LPS inhibits TRIM65 expression in macrophages and C57BL/6J mouse by activating the ERK1/2 signaling pathway

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Abstract. Activated macrophages serve a key role in various inflammatory diseases, such as atherosclerosis and septic shock. Tripartite motif-containing protein 65 (TRIM65) has been previously reported to participate in tumor progression and lung inflammation. However, the molecular mechanisms that controls its expression under inflammatory conditions and its consequences in activated macrophages remain poorly understood. The present study first collected the tissues of C57BL/6J mice, smooth muscle cells, macrophages and endothelial cells to determine the expression and distribution of TRIM65 by reverse transcription-quantitative (RT-q) PCR and western blotting. Mouse and human macrophages were treated with LPS and C57BL/6J mice were intraperitoneally injected with LPS followed by isolation of spleen, lung, aorta and bone marrow. Following treatment, TRIM65 mRNA and protein level was examined by RT-qPCR and western blotting. The results showed that TRIM65 was highly expressed in organs of the immune system, such as the spleen, lymph node and thymus, but lowly expressed in heart, liver, brain and kidneys. TRIM65 was also highly expressed in macrophages and endothelial cells. TRIM65 mRNA and protein expression levels were found to be decreased in LPS-treated macrophages *in vitro* and in tissues isolated from C57BL/6J mice intraperitoneally injected with LPS *in vivo*. In addition, to identify the signaling pathways by which LPS regulates TRIM65 expression, inhibitors of MAPK and Akt signaling pathways were used to treat macrophages followed by examination the expression of TRIM65 by western blotting. The results demonstrated that LPS-inhibited TRIM65 expression was blocked by treatment with the ERK1/2 inhibitor U0126. Moreover, the RT-qPCR results showed that TRIM65 knockout potentiated LPS-induced expression of inflammatory cytokines in macrophages. Taken together, data from the present study suggest that LPS decreased TRIM65 expression in macrophages and C57BL/6J mouse by activating the ERK1/2 signaling pathway, whilst TRIM65 knockout promoted macrophage activation. This information may facilitate the development of potential therapeutic strategies for the prevention and treatment of inflammatory diseases, such as atherosclerosis.

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Abbreviations: AP-1, activator protein-1; BMDMs, Bone marrow-derived macrophages; ECs, endothelial cells; hBMDMs, Human blood monocyte-derived macrophages; LPS, lipopolysaccharide; ND, Normal diet; PMs, peritoneal macrophages; SMCs, smooth muscle cells; Sp1, specificity protein 1; TRIM65, Tripartite motif-containing protein 65; WD, western diet

Key words: lipopolysaccharide, macrophage, tripartite motif-containing protein 65, expression, ERK1/2 signaling

Introduction

Macrophages serve a key role in host defense against infections by viruses and microorganisms (1). However, activated macrophages can also contribute to tissue damage and repair by secreting a variety of growth factors, cytokines and proteolytic enzymes (1,2). Overproduction of these inflammatory mediators may promote the development of inflammatory diseases, including atherosclerosis, septic shock and even cancer (1). Atherosclerosis has been defined to be a chronic inflammatory disease and is one of the leading causes of cardiovascular diseases, such as myocardial infarctions and coronary artery disease. As an important constituent of atherosclerotic lesions, macrophages serve an important part in the progression of atherosclerosis by forming the foam cells

and regulating inflammation (1,3). Macrophages treated with lipopolysaccharide (LPS) has been frequently used as a model of activated macrophages experimentally (1,4). LPS can activate a number of intracellular signaling pathways, including ERK1/2, c-JNK and p38, which belongs to the MAPKs family of signaling cascades (1). In addition, LPS can activate PI3K/AKT, which ultimately leads to the activation of NF-κB and activator protein-1 (AP-1) to release various inflammatory factors, including TNFα, IL-1β and IL-6 (5-7). Activated MAPKs can also regulate the activity of other transcription factors such as Sp1 to control the expression of downstream targets such as TRIM59 (1,8).

Tripartite motif-containing (TRIM) 65 belongs to a member of the TRIM protein family (9,10). Structurally, it contains a RING-finger domain, a B-box domain and a coiled-coil domain in the N-terminus (9,10). The presence of a RING domain can mediate the conjugation of proteins with ubiquitin. The B-box domains have been shown to contribute to innate resistance to HIV, and the coiled-coil domains mediate homomeric and heteromeric interactions among TRIM family members and other proteins, in particular self-association (11). Previous studies found TRIM65 to serve a key role in tumor progression by promoting hepatocellular carcinoma via ubiquitylation of Axin1 and supporting bladder urothelial carcinoma cell aggressiveness by promoting ANXA2 ubiquitination and degradation (12-15), melanoma differentiation-associated protein 5-mediated antiviral innate immunity by promoting K63 polyubiquitination of MDA5 (16,17) and modulating the miRNA pathway by ubiquitination of TNRC6 (13,18). Furthermore, TRIM65 has been found to suppress LPS-induced lung inflammation by targeting vascular cell adhesion molecule 1 (19). TRIM65 has also been observed to function as an endogenous negative regulator of NACHT domain-, leucine-rich repeat- and PYD-containing protein 3 inflammasome activation in macrophages (20). However, the molecular mechanism that controls its expression and physiological function in activated macrophages remain poorly understood.

Therefore, the present study aimed to investigate the possible effect and mechanism of LPS on TRIM65 expression *in vitro* and *in vivo* and further to evaluate the potential role of TRIM65 on macrophages activation.

Materials and methods

Cell culture. The murine macrophage cell line RAW264.7 (cat. no. SCSP-5036) were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. They were cultured in complete RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Shanghai ExCell Biology, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C in a humidified incubator with 5% CO_2.

Human umbilical vein endothelial cells (HUVECs) (cat. no. PCS-100-013; passage 3), human aortic endothelial cells (HAECs; cat. no. PCS-100-011, passage 3) and human aortic smooth muscle cells (HASMCs; cat. no. QC470, passage 2) were gifts from Professor Yuanli Chen (College of Food and Biological Engineering, Hefei University of Technology, Hefei), human cerebral microvascular endothelial cells (HCMEC/D3) were purchased from BeNa Culture Collection: Beijing Beina Chunglian Biotechnology Research Institute (cat. no. BNCC337728, passage 3), mouse cerebral microvascular endothelial cells (bEnd.3; cat. no. TCM40) and THP-1 cells (a human monocytic cell line; cat. no. SCSP-567, passage 5) were also obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences.

HUVECs, HAECs and HCMEC/D3 were cultured in Endothelial Cell Medium (cat. no. 1001; ScienCell Research Laboratories, Inc.) supplemented with 5% FBS (Shanghai ExCell Biology, Inc.) and 1% penicillin/streptomycin at 37˚C in a humidified incubator with 5% CO_2 . HASMC and bEnd.3 cells were cultured in complete DMEM (Gibco; Thermo Fisher Scientific) containing 10% FBS (Shanghai ExCell Biology, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C in a humidified incubator with 5% CO_2.

Peritoneal macrophages (PMs) were collected from the abdomen of C57BL/6 mice (WT or TRIM65 knockout, male, n=4 per group) by rinsing with PBS as described previously (21). In brief, 8-week-old C57BL/6 male mice were injected intraperitoneally with 3 ml 4% thioglycolate solution (cat. no. 211716; BD Biosciences) at day 0 and maintained with access to water and normal chow for 5 days in SPF units of the Animal Center at Nanchang University (with a 12-h light cycle from 8 a.m. to 8 p.m. and at 23±3˚C with 60-70% humidity). The mice were sacrificed in a CO_2 chamber with the flow displacement rate of 30% container volume per minute on day 5 and the PMs were collected from mouse abdomen by lavage with 10 ml PBS. After removal of erythrocytes with Red Blood Cell Lysis Buffer (cat. no. R1010; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's protocol, PMs were collected by centrifugation (500 x g for 5 min) at 4˚C, and re-suspended and cultured in complete RPMI-1640 medium for 2 days at 37˚C in a humidified incubator with 5% CO_2 followed by treatment with LPS after the 2 days.

Mouse aortic SMCs (MASMCs) were isolated from the aortas of male C57BL/6 mice (8 weeks) as described previously (22,23). Briefly, aortas were carefully dissected and cleaned of connective tissue, and then were cut into 1-2 mm pieces followed by digestion for 6 h at 37˚C in DMEM containing 200 U/ml type-II collagenase. After 6 h, MASMCs were harvested by centrifugation at 200 x g for 5 min at room temperature and cultured in DMEM containing 20% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin at 37˚C in a humidified incubator with 5% CO_2.

Bone marrow cells were isolated from mouse tibias and femurs and the erythrocytes were removed with Red Blood Cell Lysis Buffer (cat. no. R1010; Beijing Solarbio Science & Technology Co., Ltd.). The remaining cells, termed BM were collected by centrifugation (500 x g for 5 min at 4˚C) and then re-suspended in PBS twice to wash out the Red Blood Cell Lysis Buffer and this was followed by extraction of RNA and protein. Bone marrow-derived macrophages (BMDMs) were derived from BM in DMEM containing 10% FBS and 50 ng/ml macrophage-colony stimulating factor (cat. no. HY-P7085A; MedChemExpress) for 7 days at 37˚C in a humidified incubator with 5% CO_2 (1).

Human blood monocyte-derived macrophages (hBMDMs) were acquired as described previously with 10 ml blood

from a healthy volunteer (female aged 35) (1). Briefly, after the removal of red blood cells by incubating the blood with a dextran sedimentation mixture (cat. no. D2870; Beijing Solarbio Science & Technology Co., Ltd.), the remaining cells were collected by centrifugation (250 x g for 5 min at 4˚C) and re-suspended in serum-free RPMI1640 medium. The suspension was then overlaid onto the top of a Ficoll solution (cat. no. P8610; Beijing Solarbio Science & Technology Co., Ltd.). After gradient centrifugation for 20 min at 1,000 x g at room temperature, monocytes in the interface layer were collected and washed 3 times with serum-free RPMI-1640 medium followed by culture in complete RPMI-1640 medium containing 50 ng/ml macrophage-colony stimulating factor (cat. no. HY-P7050A; MedChemExpress) for 7 days at 37˚C in a humidified incubator with 5% CO_2 to differentiate into macrophages. Studies with human samples were approved by the Ethics Committee of Second Affiliated Hospital of Nanchang University (Nanchang, China) and complied strictly with the 2008 Declaration of Helsinki Principle. Written informed consents were signed by the participant prior to the collection of samples in the present study.

Cell treatment. To determine the effect of LPS (cat. no. L3129; MilliporeSigma) on TRIM65 expression in macrophages, RAW264.7, PMs, THP-1 derived macrophages and hBMDMs were treated with 0.1, 0.2, 0.5 and 1 μg/ml LPS or with 0.2 μg/ml LPS for 3, 6, 12 and 24 h in RPMI-1640 or DMEM serum-free medium at 37˚C in a humidified incubator with 5% CO_2 followed by the extraction of protein or RNA. LPS has been recognized to be a viable reagent for inducing macrophage activation and all the doses of LPS in study used exerted no toxicity on the macrophages (1,2). Further to our previously reported results (1) and the maximum inhibition of 0.2 μg/ml LPS on TRIM65 expression in primary PMs, 0.2 μg/ml LPS was chosen for treating all the macrophages for 24 h at 37˚C instead of the lowest concentration of 0.1 μg/ml and the highest concentration 1 μg/ml.

To identify the signaling pathways by which LPS-inhibited TRIM65 expression, RAW264.7 cells were pretreated with U0126 (cat. no. HY-12031; MedChemExpress), SB203580 (cat. no. HY-10256; MedChemExpress), SP600125 (cat. no. HY-12041; MedChemExpress), LY294002 (cat. no. HY-10108; MedChemExpress) and Wedelolactone (cat. no. W4016; MilliporeSigma) for 2 h at 37˚C and then were treated with 0.2 μg/ml LPS for 12 h at 37˚C in a humidified incubator with 5% CO_2 followed by the extraction of protein or RNA.

To determine the role of TRIM65 in macrophage activation, the PMs isolated from WT and TRIM65 knockout ($TRIM65^{-/-}$) mice were treated with 10 ng/ml LPS for 4 h at 37˚C in a humidified incubator with 5% CO_2 followed by extraction of RNA.

To induce monocyte/macrophage differentiation, THP-1 cells at a density of $2.5x10^5$ cells/cm^2 were treated with 100 nM PMA (cat. no. 19-144; MilliporeSigma) and cultured overnight in complete RPMI-1640 medium. PMA was then removed by aspiration and the adhesive cells were washed twice with PBS. The derived THP-1/PMA macrophages were cultured in complete RPMI- 1640 medium at 37˚C in a humidified incubator with 5% CO_2 for another 2 days followed by treatment by LPS.

Animals and in vivo study. WT mice with C57BL/6 background (male, 7-week-old, n=20) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). Apolipoprotein E-knockout ($ApoE^{-/-}$) mice on a C57BL/6 J background (8-week-old, two female and 1 male) were obtained from Charles River Laboratories, Inc. A total of 8 male $ApoE^{-/-}$ mice were produced by interbreeding and used in the present study. Heterozygous TRIM65-knockout ($TRIM65^{+/-}$) mice on a C57BL/6 background (8-week-old, two female and 1 male) were purchased from Cyagen Biosciences, Inc., whereas TRIM65 knockout homozygous ($TRIM65^{-/-}$) mice (male, 8-week-old, n=4) were generated by interbreeding and used in this study. All mice were housed in SPF units of the Animal Center at Nanchang University (with a 12-h light cycle from 8 a.m. to 8 p.m. at 23±3˚C with 60-70% humidity) and maintained on a standard rodent diet with free access to water in plastic bottles. Animal health and behavior were monitored every day. The protocols for animal experiments were approved by the Ethics Committee of Nanchang University (approval nos. 20X049 and X20200703) and complied with the Guide for the Care and Use of Laboratory Animals published by NIH (1). All the mice were sacrificed at the end of the experiments or upon reaching humane endpoints. The humane endpoints set for the present study included loss of appetite, persistent weight loss, dyspnea, persistent convulsions and severe hypothermia that could not be recovered by warming. Mortality in mice was verified by the loss of heartbeat and dilation of pupils.

The male WT mice were allowed to acclimatize to their housing environment for ≥7 days before the experiments, where ≤ five mice were kept per plastic cage with corn cob bedding material. The mice were then divided into two groups at random (n=6), before they underwent the following treatment scheme at 8 a.m. on day 0: Mice in the control group were fed with normal chow and injected intraperitoneally with saline (100 μl/mice); whereas mice in the LPS group were fed with normal diet and injected intraperitoneally with LPS (20 mg/kg). After treatment for 12 h, the mice were sacrificed in a CO_2 chamber with the flow displacement rate of 30% container volume per minute at 8 p.m. on day 0. Subsequently, the spleen, lung, aorta and bone marrow of mice were all collected. Protein and RNA samples were then extracted, which were used for the determination of TRIM65 protein and mRNA expression by western blotting and RT-qPCR.

To determine the expression level of TRIM65 in the aorta during the progress of atherosclerosis, $ApoE^{-/-}$ mice (~12-week-old, male) were randomly divided into two groups (four mice/group) and were fed with either Normal diet (ND) or western diet (WD: 0.5% cholesterol and 21% fat) at week 0. On week 16, all of the mice were anesthetized and euthanized in a CO_2 chamber with the flow displacement rate of 30% container volume per minute, followed by the collection of mouse aortas and the measurement of TRIM65 mRNA expression by RT-qPCR.

Identification of the $TRIM65^{-/-}$ mice genotype by PCR. DNA were extracted from 0.5 cm mouse tails from mice with mouse tissue lysis buffer (cat. no. 19697ES70; YEASEN Biotechnology (Shanghai) Co., Ltd.), before PCR was performed using 2X Rapid Taq Master mix (cat. no. P222-01; Vazyme Biotech Co., Ltd.). The primers were as follows: Mouse

TRIM65 forward, 5'-CTGGAACTCCCATCTGCTCTGCT-3' and reverse, 5'-GGGAGGAGTGTGGACAGGACAGTT-3' and mouse TRIM 65-Wt/He forward, 5'-CAGGAGATTCAG TAGCCTGCTTCAGG-3' and reverse, 5'-GGGAGGAGT GTGGACAGGACAGTT-3'. The following thermocycling conditions were used: Initial denaturation at 94˚C for 5 min, followed by 35 cycles at 94˚C for 30 sec, 60˚C for 30 sec, 72˚C for 1 min and then 72˚C for 10 min. The gel electrophoresis was performed with 1X TAE (cat. no. T1061; Beijing Solarbio Science & Technology Co., Ltd.) in 1% agarose gel with YeaRed Nucleic Acid Gel stain (cat. no 10202ES76; Shanghai Yeasen Biotechnology Co., Ltd.). If the PCR revealed one band at 660 bp, then the mice would be considered to be of the TRIM65$^{-/-}$ genotype. By contrast, if the result of PCR revealed two bands with lengths of 660 and 909 bp, then the mice would be identified to be of the TRIM65$^{+/-}$ genotype. If the result of PCR showed one band at 909 bp, then the mouse would be considered as WT.

Determination of TRIM65 protein expression by western blotting. After treatment, total protein samples were extracted from tissues or cells using a protein lysis buffer (RIPA; cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) containing PMSF and cocktail (10 µg/ml), before being centrifuged at 10,000 x g for 10 min at 4˚C. After detection of protein content using a BCA assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.), TRIM65 protein level was examined in 30 µg total protein of each sample using western blotting as described previously (2,21). Briefly, proteins from each sample were separated by SDS/PAGE (10% gel) and transferred onto a nylon-enhanced nitrocellulose membrane. The membrane was then blocked with a solution of 5% skimmed milk in PBS for 1 h at room temperature, before being incubated with primary antibodies against TRIM65 (1:1,000; cat. no. HPA021578; Sigma-Aldrich; Merck KGaA), p-ERK (1:1,000; cat. no. 4370S; Cell Signaling, Inc.), ERK (1:1,000; cat. no. 4695S; Cell Signaling, Inc.) and GAPDH (1:5,000, cat. no. 60004-1-Ig; ProteinTech Group, Inc.) or β-actin (1:5,000; cat. no. 66009-1-Ig; ProteinTech Group, Inc.) at 4˚C overnight. This was followed by washing three times for 10 min each with a solution of 0.5% Tween-20 in PBS (PBS-T). The membranes were then exposed to HRP-conjugated goat anti-rabbit (1:5,000; cat. no. 31460; Thermo Fisher Scientific, Inc.) or anti-mouse (1:5,000; cat. no. 31430; Thermo Fisher Scientific, Inc.) IgG secondary antibodies and incubated for 1 h at room temperature. After three washes with PBS-T (10 min each), the membrane was incubated for 5 min in a mixture of equal volumes of Western blot chemiluminescence reagents 1 and 2 of the Ultra High Sensitivity ECL Kit (cat. no. GK10008; GlpBio Technology). The protein bands were visualized using an ECL detection system (Tanon Science and Technology Co., Ltd.) and analyzed using the Image J software (version 1.46r; National Institutes of Health).

Examination of TRIM65, TNFα, IL-1β and IL-6 mRNA expression by RT-qPCR. After treatment, tissues or cells were lysed before RNA was extracted using the M5 HiPer Total RNA Extraction Reagent (cat. no. MF034-01; JHM IT Group) as described previously (1). After quantification, total RNA (2 µg) was used to synthesize cDNA with HiScript Q RT SuperMix for qPCR (+gDNA wiper; cat. no. R123-01; Vazyme Biotech Co., Ltd.). The following conditions were used: Initial wiper gDNA at 42˚C for 2 min, followed by 50˚C for 15 min and 85˚C for 5 sec. TRIM65, TNFα, IL-1β and IL-6 mRNA expression was examined by RT-qPCR using a 2x Universal Blue SYBR Green qPCR Master Mix (cat. no. G3326; Wuhan Servicebio Technology Co., Ltd.) with primers listed in Table I. The following thermocycling conditions were used: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 30 sec, before the melting curve was made according to the default Settings of the instrument. GAPDH was used as the internal reference and the $2^{-\Delta\Delta Cq}$ method (24) was used to calculate the relative expression.

Statistical analysis. All data are presented as the mean ± standard error of mean. All statistical analyses were conducted with the GraphPad Prism 7 software (Dotmatics) and the statistical significance of differences was assessed using the unpaired Student's t-test between two groups or one-way analysis of variance followed by Tukey's post hoc test among > two groups (Figs. 1-4). Statistical significance of differences in Fig. 5 was analyzed using two-way ANOVA followed by Sidak's post hoc tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression profile of TRIM65 in tissues and cells. Mouse tissues were first isolated to examine their TRIM65 expression profile by western blotting and RT-qPCR *in vivo*. As shown in Fig. 1A and B, TRIM65 is highly expressed in spleen, axillary lymph node, lung, thymus and aorta, but is expressed at low levels in the heart, liver, brain and kidneys.

Subsequently, TRIM65 expression in macrophages, endothelial cells (ECs) and smooth muscle cells (SMCs) was measured because they have all been reported to serve key roles in the progression of atherosclerosis (1,25). The results of Fig. 1C-F suggest that TRIM65 is highly expressed not only in monocytes or macrophages (THP1, THP1/PMA, RAW264.7, PM, BMDM and BM), but also in ECs (HAEC, HUVEC, HCMEC and bEnd.3). However, the expression level of TRIM65 in SMCs appeared to be species-dependent, with moderate expression in mice and negligible expression in human.

LPS reduces the expression of TRIM65 in macrophages in vitro. The effect of LPS on macrophage TRIM65 expression was then examined. Both RAW264.7 macrophages and PMs were treated with LPS at different doses for 24 h or at 0.2 µg/ml for the indicated duration. TRIM65 protein and mRNA expression was then examined by western blotting or RT-qPCR. The results in Fig. 2A and C revealed that different doses of LPS all markedly decreased TRIM65 protein and mRNA expression, in both cell types. As shown in Fig. 2B and D, the inhibition of LPS on TRIM65 expression occurred at 3 h, minimized at 12 h, and sustained until 24 h of treatment, in both cell types.

Furthermore, to define if this LPS-mediated inhibition on TRIM65 expression is dependent on species, LPS was used to treat the THP-1-derived macrophages. Consistent with results observed on mouse macrophages, LPS significantly

Table I. Sequences of primers for reverse transcription-quantitative PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
Mouse TRIM65	AAGAGAAGAGCCTCCCCAAG	GGTCTCTGGGTCAAAGGTCA
Mouse TNFα	ATGGCCTCCCTCTCATCAGT	TTTGCTACGACGTGGGCTAC
Mouse IL-1β	AACCTGCTGGTGTGTGACGTTC	CAGCACGAGGCTTTTTTGTTGT
Mouse IL-6	GGGACTGATGCTGGTGACAA	TCCACGATTTCCCAGAGAACA
Mouse GAPDH	ACCCAGAAGACTGTGGATGG	ACACATTGGGGGTAGGAACA
Human TRIM65	CCTGGAAATACAGCACACGA	AAGGTCTGCTCATCCACCTG
Human GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT

TRIM65, Tripartite motif-containing protein 65.

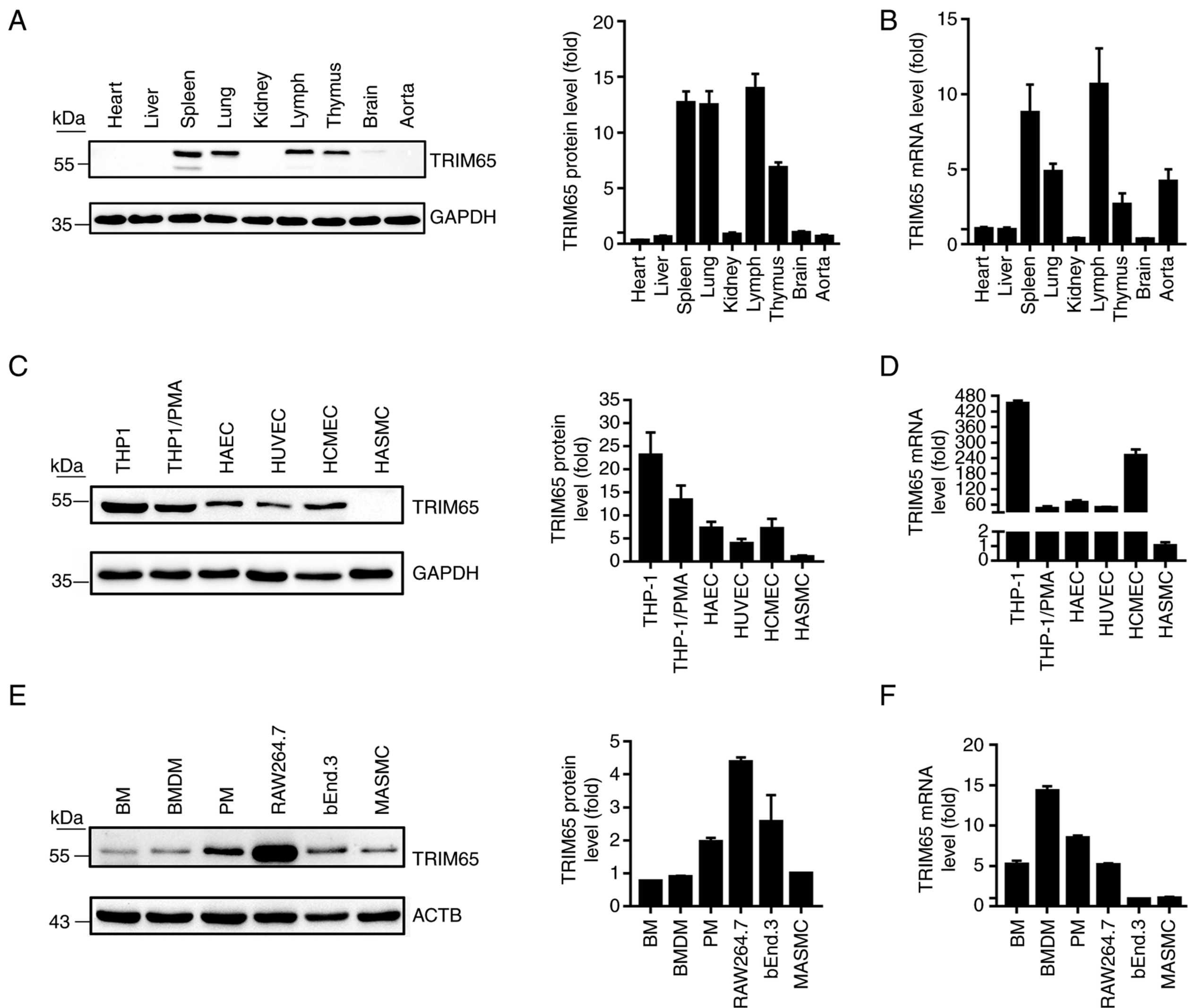

Figure 1. TRIM65 expression levels in mouse tissues and cell lines. Total protein or RNA samples were extracted from tissues or cell lines, and TRIM65 expression was examined by western blotting and RT-qPCR. (A) TRIM65 protein and (B) mRNA expression in mouse heart, liver, spleen, lung, kidney, lymph, thymus, brain and aorta tissues. (C) TRIM65 protein and (D) mRNA expression in a panel of human cell lines, namely THP1, THP1/PMA, HAEC, HUVEC, HCMEC and HASMC. (E) TRIM65 protein and (F) mRNA expression in a panel of mouse cell lines, namely BM, BMDM, PM, RAW264.7, bEnd.3 and MASMC. HUVEC, human umbilical vein endothelial cells; TRIM65, Tripartite motif-containing protein 65; PMA, phorbol 12-myristate 13-acetate; HAEC, human aortic endothelial cells; HCMEC, human cerebral microvascular endothelial cells; HASMC, human aortic smooth muscle cells; BM, bone marrow; BMDM, bone marrow-derived macrophages; PM, peritoneal macrophages; MASMCs, mouse aortic smooth muscle cells.

decreased TRIM65 protein and mRNA expression in THP-1 derived macrophages (Fig. 2E and F). To further examine whether LPS can alter TRIM65 expression in primary macrophages, hBMDMs were also treated with LPS followed by the

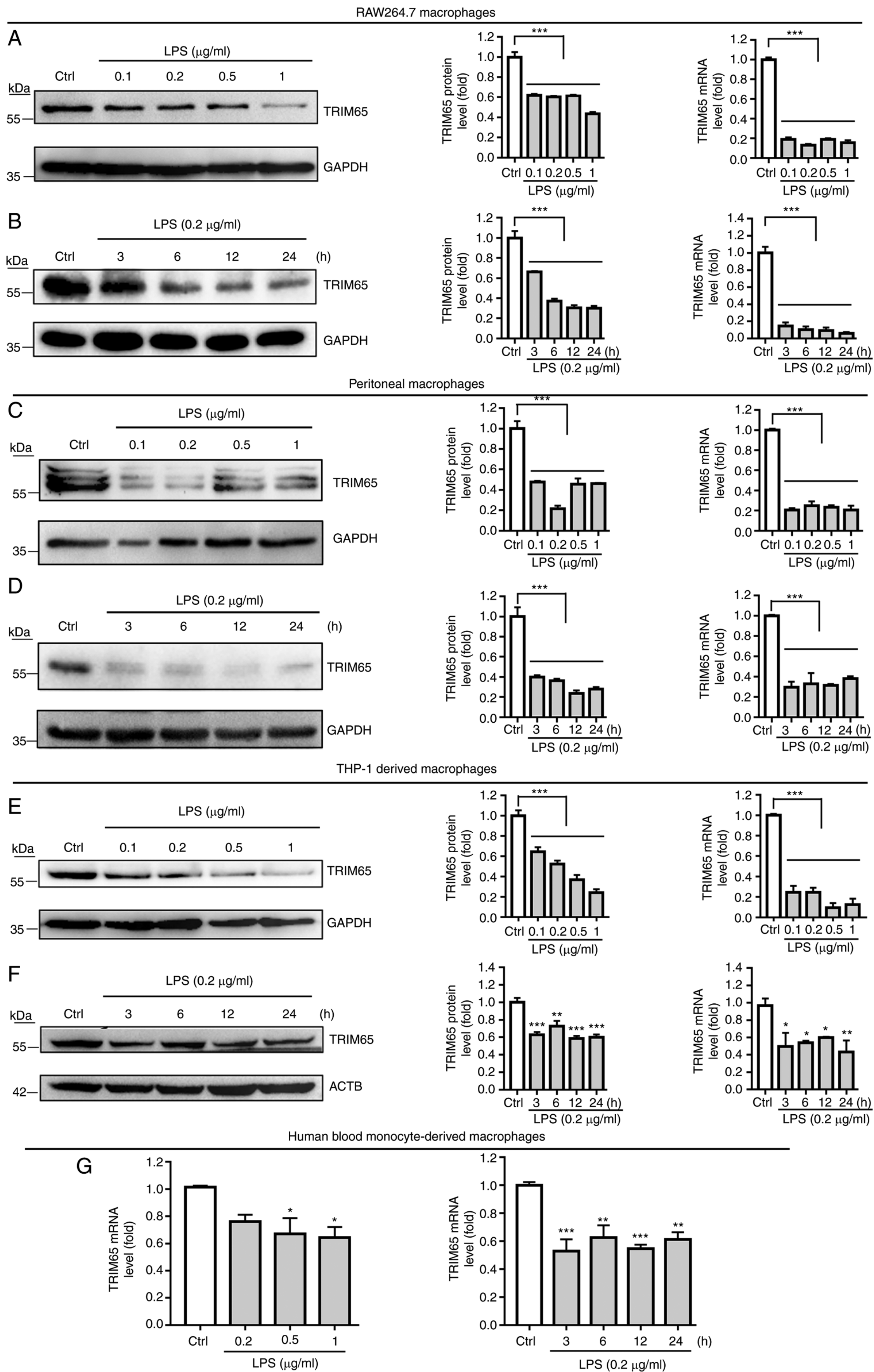

Figure 2. LPS decreases TRIM65 expression in macrophages. (A-G) Cells were stimulated with LPS at either the indicated doses for 24 h or with 0.2 μg/ml LPS for the indicated times, before TRIM65 expression was examined by western blotting or RT-qPCR. (A) TRIM65 protein and (B) mRNA expression in RAW264.7 macrophages. (C) TRIM65 protein and (D) mRNA expression in peritoneal macrophages. (E) TRIM65 protein and (F) mRNA expression in THP-1/PMA-derived macrophages. (G) TRIM65 mRNA expression in human blood monocyte-derived macrophages. *P<0.05, **P<0.01 and ***P<0.001 vs. Control. LPS, lipopolysaccharide; TRIM65, tripartite motif-containing protein 65.

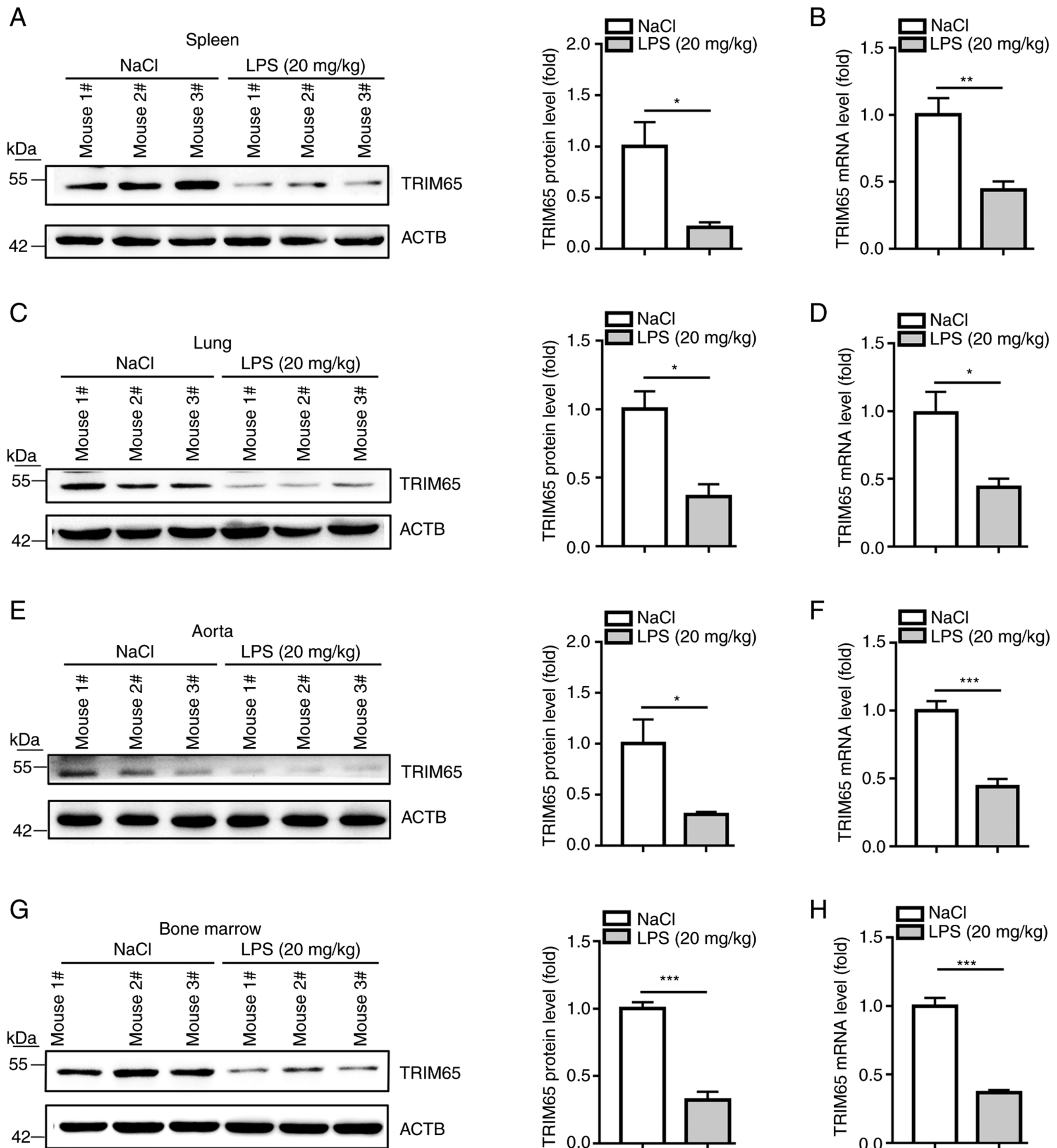

Figure 3. LPS inhibits TRIM65 expression *in vivo*. (A-G) Tissues samples of spleen, lung, bone marrow and aorta were isolated from C57BL/6 mice 12 h after saline and LPS (20 mg/kg) injection, before the extraction of total protein and RNA samples. The expression levels of TRIM65 protein or mRNA were examined by western blotting or RT-qPCR analysis. (A) TRIM65 protein and (B) mRNA expression in the spleen. (C) TRIM65 protein and (D) mRNA expression in the lung. (E) TRIM65 protein and (F) mRNA expression in the aorta. (G) TRIM65 protein and (H) mRNA expression in the bone marrow. *P<0.05, **P<0.01 and ***P<0.001 (n=6/per group). LPS, lipopolysaccharide; TRIM65, Tripartite motif-containing protein 65.

examination of TRIM65 mRNA expression with RT-qPCR. The results showed that LPS also markedly reduced TRIM65 mRNA expression in the hBMDMs (Fig. 2G). Taken together, these results suggest that LPS can inhibit TRIM65 expression in macrophages *in vitro*.

TRIM65 can be reduced by LPS in vivo. To determine if LPS can inhibit TRIM65 expression *in vivo*, protein and mRNA samples were extracted from the spleen, lung, aorta and bone marrow of mice injected with LPS (20 mg/kg). The results demonstrate that LPS significantly decreased TRIM65 protein expression in all tissues tested (Fig. 3A, C, E and G). Consistent with the protein expression changes, RT-qPCR results (Fig. 3B, D, F and H) indicate that TRIM65 mRNA expression in these tissues was likewise significantly decreased by LPS treatment. Taken together, these observations suggest that TRIM65 expression is reduced by LPS *in vivo*.

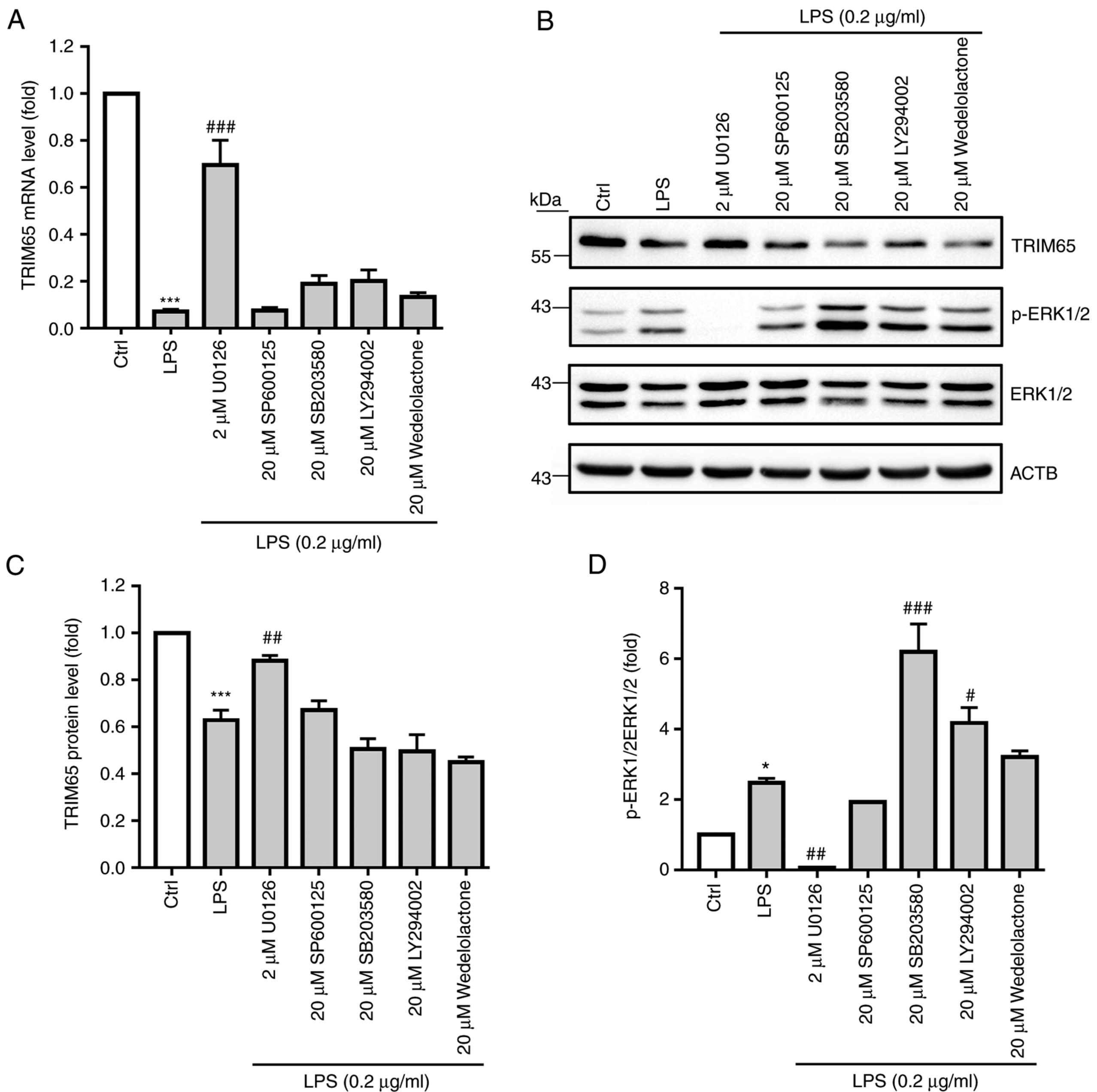

Figure 4. LPS decreases macrophage TRIM65 expression by activating the ERK1/2 signaling pathway. RAW264.7 macrophages were pre-treated with a variety of inhibitors, namely the c-JNK inhibitor SP600125, the p38 inhibitor SB203580, the ERK1/2 inhibitor U0126, the PI3K inhibitor LY294002 and the inhibitor of NF-κB kinase/NF-κB inhibitor Wedelolactone, for 2 h before they were treated with 0.2 µg/ml LPS for 12 h. After treatment, (A) TRIM65 mRNA expression was examined by RT-qPCR. (B) TRIM65 and ERK1/2 expression, in addition to ERK1/2 phosphorylation, were examined by western blot analysis. (C) TRIM65 expression and (D) p-ERK/ERK ratio were then semi-quantified from (B). *P<0.05 and ***P<0.001 vs. Ctrl, #P<0.05, ##P<0.01 and ###P<0.001 vs. LPS. LPS, lipopolysaccharide; p-, phosphorylated; Ctrl, control.

ERK1/2 signaling serve a key role in the LPS-decreased TRIM65 expression in macrophages. MAPK (p38, ERK1/2, c-JNK), PI3K and NF-κB have been reported to be the primary signaling pathways activated by LPS/toll-like receptor 4 (TLR4) signaling. To clarify if any of these pathways are responsible for LPS-decreased TRIM65 expression, RAW264.7 macrophages were pre-treated with U0126 (ERK1/2 inhibitor), SP600125 (c-JNK inhibitor), SB203580 (p38 inhibitor), LY294002 (PI3K inhibitor) or Wedelolactone (inhibitor of NF-κB kinase/NF-κB inhibitor) for 2 h, followed by LPS treatment for 12 h. LPS-reduced TRIM65 mRNA expression inhibition was significantly reversed by U0126, but not by other inhibitors (Fig. 4A). Consistent with mRNA expression, LPS-reduced expression of TRIM65 protein was likewise significantly reversed by U0126, but not by other inhibitors (Fig. 4B and C). In addition, U0126 significantly reversed, whilst SB203580 and LY294002 significantly potentiated the phosphorylation of ERK1/2 induced by LPS (Fig. 4B and D). In conclusion, these results indicate that the ERK1/2 pathway serves a crucial role in LPS-induced suppression of TRIM65 expression.

TRIM65 knockout promotes LPS-induced macrophage activation. Next, the possible role of TRIM65 in macrophage

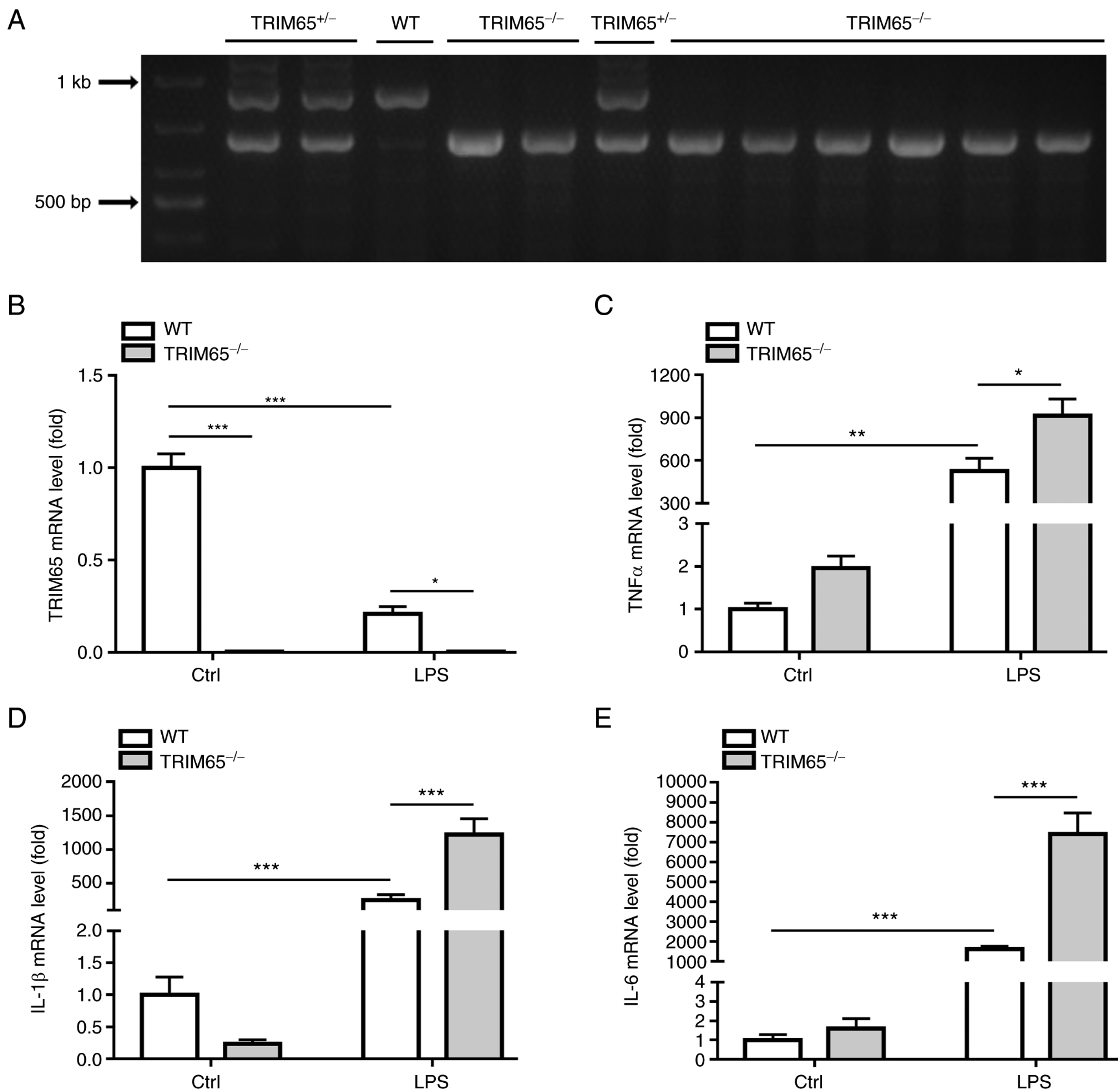

Figure 5. TRIM65-knockout potentiates LPS-induced expression of inflammatory cytokines in macrophages. (A) The genotype of WT, TRIM65$^{+/-}$ and TRIM65$^{-/-}$ mice were identified by PCR. (B-E) Peritoneal macrophages isolated from WT and TRIM65$^{-/-}$ mice were treated with 10 ng/ml LPS for 4 h followed by extraction of total RNA. The expression levels of (B) TRIM65, (C) TNF-α, (D) IL-1β and (E) IL-6 mRNA were examined by RT-qPCR. *P<0.05, **P<0.01 and ***P<0.001. LPS, lipopolysaccharide; TRIM65, Tripartite motif-containing protein 65; WT, wild-type; $^{-/-}$, knockout.

activation was examined using knockout mice. The genotype of WT and TRIM65$^{-/-}$ mice were identified by PCR (Fig. 5A) and then PMs isolated from WT and TRIM65$^{-/-}$ mice were treated with 10 ng/ml LPS for 4 h followed by the measurement of TRIM65, TNFα, IL-1β and IL-6 mRNA expression. As shown in Fig. 5B, TRIM65 mRNA expression was found to be completely abolished in PMs isolated from TRIM65$^{-/-}$ mice. In addition, LPS treatment significantly decreased the expression of TRIM65 mRNA in WT-PMs (Fig. 5B). LPS treatment also significantly increased the expression of TNFα, IL-1β and IL-6 mRNA, whilst TRIM65-knockout significantly potentiated the LPS-induced increases in these inflammatory cytokines (Fig. 5C-E).

Atherosclerosis-associated cardiovascular diseases are among the leading causes of mortality worldwide, in which macrophages have been documented to serve an important role in the development of atherosclerosis (1,25). Therefore, the expression of TRIM65 in mouse aortas collected from ApoE$^{-/-}$ mice fed a ND or WD for 16 weeks was next measured. As shown in Fig. S1, the expression of TRIM65 mRNA was found to be significantly downregulated in the aorta after feeding on a WD. Taken together, these results indicate that TRIM65 is a negative regulator of macrophage activation, which may serve a potential role in the development of atherosclerosis.

Discussion

Macrophages serve a pivotal role in the progression of inflammatory diseases, such as atherosclerosis (26-30),

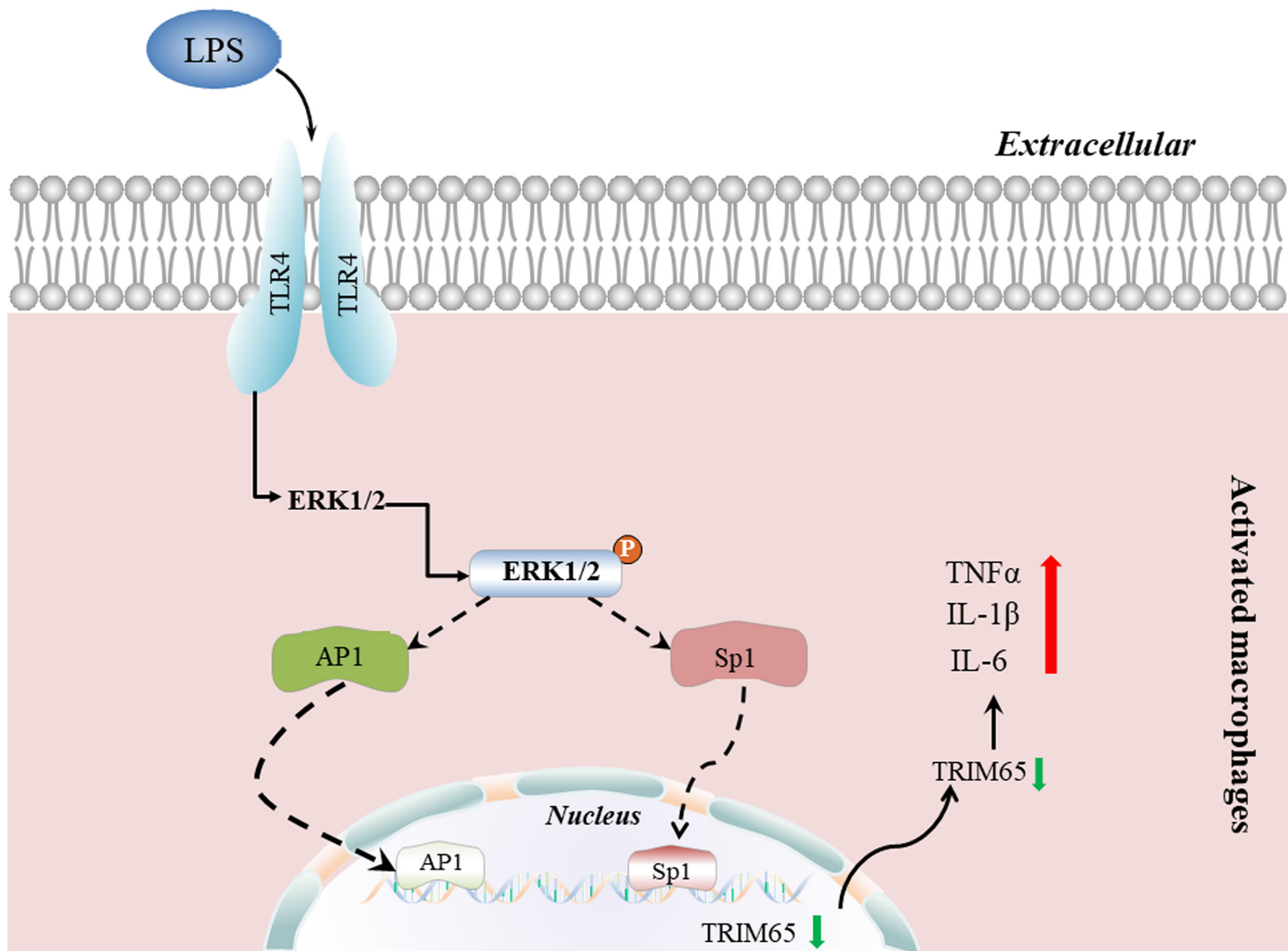

Figure 6. Proposed model of the signaling pathway by which LPS can inhibit macrophage TRIM65 expression and the possible role of TRIM65 in macrophages. LPS can activate the ERK1/2 signaling pathway to decrease the expression of TRIM65 in macrophages. This reduced TRIM65 expression can then promote macrophage activation further by increasing the production of inflammatory cytokines, including TNF-α, IL-1β and IL-6. AP-1, activator protein-1; - LPS, lipopolysaccharide; Sp1, specificity protein 1; TRIM65, Tripartite motif-containing protein 65; TLR4, Toll-like receptor 4.

cancer (30-32) and diabetes (30,33,34). Macrophages are closely associated with the inflammatory response, with M1 macrophages mainly facilitating the proinflammatory response and M2 macrophages mainly promoting the anti-inflammatory response (28,31). Therefore, improving the inflammatory environment by regulating the activation status of macrophages has been proposed to be an effective method for treating diseases such as cancer and atherosclerosis (35). As an ubiquitin E3 ligase, TRIM65 has been reported to regulate various processes, such as carcinogenesis including hepatocellular carcinoma, bladder urothelial carcinoma and colorectal cancer (12-15,36) and innate immunity (16,17). Although the significance of TRIM65 in inflammation has been acknowledged (19,20), the regulatory mechanism that controls its expression and activity in macrophages under inflammatory conditions remain unknown. In the present study, TRIM65 expression was found to be decreased by LPS treatment in macrophages and C57BL/6J mice through the ERK1/2 signaling pathway, whilst TRIM65 knockout potentiated macrophage activation by increasing the production of inflammatory cytokines induced by LPS (Fig. 6). In addition, it was found that TRIM65 mRNA expression in the aorta of ApoE$^{-/-}$ mice fed with WD was decreased. Taken together with the previous findings of anti-inflammatory characteristics of TRIM65 on LPS-induced lung inflammation by Li *et al* (19) and on MSU-induced peritonitis and gouty arthritis by Tang *et al* (20), TRIM65 may serve a particularly key role in the progression of atherosclerosis and be a potential therapeutic target for human atherosclerotic diseases.

Regarding the potential anti-inflammatory properties of TRIM65, TRIM65 is likely to be expressed highly in the immune system. Therefore, in the present study the expression profile of TRIM65 in mouse tissues was first examined. TRIM65 was found to be highly expressed in immune tissues, including the spleen, lymph node and thymus. It was also demonstrated that TRIM65 is highly expressed in monocytes/macrophages and ECs, both of which have been reported to serve important roles in inflammatory diseases, such as atherosclerosis (37). However, the expression level of TRIM65 was as high as BMDMs in mouse SMCs but negligible in human SMCs, which may be attributed to the species differences. Subsequently, possible changes in the expression of TRIM65 following inflammatory stimuli was examined further *in vitro*. Macrophages serve an important role in the inflammatory response because they can synthesize and release large quantities of inflammatory mediators and factors

following stimulation by external factors, such as LPS (38). LPS can mimic the inflammatory response *in vivo* and have been frequently used as a model of inflammatory stimulation for study (38). LPS was used for treating a wide variety of macrophages in the present study, including RAW264.7, peritoneal macrophages, THP-1-derived macrophages and hBMDMs, LPS treatment was found to significantly decrease TRIM65 expression in both murine and human macrophages. This suggest that this LPS-induced inhibition of macrophage TRIM65 expression is not species dependent. To further examine whether this can be replicated *in vivo* during inflammation, male C57BL/6 mice were injected with LPS. The results then demonstrate that TRIM65 expression was also reduced by LPS treatment *in vivo*. Taken together, these data suggest that LPS can inhibit TRIM65 expression both *in vitro* and *in vivo*.

LPS primarily interacts with monocytes and/or macrophages through the Toll-like receptor 4 (TLR4) to activate MAPKs (p38, ERK1/2, c-JNK) and PI3K signaling pathways. These in turn activate NF-κB or AP-1 to regulate the expression of downstream targets (1,4,39,40). Furthermore, activated MAPKs can phosphorylate a number of transcription factors such as Sp1 to regulate target gene expression (1,8). Therefore, to clarify the molecular mechanism underlying LPS-inhibited TRIM65, macrophages were pre-treated with inhibitors of these aforementioned signaling pathways before the extent of LPS-inhibited TRIM65 expression was measured. It was observed that LPS-decreased TRIM65 expression was reversed by ERK1/2 inhibitors but none of the other inhibitors. It was also observed that the phosphorylation level of ERK1/2 was potently inhibited by the ERK1/2 inhibitor but was increased by p38 and PI3K inhibitors, suggesting that the signaling pathway of ERK1/2, p38 and PI3K-Akt may interact with each other. Although LPS was found to inhibit macrophage TRIM65 expression by activating the ERK1/2 signaling pathway, the mechanism downstream of this activated ERK1/2 signaling in the regulation of TRIM65 expression in macrophages remains unknown. According to sequence alignment analysis, several putative AP1 or specificity protein 1 (SP1) binding sites were observed in the proximal region of the TRIM65 promoter (-1,000 to +123). AP-1 is an important transcription factor downstream of the ERK1/2 signaling pathway, which can regulate the expression of target genes such as IL-2 and JunB (41). By contrast, Sp1 is a C2H2-type zinc finger protein and an ubiquitous transcription factor belonging to the Sp/Krüppel-like factor 4 family of proteins (42). Sp1 has been reported to regulate a number of cellular processes, such as immune responses, cell differentiation, cell proliferation and apoptosis (1,43,44) Post-translational modifications, such as phosphorylation, sumoylation, acetylation, glycosylation and proteolytic processing can significantly alter Sp1 activity, resulting in either activation or repression (43,44). For example, Sp1 phosphorylation by JNK1/2 kinases increases protein stability (45). Sp1 sumoylation at Lys16 increases Sp1 degradation (46). In addition, various kinases, including ERK1/2, JNK and p38, have been found to phosphorylate Sp1 (45,47,48). A previous study (1) demonstrated that TRIM59 expression can be regulated by Sp1 and nuclear factor erythroid 2-related factor-1 in LPS-activated macrophages, which may be dependent on the activation of JNK signaling. Therefore, LPS may likely inhibit TRIM65 expression in macrophages by activating the ERK1/2 signal pathway to regulate AP1 or Sp1 activity, though this requires further investigation. In future studies, different lengths of the TRIM65 promoter according to the binding sites of AP-1 or Sp1 will need to be constructed, before preparing the Ap-1 and Sp1 expression plasmid to further examine the effect of Ap-1 and Sp1 on TRIM65 promoter activity. Additionally, the nuclear translocation of Ap-1 and Sp1 would need to be measured by western blotting and immunofluorescent staining assays. The possible interaction between Ap-1 and Sp1 on the corresponding binding sites will also need examination by chromatin immunoprecipitation assay. Finally, the possible effects of Ap-1 and Sp1 siRNA on the expression of TRIM65 in LPS-activated macrophages by will require assessment by western blotting and/or RT-qPCR.

Since LPS inhibited the expression of TRIM65 in macrophages, its role in activated macrophages was therefore explored further. TRIM65 knockout was observed to markedly enhance LPS-induced expression of proinflammatory cytokines TNFα, IL-1β and IL-6, suggesting that TRIM65 is a negative regulator of macrophage activation. These results are consistent with the previously reported anti-inflammatory characteristics of TRIM65 by Li *et al* (19). However, the mechanism by which TRIM65 can negatively regulate macrophage activation remain unclear and further investigations are required.

Collectively, the present study revealed that LPS decreased the expression of TRIM65 not only in murine and human macrophages *in vitro*, but also in spleen, lung, aorta and bone marrow of mice *in vivo*. Mechanistically, it was found that the LPS-reduced TRIM65 expression was blocked by ERK1/2 inhibitors but not by other inhibitors. Moreover, TRIM65 was found to negatively regulate macrophage activation. Taken together, these findings suggest that LPS can inhibit TRIM65 expression *in vitro* and *in vivo* by activating the ERK1/2 signaling pathway. However, reducing TRIM65 expression can potentiate macrophage activation by increasing the production of inflammatory cytokines. These data may provide an insight into the regulation of TRIM65-mediated immune responses, where TRIM65 may serve to be a potential target for the treatment of various inflammatory diseases.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XFZ, XXD and YQN conducted the experiments. HFB, MLJ and DW searched the literature and performed data analysis. PZD and YFX performed data analysis. MXJ designed the study. All authors have read and approved the final manuscript. MXJ and XFZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Studies with human samples were approved by the Ethics Committee of Second Affiliated Hospital of Nanchang University (Nanchang, China; approval no. 2019002) and complied strictly with the 2008 Declaration of Helsinki Principle. Written informed consents were signed by the one participant prior to the collection of samples. The protocols for animal experiments were approved by the Ethics Committee of Nanchang University (approval nos. 20X049 and X20200703) and complied with the Guide for the Care and Use of Laboratory Animals published by NIH.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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