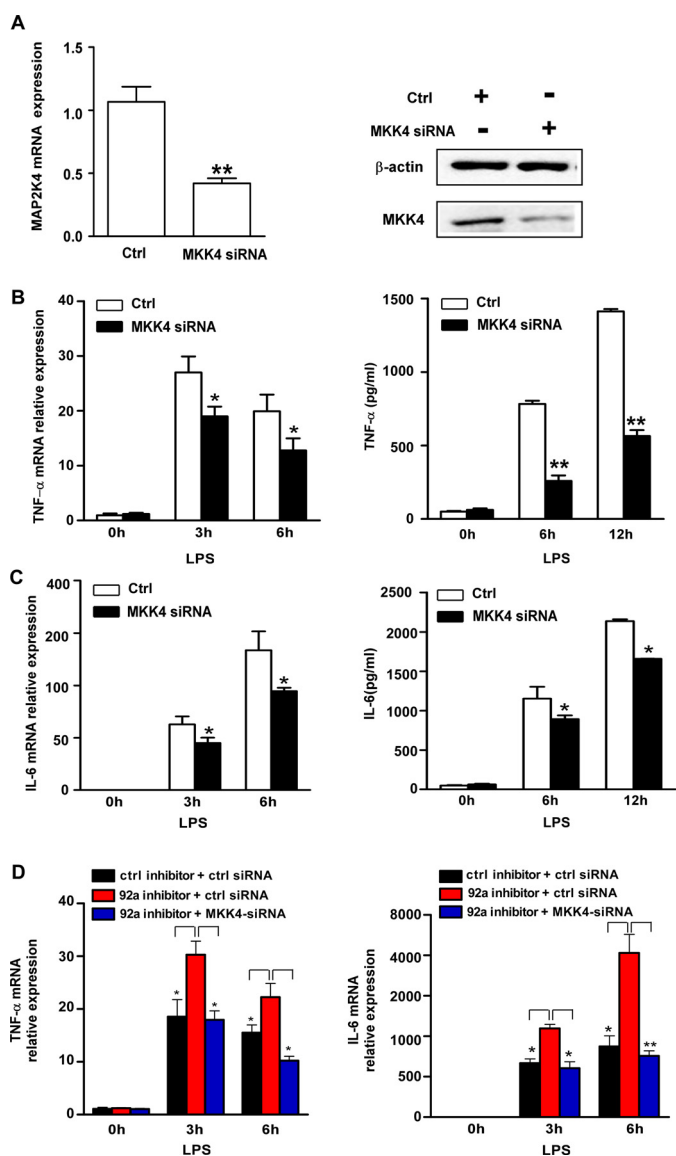
FIGURE 6. Knockdown of MKK4 inhibits LPS-induced proinflammatory cytokine production in macrophages. RAW264.7 cells were transfected with control (*Ctrl*) or MKK4 siRNA at a final concentration of 40 nM. *A*, 24 h after transfection, the mRNA and protein levels of MKK4 were evaluated by qRT-PCR and immunoblotting, respectively. *B* and *C*, 24 h after transfection, the cells were cultured with or without 100 ng/ml LPS. The mRNA levels of TNF-α (*B*) and IL-6 (*C*) were evaluated by qRT-PCR, and the production of TNF-α (*B*) and IL-6 (*C*) in the supernatants was determined by ELISA. *D*, RAW264.7 cells were transfected with control inhibitor combined with siRNA control, miR-92a inhibitor combined with siRNA control, or miR-92a inhibitor combined with MKK4 siRNA, respectively, as indicated. After 24 h, the mRNA levels of TNF-α and IL-6 were evaluated by qRT-PCR. Data are the mean ± S.D. of one representative experiment. Similar results were obtained in three independent experiments.*, $p < 0.05$ and **, $p < 0.01$.

To further demonstrate a more direct relationship between miR-92a and MKK4, we transfected RAW264.7 cells with control inhibitor combined with siRNA control, miR-92a inhibitor combined with siRNA control, or miR-92a inhibitor combined with MKK4 siRNA. These results showed that the expression of TNF-α and IL-6 was significantly enhanced by miR-92a inhibitors and that this effect could be blocked by MKK4 knockdown (Fig. 6D).

In addition, the levels of phosphorylation of JNK and c-Jun were assessed in cells transfected with the MKK4 siRNA. As shown in Fig. 7A, LPS-induced phosphorylation of JNK and c-Jun was significantly inhibited by knockdown of MKK4, an effect similar to that observed in macrophages overexpressing miR-92a.

To confirm the effects of JNK signaling pathway on the LPS-induced proinflammatory cytokine production in macrophages, RAW264.7 cells were pretreated with JNK inhibitor SP600125 and then stimulated with LPS. JNK pathway inhibition significantly decreased LPS-induced proinflammatory cytokines TNF-α and IL-6 production (Fig. 7B). These data further confirmed that JNK activation has a significant impact on proinflammatory cytokines production in LPS-stimulated macrophages.

Thus, all of the above results indicated that miR-92a-mediated posttranscriptional regulation can potentially be involved in fine-tuning TLR-induced inflammatory response in macrophages. miR-92a was down-regulated upon stimulation of TLRs, and then the decreased miR-92a enhanced the TLR-associated signaling events by increasing the target gene MKK4, a kinase shown to activate JNK/c-Jun pathway, consequently promoting the production of proinflammatory cytokines. This is a positive loop in which the TLR-triggered inflammatory response is tightly controlled (Fig. 7C).

DISCUSSION

In recent years, microRNAs have emerged as important regulators in TLR signaling. Several microRNAs have been shown to target the 3′-untranslated regions of mRNAs encoding components of the TLR signaling system (26, 50). miR-146 was shown to be induced by LPS, TNF-α, and IL-1β, and in turn, miR-146a acted as a negative regulator for fine-tuning the immune response mainly through targeting tumor necrosis factor receptor-associated factor (TRAF) 6 and interleukin-1 receptor-associated kinase (IRAK) 1 (50). Furthermore, miR-146a also increased after viral infection and was a negative regulator of the retinoic acid-inducible gene I (RIG-I)-dependent antiviral pathway (51). Considering the ability of miR-146a to regulate TRAF6 and IRAK-1, it may have an important role in LPS-induced cross-tolerance against various microbial cargos (25). Another miRNA, miRNA-21, has been implicated as a central player in controlling the inflammatory response by targeting PDCD4 that acts as a molecular switch between the proinflammatory (NF-κB) and anti-inflammatory (IL-10) responses (26). Unlike miR-146 and miR-21, miR-155, a multifunctional miRNA that is highly induced after TLR or viral treatment, exerts a complex effect on inflammation. There are several studies indicating that miR-155 can negatively regulate the TLR signaling pathway. Inhibition of miR-155 in dendritic cells resulted in the up-regulated expression of components of the p38 mitogen-activated protein kinase (MAPK) pathway by targeting TAK1-binding protein 2 (TAB2) (24). Additionally, MyD88 and IκB kinases have also been identified as targets of miR-155, further supporting it as a negative regulator of immune signaling (53, 54). On the other hand, miR-155 is required for the expression of TNF, IL-6, IL-23, and type I IFNs, possibly mediated by suppressing of suppressor of cytokine signaling 1 (SOCS1) and/or SHIP1, two negative regulators of TLR signaling pathways (23, 55, 56). Therefore, the roles of miR-155
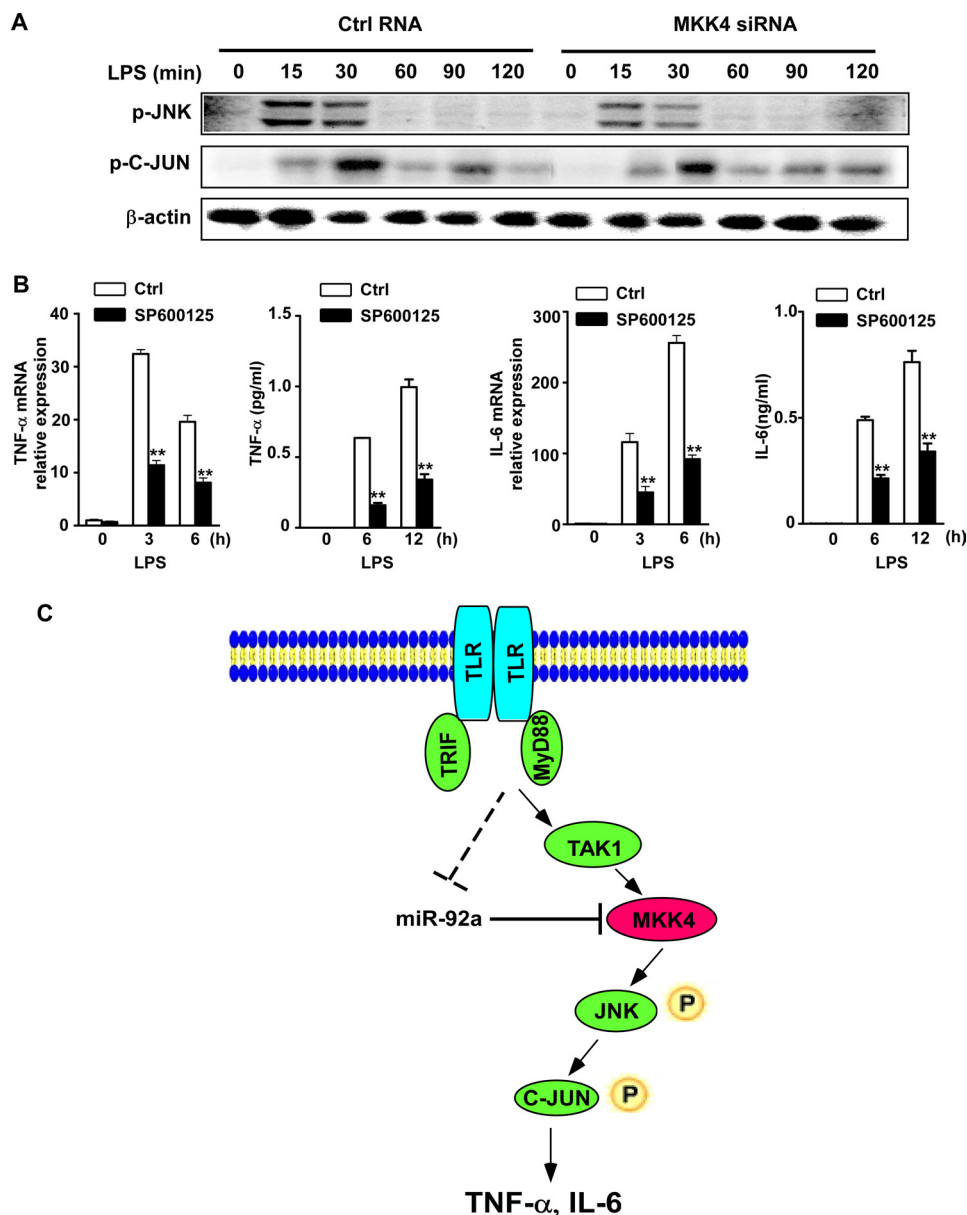
FIGURE 7. **Knockdown of MKK4 inhibits LPS-induced activation of JNK pathway.** *A*, RAW264.7 cells were transfected with control (*Ctrl*) or MKK4 siRNA. 24 h after transfection, the cells were cultured with or without 100 ng/ml LPS for different time points as indicated. p-JNK, p-c-Jun, and JNK levels were measured by immunoblotting. Data shown are one representative experiment of three. *B*, RAW264.7 cells were pretreated with SP600125 (20 μM) for 4 h and then stimulated with 100 ng/ml LPS. The mRNA levels of TNF-α and IL-6 were evaluated by qRT-PCR and normalized to β-actin, and the protein levels of TNF-α and IL-6 in the supernatants were determined by ELISA. Data are the mean $\pm$ S.D. of one representative experiment. Similar results were obtained in three independent experiments. *, $p < 0.05$ and **, $p < 0.01$. *C*, diagram depicting the signaling pathways for miR-92a in the regulation of TLR-triggered inflammatory responses in macrophages. *P*, phosphorylation.

in the immune response need to be further elucidated. In this study, we demonstrated that miR-92a was involved in the regulation of the production of proinflammatory cytokines such as TNF-α and IL-6, which were significantly inhibited when miR-92a was overexpressed. On the other hand, inhibition of miR-92a resulted in an increase in levels of TNF-α and IL-6.

MicroRNAs have been thought to target multiple mRNAs, and a single mRNA might be regulated by several miRNAs. Members in the miR-92a family (miR-25, miR-92b, miR-32, miR-363, and miR-367) that have similar seed sequences may target the same mRNA UTR. miR-25 belongs to the miR-106b-25 cluster that was involved in development and tumorigenesis with the miR-17–92a cluster (57, 58). miR-25 itself has

been demonstrated to regulate apoptosis by targeting Bim in human ovarian cancer (59) and promote apoptosis resistance in cholangiocarcinoma by targeting NF-related apoptosis-inducing ligand (TRAIL) death receptor-4 (40). In addition, miR-25 with other miRNAs collectively influences MKK4 abundance during replicative senescence (60), but its function in innate immunity is unknown.

The decreased miR-92b expression in mantle cell lymphoma augments the target gene *PRMT5* translation and leads to altered epigenetic modification of chromatin, which in turn impacts transcriptional performance of anticancer genes and growth of transformed lymphoid cells (41). Another family member, miR-32, induced by androgen, is overexpressed in

castration-resistant prostate cancer, thus leading to reduced expression of BTG2, which is associated with a short progression-free time in patients who underwent prostatectomy (42). miR-32 can also regulate the lipid metabolism of oligodendrocytes and myelin by targeting SLC45A3 (61). It is not certain whether these miRNAs take part in innate immune responses or not. In our work, we found that miR-25 decreased during activation induced by TLR3 and TLR4 agonists, and miR-92b was also reduced during activation induced by TLR2, TLR3, and TLR4 agonists. These multiple miRNAs may also participate in increasing MKK4 abundance in coordination with miR-92a.

In the present study, we found that miR-92a was present at much higher levels than the other miRNAs of its family and displayed the most drastic down-regulation after TLR ligand stimulations in the macrophages. This indicates that miR-92a may play a dominant role in the regulation of the TLR signaling. The reduction of miR-92a after ligand activation for TLRs may be both MyD88-dependent and TRIF-dependent; the exact mechanism still needs to be further investigated. MKK4 was proven to be a direct target of miR-92a; this posttranscriptional regulation contributes to the positive regulatory mechanism in properly controlling TLR-induced inflammatory response signaling. It is probable that there are some other potential targets that also modulate the LPS-triggered inflammatory response. Future works need to be done to reveal all the functions of miR-92a in controlling innate immune response.

miR-92a also belongs to the miR-17–92a cluster and is located on chromosome 13q32–33, which is highly expressed in B-cell lymphoma and different cancers (35, 36, 57). The exact biological activities of each miR-17–92 component have not been clearly dissected. However, knocking out the miR-17–92 cluster and its paralogous clusters, miR-106a-363 and miR-106b-25, both individually and in combination, has shown different knock-out phenotypes. Whether the miR-17–92 cluster is involved in the TLR-triggered innate immune response is unknown. It has been reported that following LPS stimulation, NF-κB p65 can directly bind to the promoter elements of miR-17–92, which induces the expression of the miR-17–92 cluster in human biliary epithelial cells (62). In our present work, for the first time, we demonstrated that miR-92a, one component of the miR-17–92 cluster, decreased after TLR ligand treatment and negatively regulated the production of TNF-α and IL-6, suggesting that the feedback of miR-92a expression may be required for optimal inflammatory cytokine production. The clarification of the molecular mechanisms that regulate the production of inflammatory cytokines is crucial for understanding the pathogenesis of inflammatory and autoimmune diseases and identification of potential targets for therapeutic intervention. The significance of decrease in miR-92a expression after LPS exposure still needs to be confirmed in the future.

MKK4 is a conserved gene that possesses two common binding sites of miR-92a in its 3′-UTR. We confirmed that miR-92a directly targets MKK4. MKK4 is activated by the majority of MKKKs, such as ASK, MEKK, and TAK (8), and preferentially phosphorylates the tyrosine residue of JNKs, which are required for optimal JNK activation (63). JNK can be strongly activated in multiple cell types by LPS or inflammatory cyto-

kines and regulates AP-1 transcription factor activity and the production of inflammatory cytokines (64, 65). Previous studies demonstrated that MKK7, but not MKK4, is essential for IL-1-mediated JNK activation in cultured fibroblast-like synoviocytes (52). However, the functions of MKK4 in regulating the innate immune responses in the macrophages have not been fully defined. Therefore, we examined how MKK4 regulates TLR-induced inflammatory responses in murine macrophages. In our experiments, we showed that knockdown of MKK4 in mouse macrophages significantly decreased the LPS-triggered production in TNF-α and IL-6, suggesting that MKK4 is partially required for JNK/Jun pathway activation induced by TLR4 ligation. These discrepancies may be due to different cell types and experimental systems.

In conclusion, our results demonstrated that miR-92a expression is down-regulated by stimulation of multiple TLR ligands in macrophages, which, in turn, promotes the production of proinflammatory cytokines through regulating the MKK4-mediated activation of JNK/c-Jun signaling. Our findings provide a new explanation characterizing the molecular mechanism responsible for the tight regulation of TLR-triggered macrophage activation.

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