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Tripartite Motif Containing 52 Positively Regulates NF-κB Signaling by Promoting IκBα Ubiquitination in Lipopolysaccharide-Treated Microglial Cell Activation

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

AG Pei Zhang
BE Yimin Wu
CD Ruifeng Li
D Huicheng Lv
F Baolong Yu

Department of Orthopedics, The Second Affiliated Hospital of Inner Mongolia Medical University, Hohhot, Inner Mongolia, P.R. China

Corresponding Author: Pei Zhang, e-mail: zhangpei1966@sina.com
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Background: Microglial cell activation is the first response to spinal cord injury (SCI). The purpose of the study was to investigate the role and mechanism of tripartite motif containing 52 (TRIM52) in microglial cell activation and the inflammatory response.

Material/Methods: The cerebral cortex was isolated in rats, and primary microglial cells were subsequently incubated for 7 to 9 days and activated by lipopolysaccharide (LPS). TRIM52 overexpression and interference lentivirus were constructed, and primary microglial cells were transfected. Cytokine levels of interleukin-1β and tumor necrosis factor-α were detected using enzyme-linked immunosorbent assay kits. TRIM52 mRNA expression and protein levels were examined by real-time polymerase chain reaction and nuclear factor-kappa B (NF-κB) and inhibitory kappa B-alpha (IκBα) protein expression were examined by western blot. The interaction between TRIM52 and IκBα was analyzed by co-immunoprecipitation (Co-IP) detection. Microglial marker Iba-1 and microglial cell activation marker OX-42 were detected by immunofluorescent staining.

Results: Primary rat microglial cells were successfully isolated and activated by LPS. The expression levels of cytokines and TRIM52 and nuclear accumulation of NF-κB in microglial cells all increased in a dose-dependent manner with LPS. Cytokine and nuclear NF-κB levels decreased after TRIM52 knockdown, while the opposite expression pattern was found in microglial cells transfected with TRIM52 gene overexpression lentivirus. Co-IP revealed the association between TRIM52 and IκBα, and overexpressed TRIM52 promoted the ubiquitination of IκBα and significantly reduced its protein expression.

Conclusions: TRIM52 activated the NF-κB signaling pathway by promoting IκBα ubiquitination, thereby regulating LPS-induced microglial cell activation and the inflammatory response.

MeSH Keywords: Lipopolysaccharides • Microglia • Receptor Activator of Nuclear Factor-kappa B

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Background

Spinal cord injury (SCI) is a disastrous, life-altering occurrence, which is estimated by the World Health Organization to affect 250 000 to 500 000 individuals each year globally. Researchers are working on developing treatments for SCI, and microglial cells have become a promising area of research [1–4].

Broadly, microglial cells contribute to the phagocytosis (ingestion) of foreign particles, antigen presentation, and the secretion of growth factors and anti-inflammatory cytokines [5,6]. Spinal microglial cells originate from primitive yolk sac progenitors. These microglial cells are a key component in the formation of scars after SCI, and have been found to be involved in the inflammatory response after injury [2]. After SCI, microglial cells are activated to secrete a large number of neurotoxic amino acids, such as aspartic acid [7], and inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [8]. In addition, many studies have found that factor-kappa B (NF-κB) and inhibitory kappa B (I-κBα), important regulators in inflammatory cytokine expression, are related to the inflammatory response and survival of neuronal cells after SCI [9,10]. In the resting state, IκBα and NF-κB subunits p65 and p50 exist in the cytoplasm in an inactive state. When the upstream signal activates IκB kinase (IKK), IκBα is ubiquitinated, phosphorylated, and degraded by the activated IKK, inducing the 2 subunits of NF-κB to activate and transfer from the cytoplasm to the nucleus (especially, the p65 subunit). Combined with the corresponding inflammation-related genes, subunits p65 and p50 initiate the transcription of inflammatory cytokines and induce inflammation [11].

Tripartite motif containing 32 (TRIM32) participates in antigen processing and presentation and affects neuronal differentiation, axonal regeneration, and motor function recovery after SCI [12–14]. TRIM52, a TRIM-protein family member, plays a role in autophagy, innate immunity, and carcinogenesis [15]. TRIM52 was found to be crucial for cell context-dependent proliferation in a p53-dependent manner [16]. In addition, it is a positive regulator of NF-κB signaling and plays an oncogenic role in tumorigenesis [17,18], including blocking tumor growth, inhibiting cell proliferation, and promoting cell apoptosis in tumors. Investigating the role and mechanism of TRIM52 in microglial cell activation and the inflammatory response is critical to the development of SCI treatment.

Lipopolysaccharide (LPS) is often used for microglial cell activation, neuroinflammation, neurotoxicity, and cytokine secretion [19–21]. In this study, different concentrations of LPS were applied to the primary microglial cells of treatment rats (0, 20, 50, and 100 ng/mL). Microglial activity and inflammatory activity were analyzed in TRIM52-knockdown and overexpressed microglial cells, which may explain the effect of TRIM52 on microglial cell activation after SCI.

Material and Methods

Isolation of primary microglial cells

The cerebral cortex was separated from the brains of neonatal Sprague-Dawley rats, then washed and cut into pieces in precooled Earle's balanced salt solution (EBSS). After EBSS was removed, the tissues were digested in 3 mL of 0.2% collagenase I for 15 min, with shaking of the tube every 5 min during digestion until the agglomeration appeared. Dulbecco's modified Wagle's medium (DMEM) was subsequently added. Following centrifugation at 15 000 r/min for 5 min, the pellet was collected and air-dried at least 30 times. After gradient centrifugation at 1000 r/min for 5 min, the pellet was resuspended. An amount of 1 mL of cell suspension was seeded in a T75 cm flask with DMEM (9 mL) and then incubated in 5% CO_2 at 37°C. After 3 days, the medium was refreshed, and the microglial cells were collected and cultured for 7 to 9 days.

LPS-induced microglial cell activation

The collected microglial cells were resuspended and seeded into 24-well (10^6 cells per well) plates. After 48 h, the cells were incubated without (0 ng/ml) or with LPS (20, 50, and 100 ng/mL) (Sigma-Aldrich) in medium.

TRIM52 overexpression and interference lentivirus construction

The TRIM52 (NM_001106056.1) coding sequence was synthesized and validated, and then subcloned into a pLVX-Puro vector (Addgene, Cambridge, MA, USA) via the EcoR I/BamH I sites. The TRIM52 overexpression plasmids (oeTRIM52) were purified using a commercial kit (Solarbio, Beijing, China). The sequences of TRIM52 siRNA (siTRIM52) were synthesized and inserted into the pLKO.1-puro vector (Addgene). The sequences were as follows:
siTRIM52-1 (site at 510-528), 5'-CCACCAATACCATCACTAT-3';
siTRIM52-2 (site at 706-724), 5'-CCACAATGCCGAAGGACAT-3';
siTRIM52-3 (site at 771-789), 5'-CCATATAATTCGCCAGATT-3'.

Cell transfection

Primary microglial cells in logarithmic growth were trypsinized and counted for 1×10^6 cells/mL suspension. Then, 2 mL of suspension was inoculated into 6-well plates for overnight culture in 5% CO_2 at 37°C. Next, siTRIM52-1, siTRIM52-2, siTRIM52-3, siNC, oeTRIM52 (multiplicity of infection [MOI]=5, 5 μL), and empty plasmids (vector, MOI=5, 5 μL) were transferred to cells with 60-70% confluency using Lipo2000. After 24 h of transfection, serum-free transfer solution was replaced by complete medium to culture for 48 h.

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Enzyme-linked immunosorbent assay

After treatment followed by centrifugation at 2000 to 3000 rpm/min for 20 min, the cell culture supernatants were reserved. Following the manufacturer's instructions, cytokine levels (IL-1β and TNF-α) were assayed using rat-specific ELISA kits (#K0223, Thermo Fisher Scientific, Waltham, MA, USA).

Real-time polymerase chain reaction assay

The total RNA in cells was extracted using TRIzol® reagent (Invitrogen, 1596-026). After quantification and integrity analysis, RNA was reverse transcribed using a reverse transcription kit (#K1622, Fermentas, Lithuania). Then, real-time polymerase chain reaction (RT-PCR) was conducted using the ABI 7300 system (Applied Biosystems, Waltham, MA, USA) with GAPDH as the endogenous control: 10 min initial denaturation at 95 °C was followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. The following primer sequences were applied:
TRIM52-Forward (F): 5'-GCCCTATGCAGTCACTTC-3';
TRIM52-Reverse (R): 5'-CCGGTCCTGTTCATCTTC-3';
NF-κB-F: 5'-AAACCAAAGCCCTGAAAG-3';
NF-κB-R: 5'-AGCAGTGACAACACAAAG-3';
IκBα-F: 5'-AATCCTGACCTGGTCTCGC-3';
IκBα-R: 5'-CGTAGGGCAACTCATCTTCC-3';
GAPDH-F: 5'-GGAGTCTACTGGCGTCTTCAC-3';
and GAPDH-R: 5'-ATGAGCCCTTCCACGATGC-3'.

Western blot analysis

After collection using a radioimmunoprecipitation assay (RIPA) buffer (R0010, Solarbio) and quantification using a bicinchoninic acid (BCA) assay kit (PICPI23223, Thermo Fisher Scientific), total protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (Millipore, USA). After blocking in dried skimmed milk powder, the membranes were probed with anti-TRIM52 (1: 500, H00084851-M01, Novus), anti-NF-κB (1: 2000, Ab16502, Abcam), anti-H3 (1: 1000, Ab1791, Abcam), or anti-GAPDH (1: 2000, #5174, Cell Signaling Technology) antibodies at 4 °C overnight. The blots were then washed and incubated with a horseradish peroxide-conjugated secondary antibody (1: 1000, Beyotime, China). Later, the membranes were developed with a chemiluminescent reagent (Millipore, WBKLS0100), and visualized by an enhanced chemiluminescence method (Tanon, China). Beta(β)-actin served as an endogenous reference.

Co-immunoprecipitation detection

For co-immunoprecipitation (Co-IP) detection, the total proteins of microglial cells were grouped to incubate with rabbit-IgG (Sc-2027, Santa Cruz Biotechnology), IP-indicated antibodies, and untreated proteins as an input control. The mixtures were then incubated with Protein A/G PLUS-Agarose to form an immune complex. After centrifuging at 3000 rpm and 4 °C for 4 min, 1 mL of lysate was added to wash the Protein A and G PLUS-Agarose beads, and protein loading buffers were added to boil for 5 min. Following centrifugation at 3000 rpm for 1 min, the supernatant was collected for western blot analysis. Anti-TRIM52 (sc-135589, Santa Cruz Biotechnology) and anti-IκBα (#4812, Cell Signaling Technology) antibodies were applied for IP detection, while anti-TRIM52 (H00084850-M01, Novus) and anti-IκBα (#4812, Cell Signaling Technology) were used in western blot analysis.

Immunofluorescence

Primary microglial cells were washed and fixed in paraformaldehyde. After permeabilization with 0.5% Triton X-100, the cells were blocked in BSA. Subsequently, the cells were treated with anti-Iba-1 (Ab178847, Abcam) and anti-OX-42 (Ab1211, Abcam) antibodies. Next, the cells were incubated with fluorescein-labeled secondary antibodies (A0423 or A0428, Beyotime) for 2 h at room temperature, and sealed with mounting medium (P0126, Beyotime). Images were collected with a laser scanning microscope.

Statistical analysis

All experiments were independently repeated 3 times. Data are presented as mean±standard deviation (SD). Data analysis was performed using GraphPad prism v.7.0 (Graphpad, USA). The *t* test was used to evaluate the difference between 2 groups, while one-way analysis of variance with post hoc tests was used to compare among multiple groups. The criteria for statistical significance was a *P* value less than 0.05.

Results

Microglial cell isolation and activation by LPS

Primary rat microglial cells were isolated, and immunofluorescent staining of Iba-1 microglial cell markers was positive (Figure 1A), identifying them as microglial cells. After treating rat primary microglial cells with different concentrations of LPS, the expression of microglial cell activation marker OX-42 was significantly increased (Figure 1B).

LPS-induced inflammatory response and NF-κB signaling pathway activation

Cytokine levels (Figure 1C, 1D) and TRIM52 protein levels were significantly increased by LPS in a dose-dependent manner in microglial cells (Figure 1E). In addition, NF-κB signaling was

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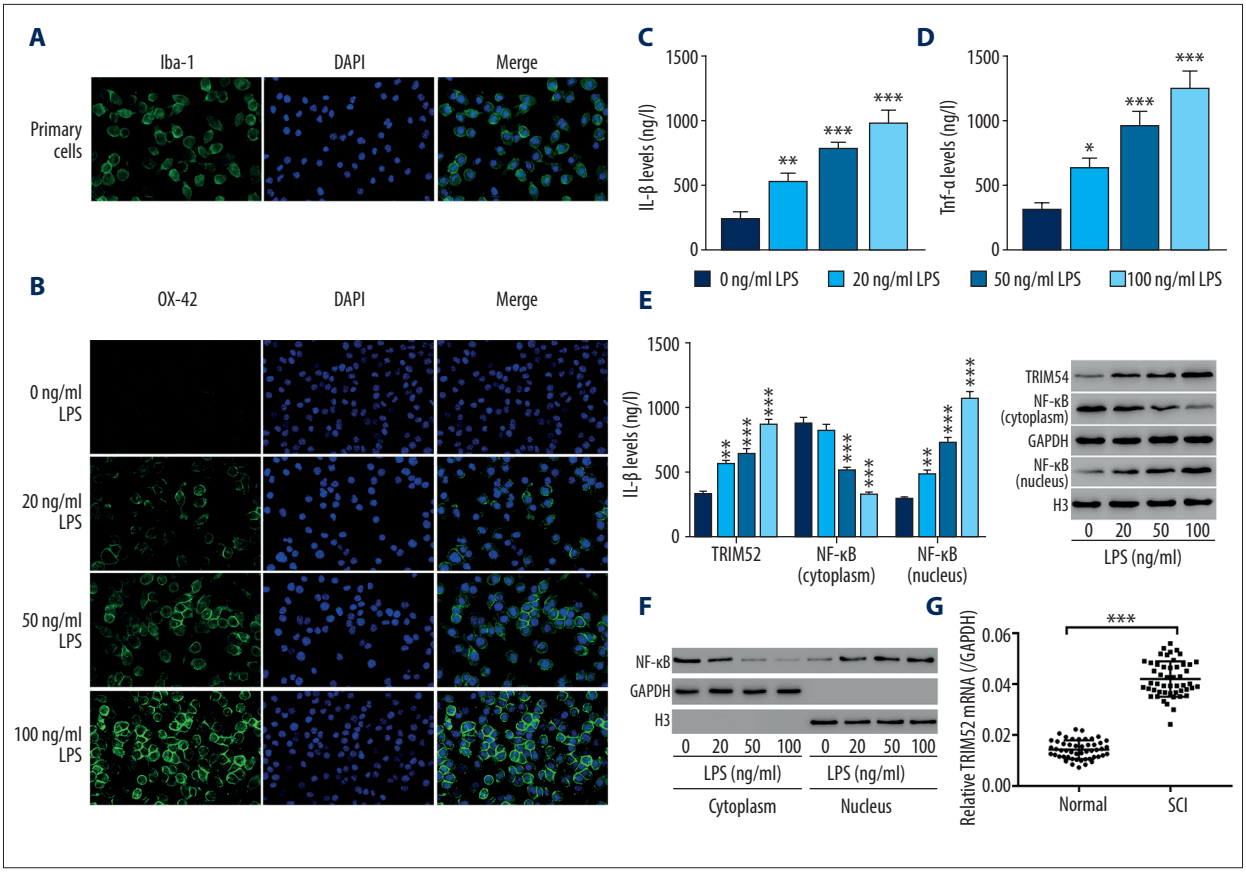

Figure 1. Tripartite motif containing 52 (TRIM52) was highly expressed in lipopolysaccharide (LPS)-activated primary microglial cells. (**A**) Primary rat microglial cells were isolated, and immunofluorescent staining of Iba-1 microglia markers was positive. Rat primary microglial cells were treated with different concentrations of LPS (0, 20, 50, and 100 ng/ml) for 6 h. (**B**) Immunofluorescence staining was used to detect the expression of microglial activation marker OX-42. (**C, D**) ELISA was used to detect the levels of IL-1β (**C**) and TNF-α (**D**) in the cell supernatant. (**E**) Western blot was used to detect TRIM52 and NF-κB (cytoplasm and nuclear) protein expression. (**F**) The efficient isolation of cytosolic and nuclear proteins was shown by western blot. (**G**) The expression of TRIM52 in SCI patients was detected. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs.* 0 ng/mL LPS or 20, 50, and 100 ng/ml.

activated, whereby the nuclear NF-κB level was increased, in LPS-treated microglial cells (Figure 1E). And the result of western blot analysis showed the efficient isolation of cytosolic and nucleus proteins (Figure 1F). This shows that LPS treatment induced microglial activity and inflammation. TRIM52 expression was significantly increased and the NF-κB signaling pathway was activated in activated microglial cells. From our results and those of a previous study [22], 100 ng/mL of LPS was selected for subsequent experiments. Furthermore, compared to healthy patients, TRIM52 was significantly highly expressed in the tissues of SCI patients (Figure 1G).

Knockdown of TRIM52 inhibited LPS-induced inflammatory changes and NF-κB signaling activation

The TRIM52 gene of rat primary microglial cells was interfered with siRNA mediated by lentiviral vectors. After treatment with LPS at 100 ng/mL and TRIM52 downregulation, it was found that downregulating TRIM52 (Figure 2A, 2B) inhibited LPS-induced cytokines IL-1β and TNF-α levels (Figure 2C). Moreover, downregulation of TRIM52 significantly inhibited LPS-induced NF-κB signaling pathway activation in microglial cells (Figure 2D). And the result of western blot analysis showed the efficient isolation of cytosolic and nucleus proteins (Figure 2E). These results showed that downregulating TRIM52 can inhibit an LPS-induced inflammatory response, and that TRIM52 may be related to NF-κB signaling.

LPS-induced inflammatory changes and NF-κB signaling activation after TRIM52 overexpression

Rat primary microglial cells were successfully transfected with TRIM52 gene overexpression lentivirus (Figure 2A, 2B) and treated with 10 μmol/L NF-κB inhibitor pyrrolidine dithiocarbamate

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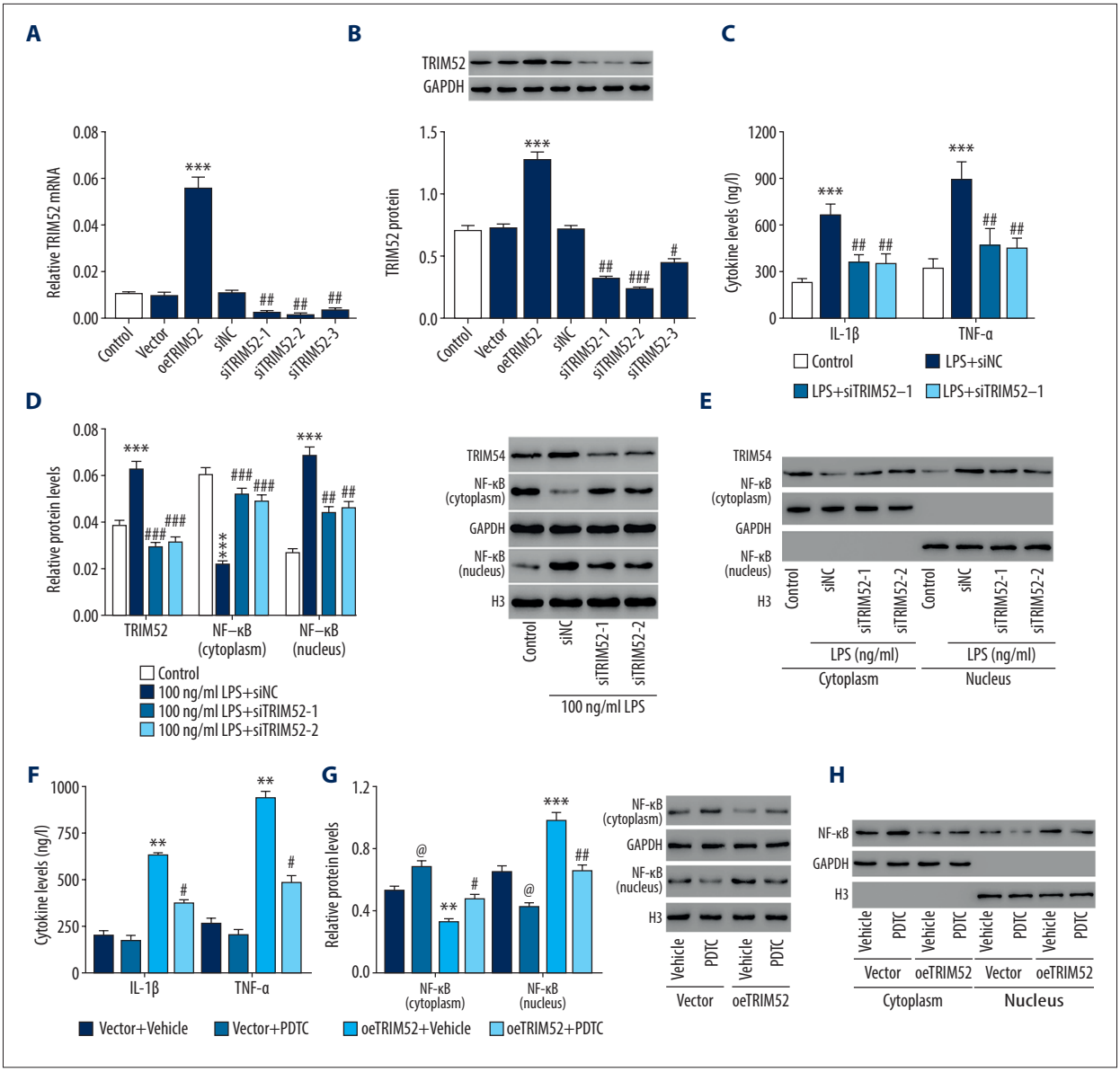

Figure 2. Knockdown of tripartite motif containing 52 (TRIM52) significantly inhibited lipopolysaccharide (LPS)-induced inflammatory response, likely through inhibition of NF-κB signaling activation. Construction of TRIM52 interference and overexpression lentivirus transfected rat microglial cells *in vitro*: Q-PCR (**A**) and western blot (**B**) were used to detect TRIM52 interference and overexpression efficiency *** $P<0.001$ *vs.* Vector; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ *vs.* siNC. The TRIM52 gene interfered with lentivirus pre-transfected rat primary microglial cells and was treated with LPS at 100 ng/ml for 6 h. (**C**) ELISA was used to detect the levels of IL-1β and TNF-α in the cell supernatant. (**D**) Western blot was used to detect protein expressions of TRIM52 and NF-κB (cytoplasm and nuclear). *** $P<0.001$ *vs.* Control; ## $P<0.01$, ### $P<0.001$ *vs.* 100 ng/mL LPS+siNC. (**E**)4The efficient isolation of cytosolic and nuclear proteins was shown by western blot. TRIM52 gene-overexpressing lentivirus transfected rat primary microglial cells were treated with 10 μmol/L NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). (**F**) ELISA was used to detect the levels of IL-1β and TNF-α in the cell supernatant. (**G**) Western blot was used to detect NF-κB (cytoplasm and nuclear) protein expression. (**H**) The efficient isolation of cytosolic and nuclear proteins was shown by western blot. ** $P<0.01$, *** $P<0.001$ *vs.* Vector+Vehicle; # $P<0.05$, ## $P<0.01$ *vs.* oeTRIM52+Vehicle; @ $P<0.05$ *vs.* Vector+Vehicle.

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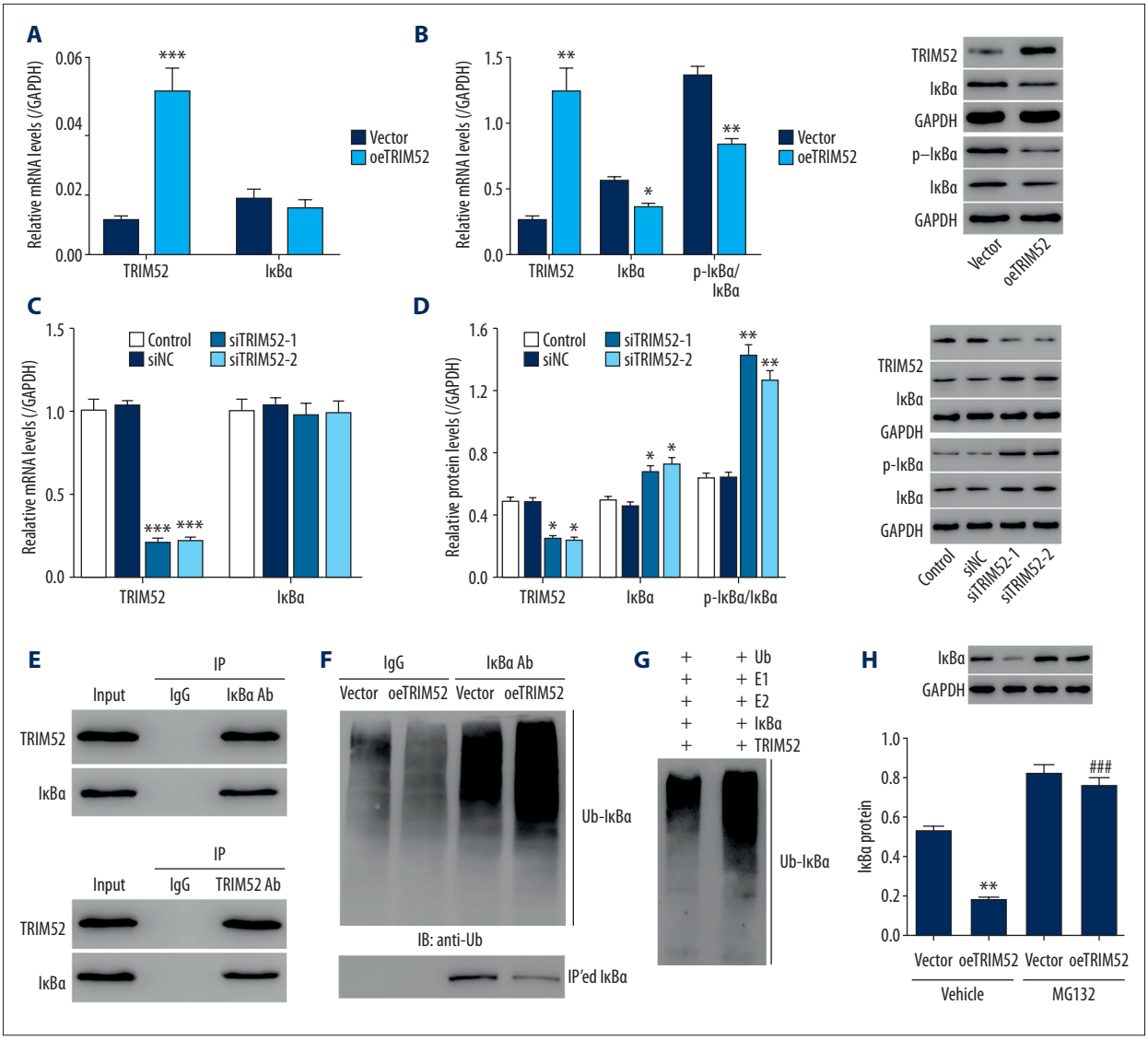

Figure 3. Tripartite motif containing 52 (TRIM52) activated the NF-κB signaling pathway through promotion of IκBα protein ubiquitination. TRIM52 gene overexpression lentivirus transfected rat primary microglial cells: (**A**) The mRNA expression of TRIM52 and IκBα was detected by Q-PCR. (**B**) The protein levels of TRIM52, IκBα, and p-IκBα were detected by western blot. TRIM52 gene interference lentivirus transfected rat primary microglial cells: (**C**) The mRNA expression of TRIM52 and IκBα was detected by Q-PCR. (**D**) The protein levels of TRIM52, IκBα, and p-IκBα were detected by western blot. (**E**) Co-IP detected TRIM52 and IκBα interaction in rat primary microglia. (**F**) Overexpression of TRIM52 gene in rat primary microglia. Western blot was used to detect IκBα ubiquitination. (**G**) Co-IP detected TRIM52 and IκBα interaction by E3 ligase activity. (**H**) TRIM52 gene overexpression in rat primary microglial cells was treated with 10 μmol/L MG132 (proteasome inhibitor). Western blot was used to detect IκBα protein expression. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs.* Vector, siNC or Vehicle+Vector; ### $P<0.001$ *vs.* Vehicle+oeTRIM52.

(PDTC). We found that PDTC significantly inhibited expression levels of TRIM52-induced cytokines (Figure 2F) and NF-κB signaling was activated by TRIM52, which was inhibited by PDTC (Figure 2G). Also, the result of western blot analysis showed the efficient isolation of cytosolic and nucleus proteins (Figure 2H). This indicated that TRIM52 may stimulate NF-κB signaling, which subsequently regulates the inflammatory response.

Regulation of the inflammatory response in microglial cells by TRIM52-promoted ubiquitination of IκBα

Overexpression of TRIM52 was found to significantly inhibit IκBα phosphorylation and IκBα protein expression without affecting mRNA levels (Figure 3A, 3B), whereas TRIM52 knockdown had the opposite effect (Figure 3C, 3D). Co-IP revealed the interaction between TRIM52 and IκBα (Figure 3E), and

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overexpression of TRIM52 significantly promoted the ubiquitination of IκBα (Figure 3F). Furthermore, we found that the regulation of NF-κB signaling by TRIM52 was due to E3 ligase activity (Figure 3G). Western blot results showed that TRIM52 overexpression significantly reduced IκBα protein expression, but that the proteasome inhibitor MG132 counteracted the effects of TRIM52 (Figure 3H). It was shown that TRIM52 activated the NF-κB signaling pathway by promoting IκBα ubiquitination, thereby regulating the inflammatory response.

Discussion

Microglial cell activation is essential to the response to SCI. Previous studies have indicated that microglial cell activation is NF-κB dependent and can increase levels of inflammatory factors, such as TNF-α [23]. TRIM52, which is a positive regulator of NF-κB signaling, was investigated in this study to identify its role and underlying mechanism in microglial cell activation and inflammatory response. These results could help to illustrate the mechanism of action of TRIM52 in SCI pathophysiology.

Cytokine levels and NF-κB accumulation in nuclei have been found to increase in activated microglial cells after SCI [24]. These results were confirmed by the LPS-induced microglial cell activation in the present study. In addition, the level of TRIM52 in the present study was raised with LPS concentration. Although there is currently no evidence of a direct link between TRIM52 and microglial cell activation after SCI, TRIM52 is closely associated with NF-κB signaling and the inflammatory response. Previous studies have reported that some TRIM proteins, including TRIM8 [25], TRIM22 [26], and TRIM52 [18,27], could regulate NF-κB transcription and cytokine-induced (including IL-1β and TNF-α) NF-κB activation. The activated NF-κB signaling pathway was also found to contribute to TRIM52-mediated regulation of tumorigenesis in hepatocellular carcinoma [28] and ovarian cancer [17]. Our results showed that the LPS-induced inflammatory response by IL-1β and TNF-α, and NF-κB signaling were inhibited after TRIM52 knockdown and activated after TRIM52 overexpression. These results demonstrate the importance of TRIM52 for microglial cell activation.

Additionally, the transient activation of cytoplasmic NF-κB from an inactive form is regulated by ubiquitination of IκBα, which could enable the translocation of NF-κB from the cytoplasm to nucleus to be activated [29]. BAY 11-7082 and MG132 are pharmacological NF-κB inhibitors. BAY 11-7082 exerts a neuroprotective effect by inhibiting IκBα phosphorylation and NF-κB activation in cases of inflammation, neoplastic disease, and neurodegenerative disease [30]. Our results indicate that overexpression of TRIM52 significantly promoted IκBα ubiquitination without affecting mRNA levels. Previous studies have suggested that the NF-κB pathway may be promoted by TRIM71 activation via an increase in the level of ubiquitination and IκBα degradation by means of the RING domain [31]. Similar results have indicated that the RING domain of TRIM52 is crucial for NF-κB activation and that increased TRIM52 levels could be induced by IL-6 [32]. Therefore, TRIM52 would contribute to the NF-κB signaling pathway by promoting IκBα ubiquitination, thereby regulating the inflammatory response.

Currently, the regulatory mechanism of TRIM52 on NF-κB activity remains largely unknown. There are several other possible connections between TRIM proteins and NF-κB. For example, Li et al. indicated that cytokine-induced (including cytokines TNF-α and IL-1β) NF-κB activation is regulated by TRIM8 via TGF-β activated kinase 1 polyubiquitination [33]. Therefore, the regulatory mechanism between TRIM52 and NF-κB signaling requires further study.

Conclusions

The expression level of TRIM52 is associated with LPS-induced microglial cell activation and inflammatory changes, and TRIM52 activates the NF-κB signaling pathway by promoting IκBα ubiquitination. The putative functional factors and regulatory signaling pathways identified in this study provide important leads for designing future studies to increase our understanding of the mechanism of SCI and aid in the development of a novel therapeutic strategy.

Conflicts of interest

None.

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