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TRIM59 expression is regulated by Sp1 and Nrf1 in LPS-activated macrophages through JNK signaling pathway

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Abstract

Activated macrophages play an important role in many inflammatory diseases including septic shock and atherosclerosis. TRIM59 has been showed to participate in many pathological processes, such as inflammation, cytotoxicity and tumorigenesis. However, the molecular mechanisms controlling its expression in activated macrophages are not fully understood. Here we report that TRIM59 expression is regulated by Sp1 and Nrf1 in LPS-activated macrophages. TRIM59 is highly expressed in macrophages, and markedly decreased by LPS stimuli *in vivo* and *in vitro*. TRIM59 promoter activity is also significantly suppressed by LPS and further analysis demonstrated that Sp1 and Nrf1 directly bound to the proximal promoter of TRIM59 gene. LPS treatment significantly decreased Sp1 expression, nuclear translocation and reduced its binding to the promoter, whereas increased Nrf1 expression, nuclear translocation and enhanced its binding to the promoter. Moreover, LPS-decreased TRIM59 expression was reversed by JNK inhibitor. Finally, TRIM59 level is significantly decreased during atherosclerosis progression. Taken together, our results demonstrated that TRIM59 expression was precisely regulated by Sp1 and Nrf1 in LPS-activated macrophages, which may be dependent on the activation of JNK signaling pathway and TRIM59 may be a potential therapeutic target for inflammatory diseases such as atherosclerosis.

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Author contributions

Yanying An, Zhihao Xu and Jiashu He conducted biochemical and *in vivo* experiments. Yuqi Ni, Shuizhen Shi and Yu Liu conducted molecular biology experiments. Ke-Yu Deng, Mingui Fu and Hong-Bo Xin edited the paper before submission. Meixiu Jiang designed experiments and prepared the paper.

Declaration of Competing Interest

The Authors declare that there are no competing interests associated with the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109522.

Keywords

Macrophage; TRIM59; Sp1; Nrf1; Atherosclerosis

1. Introduction

Inflammation is the initial host immune reaction and is mediated by activating various immune cells, such as macrophages, which play an important role in host defense against microbial and viral infections [1,2]. Activated macrophages by inflammatory stimuli elaborate a large array of cytokines, growth factors and proteolytic enzymes that are critical for tissue damage and repair [3–5]. However, overproduction of these inflammatory mediators may lead to pathogenesis of many inflammatory diseases, such as septic shock, cancer and atherosclerosis [6,7]. Atherosclerosis is recognized as a chronic inflammatory disease and is the primary cause of most cardiovascular diseases, such as coronary artery disease (CAD), myocardial infarctions and strokes [8,9]. As the major component in atherosclerotic lesions, macrophages play a critical role in the development of atherosclerosis [10].

Lipopolysaccharide (LPS/endotoxin), the major constituent of the outer membrane of gram-negative bacteria, was used to induce activation of macrophages experimentally [2]. Mechanically, LPS mainly interacts with monocytes and/or macrophages *via* Toll-like receptor 4 (TLR4) to activate a number of intracellular signal pathways including PI3K/Akt, mitogen-activated protein kinases (MAPKs) cascades (ERK1/2, c-JNK, and p38), as well as nuclear translocation of NF-κB and AP-1 [11–13], which leads to release of multiple inflammatory mediators [14–16]. Furthermore, the activated MAPKs can phosphorylate some transcription factors to regulate target genes expression [17].

TRIM59 is a member of the tripartite motif-containing (TRIM) protein superfamily. It has a TRIM or RBCC motif consisting of a RING-finger domain (R), a B-box domain (B), and a coiled-coil domain [18,19]. Previous studies show that TRIM59 participates in many pathological processes, such as inflammation [5,20], cytotoxicity [21] and especially tumorigenesis [19,22]. TRIM59 interacts with evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) and negatively regulates NF-κB and IRF-3/7-mediated signal pathways [23]. TRIM59 also regulates autophagy through modulating both transcription and ubiquitination of beclin 1 (BECN1) [24]. Moreover, TRIM59 plays a vital role in mouse early embryonic development stage, in which TRIM59 deficiency leads to embryonic lethality in mice by impairing gastrulation development [25]. Our previous study showed that TRIM59 expression was inhibited by LPS and TLR3 ligands in macrophages [5], but the underlying mechanisms remain to be elucidated.

Herein, we report that TRIM59 expression is controlled by Sp1 and Nrf1 in LPS-activated macrophages, which may be dependent on the activation of JNK signaling pathway. Our results also indicate that TRIM59 is highly expressed in macrophages, but significantly decreased *in vivo* and *in vitro* by LPS stimuli and during atherosclerosis progression. Combined with the anti-inflammatory properties of TRIM59 discovered in our previous work [5] and the function of inhibiting macrophage foam cells formation (data not shown),

these results indicate that TRIM59 may be a potential therapeutic target for inflammatory diseases such as atherosclerosis.

2. Materials and methods

2.1. Materials

LPS (L3129) and Wedelolactone (W4016) were purchased from Sigma. U0126 (HY-12031), SP600125 (HY-12041), SB203580 (HY-10256) and LY294002 (HY-10108) were obtained from MCE. Anti-TRIM59 (HPA017750, Sigma), Sp1 (CSB-PA004135, CusAb), Nrf1 (8052, CST, for Western blot; 12,936–1-AP, Proteintech, for CHIP array), TBP (ab818, Abcam) and Tubulin (sc-5286, Santa Cruz) antibodies were purchased as indicated.

2.2. Cell culture

RAW264.7 cells, a murine macrophage cell line, were purchased from Cell Bank of Chinese Academy of Sciences and cultured in complete RPMI medium containing 10% fetal bovine serum (Gibco, NY, USA), 50 μg/ml of penicillin/streptomycin and 2 mM of glutamine. Cells were switched to serum-free medium at ~90% confluence for 2 h followed by indicated treatment. HEK (human embryonic kidney)-293 T cells were also purchased from Cell Bank of Chinese Academy of Sciences and cultured in complete DMEM (Dulbecco′s modified Eagle′s medium). Cells at 95% confluence were used for transfection and analysis of promoter activity. NCTC1469, HUVEC, HCMEC/D3, bEnd.3 and HASMC were also purchased from Cell Bank of Chinese Academy of Sciences.

To collect peritoneal macrophages, C57BL/6 male mice (8-week males) were i.p. injected with 3 ml 4% thioglycolate solution and maintained with access to water and normal chow for 5 days. The animals were anesthetized within a CO_2 chamber. The peritoneal macrophages were collected from mouse abdomen by lavage with PBS. Cells were cultured in complete RPMI medium for 2 h. After removal of all the floating cells, the adhesive cells (macrophages) were cultured in complete RPMI medium for an additional 2 days, followed by treatment in serum-free medium. Bone marrow cells were collected from mouse femurs and tibias. After removal of erythrocytes, bone marrow cells were extracted RNA and protein. Bone marrow-derived macrophages (BMDMs) were obtained from bone marrow cells and cultured in DMEM medium containing 10% FBS and 30% of L929-conditioned medium for 7 days.

Human blood monocyte-derived macrophages (hBMDMs) were obtained as described previously with blood obtained from healthy donors [26–28]. Briefly, after removal of red blood cells by incubating blood with dextran sedimentation mixture, the remained cells were collected by centrifugation (250 *g* for 5 min at 4 °C) and re-suspended in serum-free RPMI 1640 medium. The suspension was overlaid on the top of a Ficoll Hypaque solution. After gradient centrifugation for 20 min at 1000 *g*, monocytes in the interface layer were collected and washed twice with serum-free medium followed by culture in complete RPMI 1640 medium containing 50 ng/ml M-CSF for 7 days to differentiate into macrophages.

2.3. Animals and In vivo study

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by NIH and all the experimental protocols were approved by the Ethics Committee of Nanchang University. ApoE$^{-/-}$ mice on C57BL/6 J background were purchased from company of Charles River (China) and then bred in the transgenic animal Facility of Nanchang University.

To investigate the effect of LPS on TRIM59 expression *in vivo*, C57BL/6 J wild-type mice (~8-week-old, male) were randomly divided into two groups (5 mice/group), and i.p. injected with saline (control group) and LPS (20 mg/kg) for 16 h. After treatment, the animals were killed in a CO_2 chamber followed by collection of tissue samples (spleen, liver, bone marrow and aorta). Total cellular RNA and proteins were extracted from tissue samples and used for determination of TRIM59 mRNA and protein expression.

To determine the effects of western diet (WD: 0.5% cholesterol and 21% fat) on TRIM59 expression in mice aortas and macrophages, ApoE$^{-/-}$ mice (~12-week-old, male) were randomly divided into two groups (4 mice/group), and fed a Normal diet (ND) or WD for 10 weeks. The mice were injected intraperitoneally with 4% thioglycollate solution 5 days before the end of the experiments. At the end of the experiment, all of the mice were anesthetized and euthanized in a CO_2 chamber followed by collection of mouse aortas and peritoneal macrophages and determined the TRIM59 expression by Western blot and qPCR.

2.4. Human peripheral blood monocytes sample collection

The study with human samples was approved by the Ethical Review Board of Second Affiliated Hospital of Nanchang University (Nanchang, China) and adhered strictly to the Declaration of Helsinki Principle 2008. All samples were collected after the informed written consents were signed by patients and their family members. To determine the expression changes of TRIM59 in patients with hypercholesterolemia, 8 healthy donors (plasma total cholesterol < 5.17 mmol/l) and 6 patients with hypercholesterolemia (plasma total cholesterol > 6.2 mmol/l) enrolled in the study. Peripheral blood monocytes were isolated with Percoll and Ficoll density gradients [29] from the blood of these donors and washed twice with serum-free medium followed by RNA extraction and examined the expression of TRIM59 mRNA by qPCR.

2.5. Extraction of total or nuclear proteins for determination of TRIM59, Sp1 and Nrf1 protein expression by Western blot

After treatment, cells or tissues were lysed in an ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 50 μg/ml of aprotinin/leupeptin). The cellular lysate was centrifuged with a Microfuge for 15 min at 4 °C, and the supernatant was collected as whole cellular extract.

Extraction of nuclear proteins was performed as described previously [30]. After treatment, macrophages in 60-mm dishes were lifted using a cell scraper, washed twice with cold PBS, re-suspended in 400 μl of cold fresh buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM

EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and incubated for 15 min on ice, followed by addition of 50 μl of 10% NP-40 and vortex for 10 s. After spinning for 30 s at 16,200 g at 4 °C, the pellet was saved and re-suspended in 100 μl cold fresh buffer B (20 mM HEPES, pH 7.91, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin/aprotinin) and rocked for 15 min on a rocker at 4 °C. The mixture was centrifuged again for 5 min at 16,200 g at 4 °C, and the supernatant containing nuclear proteins was collected and divided into aliquots to keep at −80 °C. Expression of TRIM59, Sp1 and Nrf1 protein was determined by Western blot [5,28].

2.6. Isolation of total RNA and quantitative real time PCR (qPCR) analysis of TRIM59, Sp1 and Nrf1 mRNA expression

After treatment, cells or tissues were lysed in Trizol (Invitrogen). The lysate was mixed well with chloroform and centrifuged for 10 min using a Microfuge at 16,200 g at 4 °C. The top aqueous phase was collected and mixed with an equal volume of isopropanol to precipitate total cellular RNA. The cDNA was synthesized with 2 μg total RNA using an RT kit purchased from Thermo Scientific (USA). Real time PCR was performed using an SYBR green PCR master mix purchased from Roche (USA), and the PCR-specific amplification was conducted with the Applied Biosystems VIIATM real-time PCR machine (ABI, CA, USA) with the primers listed in Supplementary Table 1. Quantification was performed by the dCT method and GAPDH levels in the corresponding sample were used for normalization.

2.7. Inhibition of macrophage Sp1 and Nrf1 expression by siRNA

The siRNA against Sp1 or Nrf1 was purchased from RIB BI (China). The target sequences of Sp1 and Nrf1 (1#) were GGATGGTTCTGGTC AAATA and CAATGAGCTGCTGTCCAAATA, respectively. To selectively inhibit Sp1 or Nrf1 expression, RAW264.7 macrophages in 6-well plates were transfected with scrambled siRNA (25 nM), Sp1 siRNA (25 nM) or Nrf1 siRNA (25 nM) using Hiperfect (Qiagen) in RPMI 1640 medium. After 12 h of transfection, cells were added the same volume of medium and continued the transfection for another 12 h. The transfected cells were then switched into complete medium and cultured for another 24 h or followed by treatment overnight in serum-free medium. The cells were then lysed, and the cellular proteins or RNA were used to determine expression of TRIM59 and Sp1 or Nrf1 by Western blot or qPCR.

2.8. Preparation of plasmid DNA and determination of TRIM59 promoter activity

The Sp1 and Nrf1 expression plasmid was prepared as follows: The cDNA encoding mouse Sp1 or Nrf1 was generated by RT-PCR with total cellular RNA isolated from mouse peritoneal macrophages and the following primers for PCR: Sp1 forward, 5′-ATCTCGAGCATGAGCGACCAAGATCAC-3′; backward, 5′-CCCGGGCTAGAAACCATTGCCACT GAT-3′; and Nrf1 forward, 5′-CGGGGTACCGCATGCTTTCTCTGAAGAAA-3′; backward, 5′-TCCCCCGGGTCACTTCCTCCGGTCCTTTG-3′. After the sequence was confirmed, the PCR product was digested with *Xho*I and *Sma*I (for Sp1) or *Kpn*I and SmaI (for Nrf1), and then sub-cloned into expression vector of pEGFP-C2. The generated expression plasmids were named as pC2-Sp1 and pC2-Nrf1.

Mouse TRIM59 promoter (from −600 to +40) was constructed by PCR with genomic DNA extracted from mouse liver and the following primers: forward, 5′-CGGGGTACCTGTCTCCGAGGTCAAGTC-3′; and backward, 5′-CCGCTCGAGTGGTGCGGACACTGCGAG-3′ and named as p640TRIM59. To define the location of Sp1 or Nrf1 responsive elements, several shorter TRIM59 promoters than p640TRIM59 were constructed by PCR with p640TRIM59 DNA and forward primer at different location and the same backward primer mentioned above. The forward primers are as follows: p375TRIM59 (from −335 to +40), 5′-CGGGGTACCAGGAACCTGTGAGGTAAC-3′; p295TRIM59 (from −255 to +40), 5′-CGGGGTACCATAAGCAGTCCCTCAGCC-3′; p189TRIM59 (form −149 to +40), 5′-CGGGGTACCTGCCGCCCGTGCGCTTTC-3′; p87TRIM59 (form −47 to +40), 5′-CGGGGTACCCGCGGTTGAAAGT CGCTG-3′. After sequence was confirmed, the PCR product was digested with *Kpn*I and *Xho*I, followed by ligation with pGL4.1 luciferase reporter vector, transformed into *E Coli* for amplification. The TRIM59 promoter with deletion of the putative Sp1 binding site (from −380 to −371 or − 273 to −264) or deletion of the putative Nrf1 binding site (from −116 to −106) was constructed using the Phusion Site-Directed Mutagenesis kit from New England Biolabs with p640TRIM59 DNA and primers with the corresponding Sp1 or Nrf1 binding site deletion respectively. To analyze TRIM59 promoter activity, HEK-293 T cells or RAW264.7 cells in 24-well plates were transfected with DNA of the TRIM59 promoter or plus pC2-Sp1 or plus pC2-Nrf1 and *Renilla* (for internal normalization) using LipofectamineTM 3000 (Invitrogen). After 24 h transfection plus treatment, cells were lysed and cellular lysate was used to determine activity of firefly and *Renilla* luciferases using the Dual-Luciferase Reporter Assay system (Promega).

2.9. Transfection of peritoneal macrophages with Nrf1 expression plasmid

Peritoneal macrophages in 6-well plates were transfected with DNA for Nrf1 expression vector or pEGFP-C2 vector using LipofectamineTM 3000 (Invitrogen) in RPMI 1640 medium. After 8 h transfection, cells were switched into complete medium for another 48 h followed by treatment in serum-free medium. Cells were then lysed, and cellular proteins were used to determine expression of TRIM59 and Nrf1 by Western blot.

2.10. ChIP assay of Sp1 or Nrf1 DNA-binding activity

The ChIP assay was conducted as follows. After treatment cells were cross-linked by addition of formaldehyde, and then sonicated in a lysis buffer [50 mM Hepes-KOH (pH 7.5), 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS and protease inhibitors aprotinin/leupeptin] to fragment DNA into an average size of 500–1000 bp. The input PCR was conducted with DNA extracted from the sonicated chromatin after reversal of the cross-linking overnight at 65 °C. Immunoprecipitation was conducted with the same amount of chromatin from each sample based on the input and incubated with Sp1(Nrf1) antibodies or IgG control at 4 °C overnight. Beads were washed several times and DNA was purified and amplified by qPCR. The primers used for ChIP-qPCR analysis is provided in Supplementary Table 1. qPCR was performed with three technical and biological replicates.

2.11. Immunofluorescent staining analysis

RAW264.7 macrophages were cultured on cover slips in 24-well plates. After treatment, cells were washed twice with PBS (500 μl/well) and then fixed with 4% paraformaldehyde (PFA, 400 μl/well) for 30 min at room temperature. To determine the expression and location of Sp1 or Nrf1 protein, cells were incubated in 0.5% TritonX-100/PBS for 10 min and then blocked with 2% BSA for 2 h at room temperature, followed by incubation with an anti-Sp1 or Nrf1 rabbit polyclonal antibody (1:200 dilution) overnight at 4 °C. After removal of the primary antibody by washing with PBS for 30 min, cells were incubated with a Alexa 488-conjugated secondary antibodies (1:1000 dilution) for 45 min at 37 °C. After washing with PBS for 30 min, sections were stained with a DAPI solution to detect nucleus. Images of the sections were obtained with a fluorescence microscope (Olympus).

2.12. Data analysis

All experiments were repeated at least three times, and the representative results are presented. Values are represented as mean ± SEM. The statistical significance of differences was evaluated with the Student′s *t*-test or one-way analysis of variance (ANOVA). All statistical analyses were performed with GraphPad Prism 7 and SPSS 22.0 software. A value of $P < .05$ was considered significant.

3. Results

3.1. Tissue and cellular distribution of TRIM59

To define the tissue distribution of TRIM59 *in vivo*, we extracted total proteins and RNA from mouse tissues and examined the TRIM59 expression by Western blot and qPCR. As shown in Fig. 1A, TRIM59 is highly expressed in liver, kidney, adipose, testis and lung, and moderately expressed in spleen, lymph node, thymus and aorta, but lowly expressed in brain, heart, muscle and bone marrow.

Next, we examined TRIM59 expression in macrophages, endothelial cells and smooth muscle cells because all these cells take part in the development of atherosclerosis. As shown in Fig. 1B&C, TRIM59 was highly expressed not only in monocytes or macrophages (THP1, THP1/PMA, RAW264.7, PM and BMDM) but also in endothelial cells (HUVEC, HCMEC/D3 and bEnd.3). However, TRIM59 expression in smooth muscle cells is species dependent which is highly expressed in mouse but lowly expressed in human. The results suggest that TRIM59 may play a central role in inflammatory diseases such as atherosclerosis.

3.2. TRIM59 expression is decreased by LPS stimuli both in vivo and in vitro

To examine whether the expression of TRIM59 is inhibited *in vivo* during inflammation, male C57BL/6 J mice were intraperitoneally (i.p.) injected with LPS (20 mg/kg). At 16 h after LPS injection, we collected mouse tissues and determined expression of TRIM59 protein and mRNA in spleen, liver, bone marrow and aorta. Results in the left panels of Fig. 2 indicate that LPS significantly reduced TRIM59 protein expression in these tissues. Associated with changes in protein, the results of qPCR (right panels of Fig. 2) show TRIM59 mRNA expression in mouse tissues was also markedly reduced by LPS

administration in a similar manner. We also have determined that the expression of NOS2, TNFα and IL-6 mRNA were induced by LPS in mouse liver (Supplementary Fig. 1) and proved that LPS treatment indeed works.

Moreover, to determine if LPS reduces TRIM59 expression is in species-dependent manner, the human monocytes were isolated from blood and differentiated into macrophages which were then treated with LPS. Similar to mouse macrophages [5], the expression of human macrophage TRIM59 protein and mRNA were inhibited by LPS in dose-and time-dependent manners (Fig. 3A–D). In our previous study [5], we also found that TRIM59 expression was inhibited by TNFα and TLR3 ligands in macrophages. Taken together, TRIM59 expression was decreased by inflammatory stimuli both *in vivo* and *in vitro*, further suggesting that it may involve in the regulation of vascular inflammatory diseases such as atherosclerosis.

3.3. LPS inhibits macrophage TRIM59 expression at the transcriptional level

To further determine whether the inhibition of TRIM59 expression by LPS is at the transcriptional level, we constructed a TRIM59 promoter (p640TRIM59: from −600 to +40, Fig. 4A), which includes both several putative Sp1 binding sites (GC box) and Nrf1 binding site (ARE) based on sequence alignment analysis. The results in Fig. 4B indicate that LPS inhibited p640TRIM59 promoter activity, suggesting the inhibition occurs at the transcriptional levels.

Based on the sequence alignment analysis, several putative Sp1 or Nrf1 binding sites (GC box or ARE) were observed in the proximal region of TRIM59 promoter. To determine the role of these binding sites in LPS-inhibited TRIM59 transcription, we constructed different length of TRIM59 promoters which contains no, one or two GC box(es) or ARE, and determined the activity of these promoters (Fig. 4C). Compared to p640TRIM59, activity of p375TRIM59 was significantly decreased. Similarly, compared to p189TRIM59, activity of p87TRIM59 was also significantly decreased. In contrast, activity between p295TRIM59 and p375TRIM59 was closed. These data suggested that the existence of primary promoter regulatory elements between −600 and − 335 bp, −149 and − 47 bp plays an important role in regulating TRIM59 transcription. To further support the conclusion, we constructed TRIM59 promoters with one GC box deletion at different locations (p640TRIM59D1, p640TRIM59D2) and putative ARE deletion (p640TRIM59D3). Compared to p640TRIM59, as shown in Fig. 4D, the GC box deletion between −380 and − 371 bp greatly decreased promoter activity (> 50%) while the GC box deletion between −273 and − 264 bp rarely affected promoter activity. Deletion of ARE (from −116 to −106 bp) also substantially decreased promoter activity (> 70%). Taken together, these results suggest that GC box (−380 to −371 bp) and ARE (−116 to −106 bp) play a critical role in regulation of TRIM59 transcription.

To determine if inhibition of TRIM59 transcription by LPS depends on Sp1 expression, we transfected cells with a Sp1 expression vector at different concentrations and determined TRIM59 promoter activity in response to Sp1 expression. Exogenous Sp1 increased TRIM59 promoter activity in a dose-dependent manner (Fig. 5A). However, when the GC box (from −380 to −371) was deleted in p640TRIM59 promoter, Sp1 had little effect on the promoter activity (Fig. 5B), while the inhibitory effect of LPS on the promoter activity was

attenuated (Fig. 5C). We next examined whether mitA (mithramycin A), a well-known Sp1 inhibitor, could be able to affect the basal TRIM59 promoter activity. As shown in Supplementary Fig. 2A, mitA decreased TRIM59 promoter activity in a dose-dependent manner, supporting the concept that Sp1 plays a critical role in controlling TRIM59 transcription. Correspondingly, mitA decreased TRIM59 mRNA and protein expression (Supplementary Fig. 2B&C). These results indicate LPS inhibits TRIM59 expression at the transcriptional level, which is related to Sp1 activity.

To test if inhibition of TRIM59 transcription by LPS is also related to Nrf1 expression, we transfected cells with Nrf1 expression vector and TRIM59 promoter. In contrast to Sp1, exogenous Nrf1 decreased TRIM59 promoter activity dose-dependently (Fig. 5D). Interestingly, when ARE (from −116 to −106) was deleted, the effect of Nrf1 on promoter activity was attenuated (Fig. 5E), and the inhibitory effect of LPS on promoter activity was also blunted (Fig. 5F). These results also suggest that LPS inhibits TRIM59 expression in Nrf1-dependent manner.

3.4. LPS inhibits TRIM59 expression by inactivating Sp1 but activating Nrf1

To dissect if the inhibition of macrophage TRIM59 expression by LPS is completed by regulating Sp1 and/or Nrf1, we initially assessed the effects of LPS on Sp1 or Nrf1 expression. The results showed that LPS inhibited Sp1 mRNA expression but increased Nrf1 mRNA expression (Supplementary Fig. 3) in dose and time-dependent manners. Similarly, LPS also inhibited Sp1 protein expression (Fig. 6A, top panel) but increased Nrf1 protein expression (Fig. 6B, top panel) in whole cellular extract in a dose-dependent manner. Next, we further examined the effect of LPS on nuclear translocation of Sp1 or Nrf1. The results of Western blot analysis showed that LPS decreased Sp1 levels in nuclei (Fig. 6A, bottom panel), suggesting the reduced Sp1 nuclear translocation. The reduction of Sp1 nuclear translocation by LPS was further confirmed by the immunofluorescent staining assay (Fig. 6C). In contrast, LPS increased Nrf1 nuclear translocation which was determined by both Western blot (Fig. 6B, bottom panel) and immunofluorescent staining assay (Fig. 6D).

To further define the role of GC box and ARE in LPS-inhibited TRIM59 transcription, we determined the binding of Sp1 and Nrf1 proteins to GC box and ARE in TRIM59 promoter in response to LPS treatment by ChIP assay, respectively. The results showed that LPS reduced the interaction between Sp1 protein and GC box (Fig. 6E) but increased the interaction between Nrf1 protein and ARE (Fig. 6F).

The involvement of Sp1 in LPS-inhibited TRIM59 expression was further determined by the following experiments. RAW264.7 macrophages were transfected with Sp1 siRNA followed by LPS treatment. As showed in Supplementary Fig. 4A, Sp1 siRNA markedly reduced macrophage Sp1 and TRIM59 protein expression. Furthermore, although LPS inhibited TRIM59 protein expression in control siRNA-transfected macrophages, it slightly inhibited TRIM59 protein expression in Sp1 siRNA-transfected macrophages (Fig. 7A). Reciprocally, we transfected mouse peritoneal macrophages with GFP-Nrf1 overexpression plasmid to determine the role of activated Nrf1 expression in LPS-inhibited TRIM59 expression. Highly expressed Nrf1 reduced TRIM59 basal levels and further reduced LPS-inhibited TRIM59 protein expression (Fig. 7B). Moreover, siRNA-reduced Nrf1 expression also

markedly increased TRIM59 protein and mRNA expression (Supplementary Fig. 4B). Taken together, the results above clearly demonstrated that LPS inhibits TRIM59 expression mainly by inactivating Sp1 but activating Nrf1.

3.5. JNK pathways play a critical role in LPS-inhibited TRIM59 expression

MAPK (p38, ERK1/2, JNK), NF-κB and PI3K are considered as main pathways to be activated by LPS/TLR4. To identify if any of these pathway(s) is responsible for LPS-inhibited TRIM59 expression, RAW264.7 cells were pretreated with SP600125 (JNK inhibitor), U0126 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), Wedelolactone (IKK/NF-κB inhibitor) and LY294002 (PI3K inhibitor) for 3 h, followed by LPS stimulation. Interestingly, LPS-inhibited TRIM59 mRNA expression was substantially blocked by SP600125 but not by other inhibitors (Fig. 7C). Associated with mRNA expression, LPS-inhibited TRIM59 protein expression was also dampened by SP600125, but not by other inhibitors (Fig. 7D). Taken together, our results demonstrated that JNK pathway may play a critical role in LPS-inhibited TRIM59 expression.

3.6. The expression of TRIM59 is down-regulated during atherosclerosis progression

Atherosclerosis-related cardiovascular diseases are the leading cause of mortality worldwide and macrophages play an important role in the development of atherosclerosis, so we further detected the expression of TRIM59 in mouse aortas and macrophages collected from ApoE $^{-/-}$mice fed a normal diet or western diet (WD) for 10 weeks. As shown in Fig. 8A&B, both protein and mRNA of TRIM59 were significantly down-regulated in aorta and peritoneal macrophages after feeding a WD. Next, we examined the mRNA levels of TRIM59 in human peripheral blood monocytes between healthy donors and patients with hypercholesterolemia. Compared to healthy donors, TRIM59 mRNA levels were significantly decreased in patients with hypercholesterolemia (Fig. 8C). All these results suggest a potential role for TRIM59 in the development of atherosclerosis.

4. Discussion

Macrophages play a critical role in the development of atherosclerosis. Although inhibiting macrophage functions has been well recognized as a therapeutic strategy in atherosclerosis, the therapeutic targets are still elusive. Recently, the importance of TRIM59 in macrophages has been recognized, but the regulatory mechanism for its expression remains unclear. Our previous findings [5] on the functions of TRIM59 in anti-inflammation and its down-regulation by LPS in macrophages require further studies to unveil the signaling pathways for regulation of TRIM59 expression in immune systems. In the present study, we demonstrated that TRIM59 expression in LPS-activated macrophages is precisely regulated by Sp1 and Nrf1, which may be dependent on the activation of JNK signaling pathway (Fig. 9). As TRIM59 expression was significantly down-regulated by inflammatory stimuli and during atherosclerosis progression, combined with the anti-inflammatory properties of TRIM59 discovered in our previous work [5] and the function of inhibiting macrophage foam cells formation (data not shown), TRIM59 may play an important role in the pathogenesis of atherosclerosis and serve as a potential therapeutic target for human atherosclerotic diseases.

How does LPS regulate the expression of TRIM59 in macrophages? We observed that Sp1 and Nrf1 directly bound to the proximal promoter of TRIM59 gene. Sp1, a C2H2 type zinc finger protein and a ubiquitous transcription factor, belongs to Sp/KLF family [31]. Because the conserved DNA-binding zinc fingers, the complex of Sp1/KLF proteins interacts with similar GC-box sequences, GC-(GGGGCGGGG) and GT-(GGTGTGGGG) [32]. Sp1 is widely involved in many cellular processes, such as cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage and chromatin remodeling. Post-translational modifications, such as phosphorylation, acetylation, glycosylation and proteolytic processing can significantly affect Sp1 activity, either activation or a repression [33]. Several kinases including ERK, JNK and p38 have been found to be involved in Sp1 phosphorylation [34–36]. Ye et al. reported that LPS-induced Sp1 protein phosphorylation and degradation resulted in the reduction of Sp1 binding to its target sequence, even though LPS had little effect on Sp1 mRNA abundance in the lung [37]. In the present study, we identified Sp1 binding site, GC box (−380 to −371 bp), in TRIM59 promoter and further determined its critical role in LPS-inhibited TRIM59 transcription. Correspondingly, deletion of GC-box in TRIM59 promoter reduced its response to LPS treatment. We further demonstrated the role of Sp1 in LPS-inhibited TRIM59 expression by observing reduction of Sp1 protein expression, nuclear translocation and binding to GC box in TRIM59 promoter by LPS. However, how does LPS regulate Sp1? We believe that 1) LPS-activated JNK may phosphorylate Sp1 and resulted in the degradation of Sp1 protein; 2) LPS-activated JNK may phosphorylate some transcription factors to inhibit the transcription of Sp1. Whether or not, especially the inhibitory effect of LPS on Sp1 mRNA level in macrophages, requires further exploration.

Nrf1 belongs to the Cap'n' collar basic leucine zipper (CNC-bZIP) family of transcription factors, and is ubiquitously expressed across tissues and cell types [38,39]. Nrf1 regulates a range of cellular functions including oxidative stress response [40,41], differentiation [42,43], inflammatory response [44,45], metabolism [46–49], cholesterol handling [38] and maintaining proteostasis [50–52]. Human and rodent Nrf1 genes can be transcribed into alternatively-spliced forms, resulting in multiple protein isoforms [39,53]. Though newly synthesized Nrf1 by glycosylation approximately 120 kDa [54] is ER membrane bound, cleavage near the N terminus releases Nrf1 (85 kDa or shorter) from the ER to regulate transcription through anti-oxidant response elements (AREs) in the promoter region of the target gene [38,55]. Nrf1 also can be sorted out of the ER through the outer nuclear envelope membrane and into the inner nuclear membrane and when required, the luminal regions of Nrf1 is partially repartitioned out of membranes and retrotranslocated into the cyto/nucleoplasmic sub-cellular compartments, whereupon the protein is allowed for deglycosylation to generate an active 95 kDa transcription factor [53,56]. Nrf1 was reported to transactivate genes including enzymes involved in glutathione biosynthesis, other oxidative defense enzymes and regulate lipid metabolism [46,57]. Nrf1 has also been reported to function as a repressor of transcription. In undifferentiated odontoblast, Nrf1 interacts with C/EBP-β to repress DSPP expression [43]. Knockdown of Nrf1 blocks TGF-β-suppressed iNOS expression [44,57]. Silencing of the long isoforms of nuclear factor erythroid 2 like 1 primes macrophages towards M1 polarization by disinhibiting STAT1/3 signaling pathway [39]. Phosphorylation status is another important determinant of Nrf1

activity. CK2 or PKA can phosphorylate Nrf1 at different sites to activate or inhibit Nrf1 [52]. In our study, we observed that deletion of ARE (−116 to −106 bp) substantially decreased TRIM59 basal promoter activity (> 70%), suggesting the basal TRIM59 transcription was positively regulated by other transcriptional factors which bind the same or adjacent sequence. However, overexpression of Nrf1 or following by LPS stimulation, TRIM59 transcription was negatively regulated by Nrf1. We supposed that Nrf1 is an ER membrane bounded protein in normal situation so that it may barely bind to the ARE. However, after LPS stimulation, Nrf1 is translocated from the ER into the nucleus as a transcriptional repressor to regulate TRIM59 transcription. We will further study the molecular mechanism of the site (−116 to −106 bp) to positively regulate TRIM59 basal transcription in the future. These show that the ARE (−116 to −106 bp) in TRIM59 promoter also played a critical role in TRIM59 transcription. Mechanically, we observed that LPS increased Nrf1 protein expression/nuclear translocation and affinity for ARE in TRIM59 promoter. However, how does LPS regulate Nrf1? We believe that LPS-JNK may activate Nrf1 by phosphorylation, meanwhile LPS-activated JNK may phosphorylate some transcription factors to promote Nrf1 transcription. Whether or not, especially the increased effect of LPS on Nrf1 expression in macrophages, needs further investigation.

In summary, the present study has demonstrated that TRIM59 expression is completely regulated by Sp1 and Nrf1 in LPS-activated macrophages, which can be related to activation of JNK signaling pathway. Obviously, our study should provide an insight in TRIM59-mediated control of immune responses. Moreover, TRIM59 was highly expressed in macrophages, but was significantly down-regulated by inflammatory stimuli and during atherosclerosis progression. Combined with the anti-inflammatory properties of TRIM59 discovered in our previous work and the function of inhibiting macrophage foam cells formation (data not shown), these findings identify that TRIM59 may play an important role in the pathogenesis of atherosclerosis and serve as a potential therapeutic target for atherosclerotic diseases. We will further determine the function of TRIM59 in the development of atherosclerosis in our future work.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

TRIM59	Tripartite motif-containing protein 59

Sp1	Specificity protein 1
Nrf1	nuclear factor erythroid 2 related factor-1
TLR4	Toll-like receptor 4
mitA	mithramycin A
CNC-bZIP	Cap'n' collar basic leucine zipper
hBMDMs	Human blood monocyte-derived macrophages
BMDMs	Bone marrow-derived macrophages

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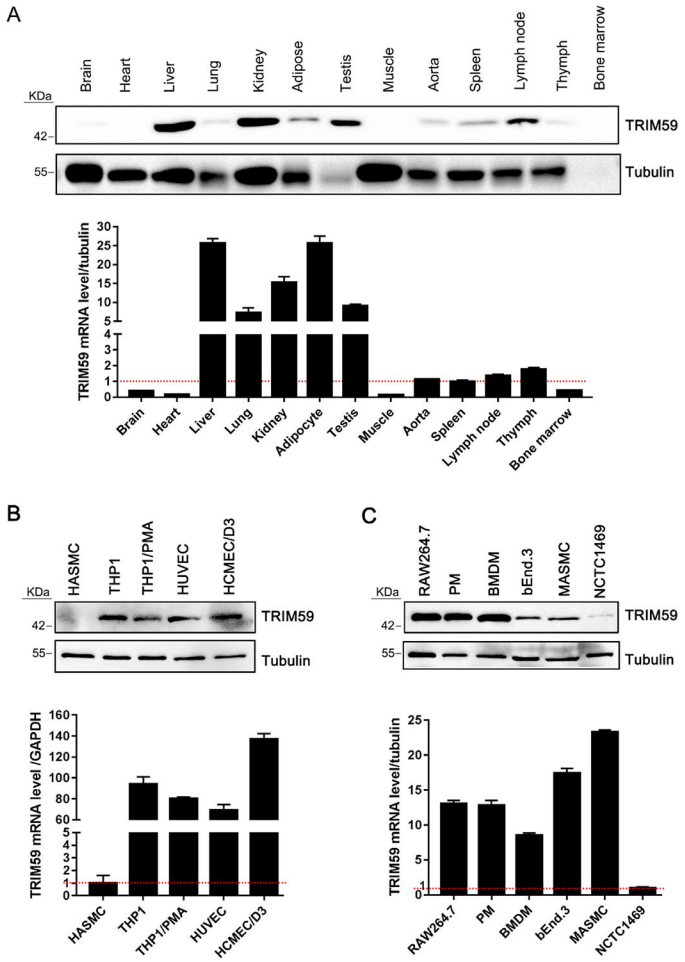

Fig. 1.

TRIM59 expression in mouse tissues and cell lines. **(A)** Total proteins and RNA were extracted from mouse tissues (8-week old). (**B**) Total proteins and RNA were extracted from human different types of cells. (**C**) Total proteins and RNA were extracted from mouse different types of cells. TRIM59 protein (30 μg/sample) and mRNA levels were determined by Western blot and qPCR analysis and normalized to tubulin.

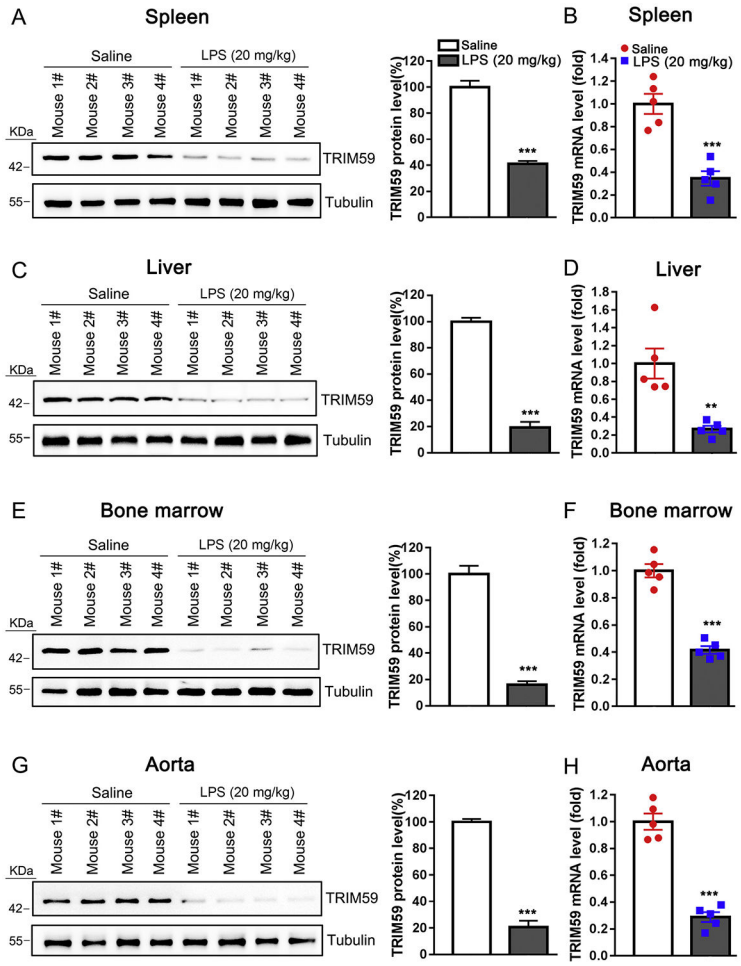

Fig. 2.

LPS inhibits TRIM59 expression *in vivo*. C57BL/6 J wild-type mice (~8-week-old) were i.p. injected with LPS (20 mg/kg). Tissues samples of spleen **(A-B)**, liver **(C-D)**, bone marrow **(E-F)** and aorta **(G-H)** were collected after 16 h of LPS injection and used to extract total proteins and RNA. Expression of TRIM59 protein or mRNA was determined by Western blot or qPCR analysis. Data are presented as mean ± SEM ($n = 5$); $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. saline group in the corresponding groups by Student′s *t*-test.

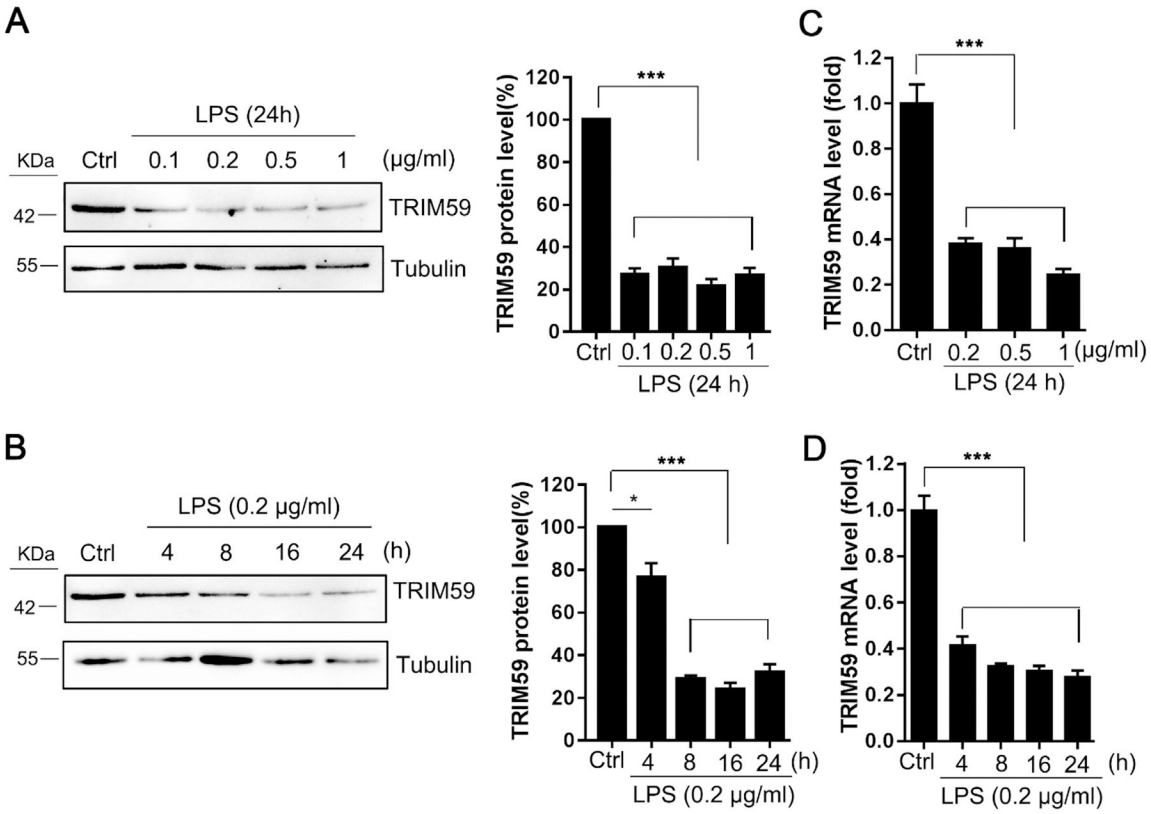

Fig. 3.

LPS inhibits TRIM59 expression in hBMDMs.**(A-D)** Human blood monocyte-derived macrophages cells (hBMDMs) were treated with LPS at the indicated concentrations for 24 h, or with 0.2 μg/ml LPS for the indicated times. Expression of TRIM59 protein **(A, B)** or mRNA **(C, D)** was determined by Western blot or qPCR analysis. Data are presented as mean ± SEM ($n = 3$); *$p < 0.05$, ***p < 0.001 *vs.* Ctrl by one-way ANOVA.

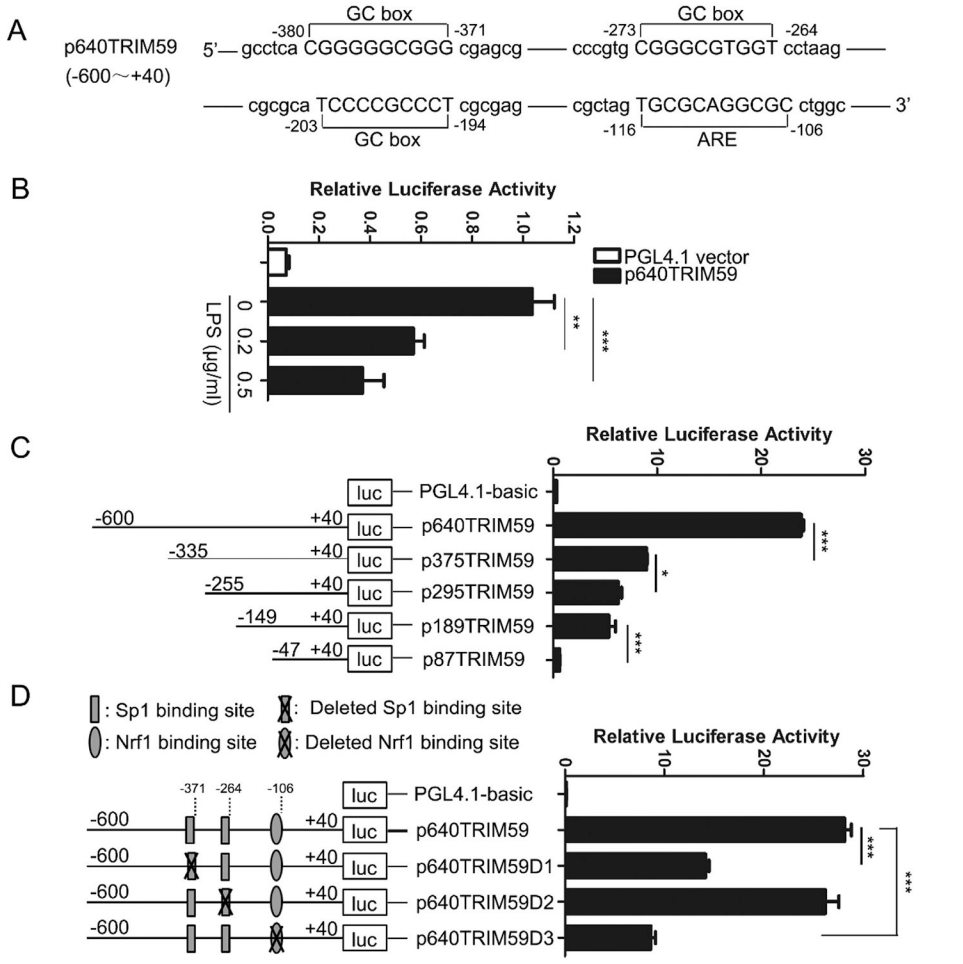

Fig. 4.

LPS inhibits TRIM59 promoter activity. **(A)** TRIM59 promoter (from −600 to +40) which includes both potential Sp1 binding sites (GC box) and Nrf1 binding site (ARE) was cloned and named as p640TRIM59. **(B)** RAW264.7 cells in 24-well plates were transfected with DNA for p640TRIM59 and *Renilla* (for internal control). After 8 h of transfection, cells were treated with LPS at the indicated concentrations for 16 h, followed by determination of activities of firefly and *Renilla* luciferases using the Dual-Luciferase Reporter Assay system. Data are presented as mean ± SEM ($n = 3$); $^{**}p < 0.01$, $^{***}p < 0.001$ by one-way ANOVA. **(C-D)** TRIM59 promoters with the indicated deletion or mutation were constructed separately. Same amount of DNA for these promoters was used to transfect HEK-293 T cells. After 4 h of transfection, cells were switched into complete medium for 20 h, followed by determination of activities of firefly and *Renilla* luciferases. Data are presented as mean ± SEM (n = 3); $^{*}p < 0.05$, ***p < 0.001 by one-way ANOVA.

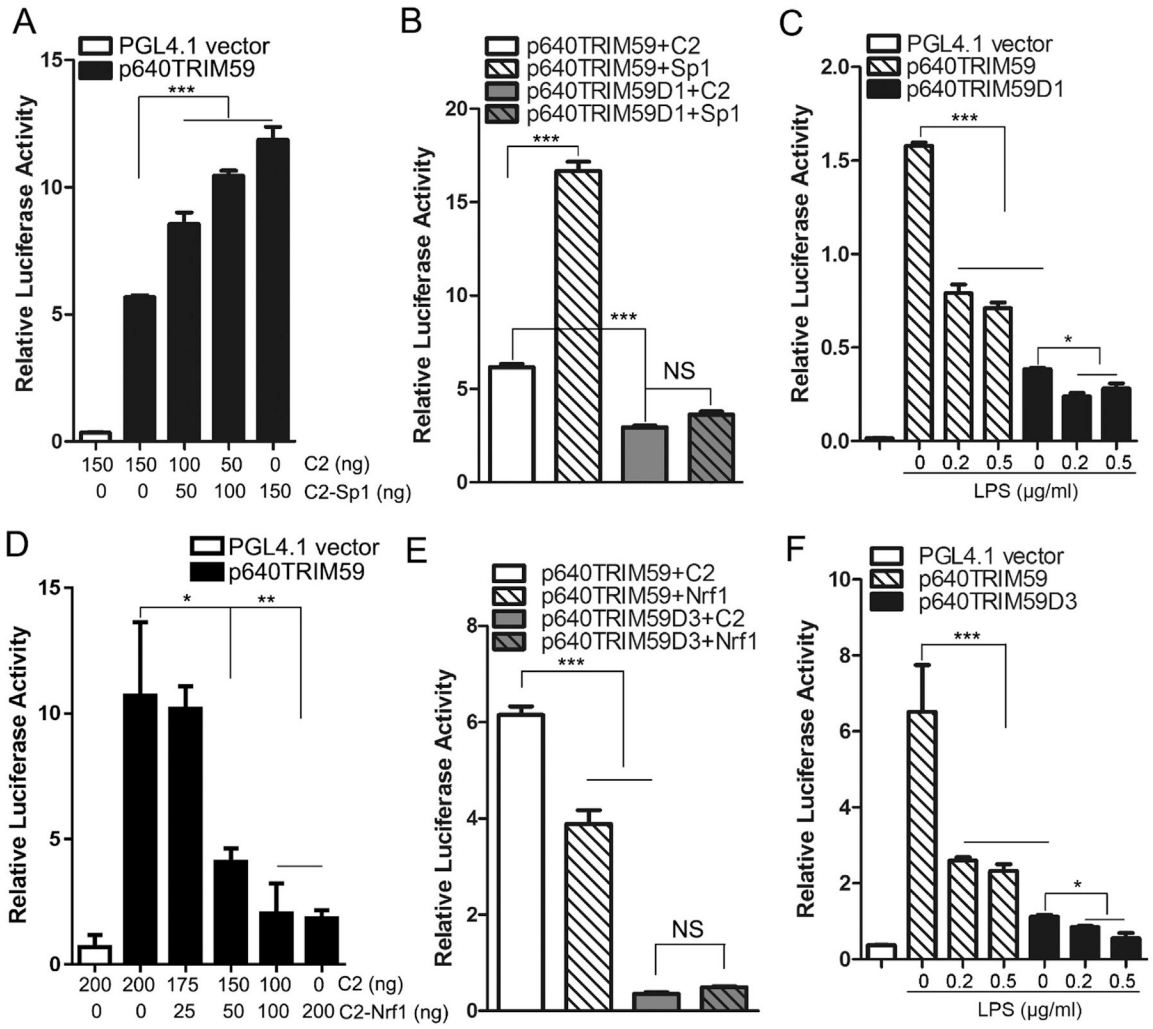

Fig. 5.

LPS inhibits TRIM59 promoter activity by regulating Sp1 and Nrf1 differently. HEK-293 T cells in 24-well plates were transfected with DNA for p640TRIM59 or plus Sp1 expression vector **(A)** or plus Nrf1 expression vector **(D)** as indicated and *Renilla*. After 4 h of transfection, cells were switched into complete medium for 20 h, followed by determination of activities of firefly and *Renilla* luciferases. *p < 0.05, **$p < 0.01$, ***$p < 0.001$ ($n = 3$). **(B, E)** HEK-293 T cells in 24-well plates were transfected with DNA for the indicated TRIM59 promoters and Sp1 or Nrf1 expression vector. Activities of firefly for the corresponding promoter and *Renilla* luciferases were determined. ***p < 0.001 (n = 3). **(C, F)** RAW264.7 cells in 24-well plates were transfected with DNA for the indicated TRIM59 promoters. After 8 h of transfection, cells were treated with LPS at the indicated concentrations for 16 h, followed by determination of activities of firefly and *Renilla* luciferases. *p < 0.05, ***p < 0.001 (n = 3).

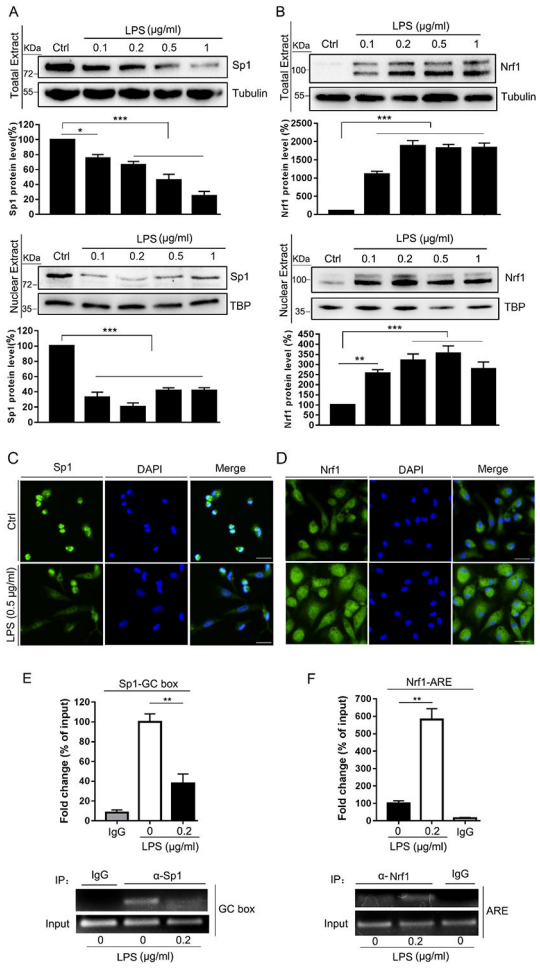

Fig. 6.

LPS inhibits Sp1 but promotes Nrf1 expression in macrophages. **(A-B)** RAW264.7 cells were treated with LPS at the indicated concentrations overnight. Total and nuclear proteins were extracted and used to determine expression of Sp1 (**A**) or Nrf1 (**B**) protein by Western blot. Data are expressed as mean ± SEM (n = 3); *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA. **(C-D)** RAW264.7 cells were treated with LPS (0.5 μg/ml) overnight, followed by determination of Sp1 protein (**C**) or Nrf1 protein (**D**) expression and localization by immunofluorescent staining. Scale bar, 50 μm. **(*E*-F)** ChIP performed with an antibody against Sp1 or Nrf1 on chromatin isolated from RAW264.7 cells treated by LPS (0.2 μg/ml) overnight. The quantity of immunoprecipitated DNA was assessed by qPCR for the predicted GC box **(E)** or ARE **(F)** present in the TRIM59 promoter. The net quantities of enriched DNA were corrected with their corresponding input DNA. Data are expressed as mean ± SEM (n = 3); **p < 0.01 by Student′s *t*-test. qPCR products then conduct electrophoresis in 2% agarose gel stained with ethidium bromide and visualized under UV light.

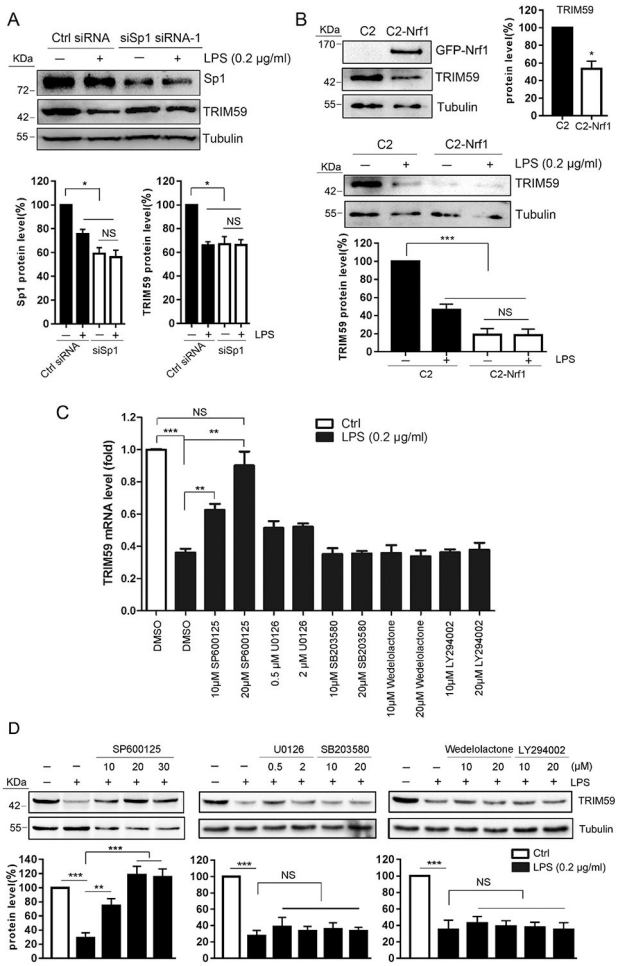

Fig. 7.

LPS inhibits macrophage TRIM59 expression in Sp1- and Nrf1-dependent manners through JNK signaling pathway. **(A)** RAW264.7 cells in 6-well plates were transfected with scrambled siRNA (25 nM) or Sp1 siRNA (25 nM) followed by treatment with LPS (0.2 μg/ml) for 24 h, and then determined of Sp1 and TRIM59 protein expression by Western blot analysis. **(B)** Peritoneal macrophages in 6-well plates were transfected with DNA for Nrf1 expression vector or pEGFP-C2 vector followed by treatment with LPS (0.2 μg/ml) overnight. Total proteins were extracted from peritoneal macrophages and used to determine expression of Nrf1 and TRIM59 protein by Western blot analysis. Data are presented as mean ± SEM (n = 3); *p < 0.05, ***p < 0.001 by Student′s *t*-test or one-way ANOVA. **(C-D)** RAW264.7 cells were pretreated with a variety of inhibitors for TLR4 signaling pathway (JNK inhibitor: SP600125; ERK1/2 inhibitor: U0126; p38 inhibitor: SB203580; IKK/NF-κB inhibitor: Wedelolactone; PI3K inhibitor: LY294002) for 3 h and then were stimulated with 0.2 μg/ml LPS for 16 h. After treatment, total RNA and proteins were extracted and used to determine TRIM59 mRNA **(C)** and protein expression **(D)** by qPCR and Western blot analysis, respectively. Data are presented as mean ± SEM (n = 3); **p < 0.01, ***p < 0.001 by one-way ANOVA.

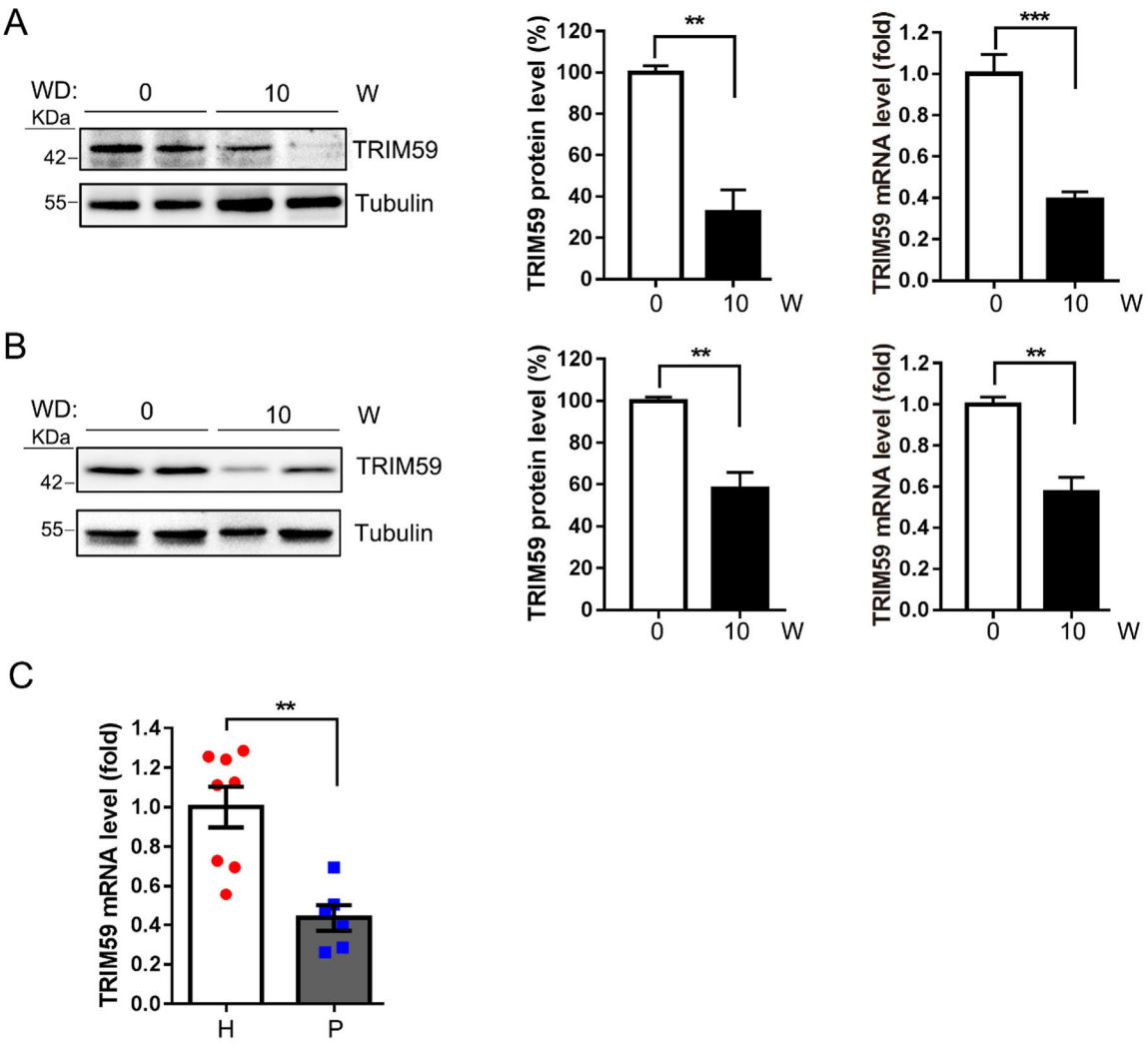

Fig. 8.

TRIM59 expression is decreased during atherosclerosis progression. **(A)** Western blot and qPCR analysis of TRIM59 protein and mRNA expression in the aortas of ApoE$^{-/-}$ mice fed a western diet for the indicated time. Data are presented as mean ± SEM (n = 4); **p < 0.01, ***p < 0.001 by Student′s t-test. **(B)** Western blot and qPCR analysis of TRIM59 protein and mRNA expression in the peritoneal macrophages of ApoE$^{-/-}$ mice fed a western diet for the indicated time. Data are presented as mean ± SEM (n = 4); **p < 0.01 by Student′s t-test. **(C)** qPCR analysis of TRIM59 mRNA level in peripheral blood monocytes of patients with hypercholesterolemia. Data are presented as mean ± SEM (n = 8 or 6); **p < 0.01 by Student′s t-test.

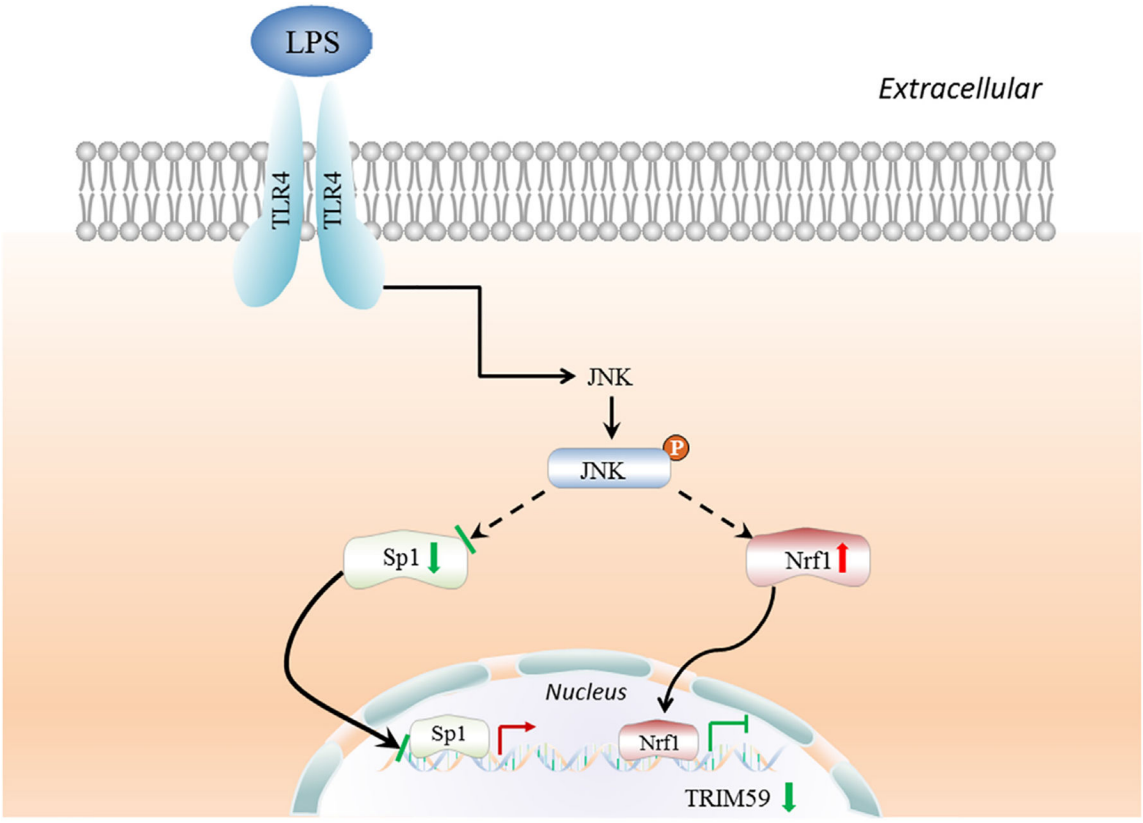

Fig. 9.

A proposed model of LPS/JNK/Sp1 or Nrf1 signaling pathways in regulation of TRIM59 expression. Sp1 promotes and Nrf1 inhibits TRIM59 expressions in LPS-activated macrophages. TRIM59 expression in LPS-activated macrophages may be dependent on the JNK signaling pathway, in which phosphorylation of JNK inhibits the expression, nuclear translocation and binding to TRIM59 promoter of Sp1, whereas increases the expression, nuclear translocation and binding to TRIM59 promoter of Nrf1.