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# TREM1 Enhances Macrophage Proinflammatory Response to LPS by Promoting NF-κB Activation via an IL-26-mediated JAK/STAT Signaling Pathway

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## ABSTRACT

Lipopolysaccharide (LPS)–induced inflammation in macrophages involves complex signaling pathways. This investigation explored the regulatory roles of triggering receptor expressed on myeloid cells-1 (TREM1) and interleukin (IL)-26 in the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and nuclear factor-kappa B (NF-κB) p65 pathways in LPS-stimulated RAW 264.7 macrophages.

RAW 264.7 cells were treated with LPS to assess TREM1 expression. TREM1 or IL-26 was silenced using short hairpin RNA (shRNA), while IL-26 was overexpressed via plasmid transfection. The JAK2 inhibitor AG490 was used to block JAK/STAT signaling. Western blot, reverse transcription–quantitative polymerase chain reaction (RT-qPCR), and enzyme-linked immunosorbent assay (ELISA) were employed to analyze the protein and mRNA levels of inflammatory markers and signaling molecules.

Results showed that LPS upregulated TREM1 expression. In addition, TREM1 knockdown suppressed p65 activation and reduced inflammatory cytokine levels. Moreover, silencing TREM1 inhibited IL-26 and JAK/STAT phosphorylation (p-JAK1, p-JAK2, p-STAT1, and p-STAT3). Similarly, IL-26 knockdown or AG490 treatment attenuated p65 activation and inflammation. Furthermore, IL-26 overexpression reversed the anti-inflammatory effects of TREM1 silencing.

Overall, TREM1 promoted LPS-induced macrophage inflammation via IL-26–mediated JAK/STAT and NF-κB pathway activation, suggesting that TREM1 and IL-26 are potential therapeutic targets.

**Keywords:** IL-26; Inflammation; JAK/STAT; Macrophages; NF-κB; TREM1

## INTRODUCTION

Mononuclear macrophages are central to the inflammatory response, acting as crucial mediators in both innate immunity and the maintenance of tissue homeostasis.[1] These cells act as key mediators, sensing pathogen-associated molecular patterns and damaged tissue signals through pattern recognition receptors. Once activated, they rapidly secrete proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6), recruiting immune cells to the injury site.[2,3] While acute macrophage-mediated inflammation protects the host, chronic activation

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contributes to diseases such as atherosclerosis, fibrosis, and autoimmune disorders.[4–6] However, the precise molecular switches governing their plasticity and inflammatory output remain incompletely understood. Unraveling these mechanisms could provide novel therapeutic targets for modulating inflammation-related diseases.

The triggering receptor expressed on myeloid cells-1 (TREM1), mainly found on neutrophils and monocytes/macrophages, is a powerful enhancer of inflammatory responses.[7] Growing evidence indicates that TREM1 is significantly involved in both infectious and noninfectious inflammatory disorders, such as sepsis, atherosclerosis, and cancer.[7–9] As an immunoreceptor, TREM1 intensifies the signaling triggered by Toll-like receptors (TLRs), resulting in a substantial increase in the production of proinflammatory mediators.[10] Past research has shown that TREM1 not only regulates innate immune activation but also engages with intracellular signaling pathways, namely the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and nuclear factor-kappa B (NF-κB) pathways.[11,12] However, the precise regulatory mechanisms by which TREM1 influences these pathways in macrophage-driven inflammation remain incompletely understood.

The JAK/STAT signaling pathway functions as a crucial regulator in cytokine-mediated signaling, coordinating immune and inflammatory processes.[13] Upon cytokine receptor engagement, JAK kinases (JAK1, JAK2, and JAK3) phosphorylate STAT proteins, enabling their dimerization, nuclear translocation, and transcriptional regulation of target genes.[13] Dysregulated JAK/STAT activation has been implicated in chronic inflammatory diseases,[14,15] highlighting its importance in immune modulation. Previous evidence suggests crosstalk between TREM1 and JAK/STAT signaling,[12] yet the molecular interplay in macrophage inflammation warrants further investigation.

Another pivotal pathway in inflammation is the NF-κB signaling cascade, particularly the p65 (RelA) subunit, which drives the expression of proinflammatory genes.[16] In unstimulated cells, NF-κB remains inactive in the cytoplasm, bound to inhibitory IκB proteins.[17] Upon activation, IκB degradation allows p65 nuclear translocation, initiating transcription of cytokines and chemokines.[17] Given that TREM1 and JAK/STAT signaling influence NF-κB activity, elucidating their synergistic or independent roles in macrophage inflammation is of significant interest.

The objective of this research was to explore how TREM1 regulates inflammatory responses in monocyte-macrophages and to uncover the underlying mechanisms, with a particular emphasis on its interactions with the JAK/STAT and NF-κB p65 signaling pathways. By identifying TREM1 as a potential regulator of these signaling pathways, our results could offer innovative perspectives on developing therapeutic approaches for macrophage-mediated inflammatory diseases.

## MATERIALS AND METHODS

### Antibodies

Antibodies used in this study were obtained from Abcam (Cambridge, MA, USA) [TREM1 (#ab90808; 1/50), H3 (#ab1791; 1/5000), β-actin (#ab8227; 1/5000), TNF-α (#ab183218; 1/2000), IL-6 (#ab290735; 1/1000), IL-1β (#ab283818; 1/1000)]; Santa Cruz Biotechnology (USA) [p65 (#sc-8008; 1/1000)]; Abmart Pharmaceutical Technology Co., Ltd (Shanghai, China) [IL-26 (#PA4913; 1/1000)]; and Thermo Fisher Scientific (Waltham, MA, USA) [phospho-JAK1 (p-JAK1; #PA5-104554; 1/2000), JAK1 (#PA5-105265; 1/2000), p-JAK2 (#PA5-105889; 1/3000), JAK2 (#PA5-11267; 1/1000), p-STAT1 (#MA5-15071; 1/1000), STAT1 (#AHO0832; 1/500), p-STAT3 (#44-384G; 1/1000), STAT3 (#MA1-13042; 1/5000), and goat anti-rabbit IgG (#31460; 1/10 000)].

### Cell Culture

RAW 264.7, mouse monocytic macrophage leukemia cells from Pricella Biotech Co., Ltd. (Wuhan, China; #CL-0190), were cultured in Dulbecco modified Eagle medium (DMEM; Pricella, Wuhan, China; PM150210) with 10% heat-inactivated fetal bovine serum (FBS; Pricella, Wuhan, China, #164210-50) and 1% penicillin/streptomycin (Pricella, Wuhan, China, #PB180120) at 37 °C in a 5% $CO_2$ humidified incubator. To explore JAK/STAT regulation, cells were preincubated with AG490 (75 μM; Sigma, St. Louis, MO, USA; HY-12000) for 2 hours before lipopolysaccharide (LPS) exposure. For LPS stimulation, $5 \times 10^5$ cells per well in 6-well plates were treated with LPS (1 μg/mL; Sigma-Aldrich, St. Louis, MO, USA, #L5293) for 0, 4, 8, 12, or 24 hours.[19] Following LPS exposure, cells were washed twice with phosphate-buffered saline (PBS) to remove residual


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LPS. We transfected RAW 264.7 cells with either (1) short hairpin RNA (shRNA) against *TREM1* or *IL26*, (2) nontargeting control shRNA, (3) *IL26* overexpression plasmid, or (4) empty vector, using Lipofectamine 3000 (Thermo Fisher, Waltham, MA, USA, #L3000001). Reverse transcription–quantitative polymerase chain reaction (RT-qPCR) was used to verify *TREM1* and *IL26* expression 48 hours post transfection.

**RT-qPCR**

Total RNA from RAW 264.7 cells was extracted using TRIzol reagent (#15596026CN; Thermo Fisher, Waltham, MA, USA). The concentration and purity of the isolated RNA were evaluated spectrophotometrically, ensuring an $A_{260}/A_{280}$ ratio of at least 1.9. Subsequently, 1 μg of total RNA was employed to synthesize cDNA under standard conditions using a reverse transcription kit (#RR037Q; TaKaRa, Tokyo, Japan). For qPCR analysis, SYBR Green Master Mix (#11201ES03; Yeason Biotech Co Ltd., Shanghai, China) was used on a real-time PCR system (Thermo Fisher, Waltham, MA, USA). Each sample was analyzed in triplicate. Relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. All primers used in this study were sourced from Genescript Biotech Co, Ltd. (Nanjing, China), and their sequences are detailed in Supplementary Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene.

**Western Blot**

RAW 264.7 cells were lysed on ice for 30 minutes in RIPA buffer (Sigma, St. Louis, MO, USA; R0278) containing protease/phosphatase inhibitors (Beyotime, Shanghai, China, P1046). After centrifugation at 12 000*g* for 15 minutes at 4 °C, the supernatant was collected. For p65 subcellular analysis, nuclear and cytoplasmic fractions were isolated using a kit from Beyotime (Shanghai, China #P0027). Protein concentration was measured by BCA assay (Beyotime, Shanghai, China; P0009). Samples with 30 μg of protein were mixed with Laemmli buffer, denatured at 95 °C for 5 minutes, and separated by 12% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Sigma; 182702) via semidry transfer, blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST; Sigma, St. Louis, MO, USA; T5912) for 1 hour at room temperature, and incubated overnight at 4 °C with primary antibodies in blocking buffer. After a 1-hour incubation with HRP-conjugated secondary antibodies at room temperature, membranes were washed with TBST. Signals were detected using an ECL substrate (Beyotime, Shanghai, China, #P0018S) on a chemiluminescence imager.

**Enzyme-linked Immunosorbent Assay**

Supernatants of RAW 264.7 cells were collected and centrifuged at 300*g* for 10 minutes at 4 °C. The levels of TNF-α, IL-6, and IL-1β were measured with enzyme-linked immunosorbent assay (ELISA) kits (COIBO Biotech Co Ltd, Shanghai, China). All operations were performed according to the manufacturers' instructions. Finally, a microplate reader was used to measure the absorbance at 450 nm. The concentrations of cytokines were computed from standard curves and adjusted according to the total protein content.

**Statistical Analysis**

Data from at least 3 independent experiments (n=3 per group) are presented as mean (SD). Unpaired 2-tailed Student *t* tests were used to compare 2 groups; 1-way analysis of variance (ANOVA) with Tukey post hoc correction was used to compare multiple groups. A *p* value less than 0.05 was considered statistically significant. Analyses were performed using SPSS version 21.0 (IBM Armonk, NY, USA), and data were visualized with GraphPad Prism version 8.0.1 (La Jolla, CA, USA).

## RESULTS

**Increased TREM1 Expression After LPS Treatment**

RAW 264.7 cells were treated with LPS for 0, 4, 8, 12, and 24 hours. Our analysis demonstrated that both TREM1 mRNA and protein levels significantly increased after 8, 12, and 24 hours of LPS treatment ($p<0.001$) (Figure 1A and 1B). Given that the 12-hour LPS treatment induced the most substantial increase in TREM1 expression, we adopted this time point for subsequent experiments.

**Suppression of p65 Signaling and Inflammation by TREM1 Inhibition**

We transfected shNC and shTREM1 vectors into RAW 264.7 cells. As expected, TREM1 inhibition led to a reduction in *TREM1* expression ($p<0.01$) (Figure 2A). LPS treatment elevated the protein levels of nuclear p65 and cytosolic p65 compared with the control group. However, in the presence of TREM1 inhibition (LPS + shTREM1), these levels decreased relative to the

LPS + shNC group (Figure 2B). ELISA and RT-qPCR data further showed that LPS increased the concentrations and mRNA levels of TNF-α, IL-6, and IL-1β, while TREM1 silencing reversed these effects ($p$<0.001) (Figure 2C–H). These results indicate that TREM1 inhibition suppresses the p65 pathway activation and inflammatory response triggered by LPS.

**Association of TREM1 Silencing with Reduced IL-26 Expression and JAK/STAT Signaling**

Western blot analysis demonstrated that compared with the shNC group, the shTREM1 group exhibited decreased protein levels of IL-26, p-JAK1, p-JAK2, p-STAT1, and p-STAT3 in RAW 264.7 cells (Figure 3). These findings suggested that TREM1 silencing was associated with reduced IL-26 expression and JAK/STAT signaling pathway activation in RAW 264.7 cells.

**Suppression of p65 Pathway and Inflammation by IL-26 Inhibition**

RT-qPCR results indicated that IL-26 inhibition successfully reduced its expression ($p$<.01) (Figure 4A). Compared with the LPS + shNC group, IL-26 silencing decreased nuclear p65 and cytosolic p65 levels, as well as TNF-α, IL-6, and IL-1β concentrations and mRNA levels ($p$<.001) (Figure 4B–H), highlighting the role of IL-26 in promoting inflammation via the p65 pathway.

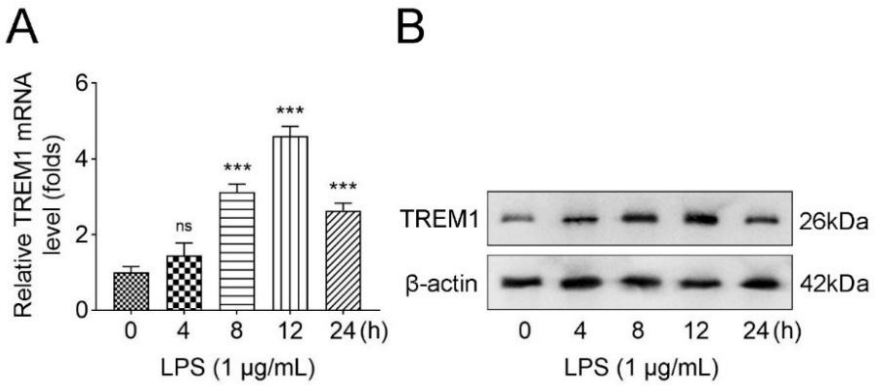
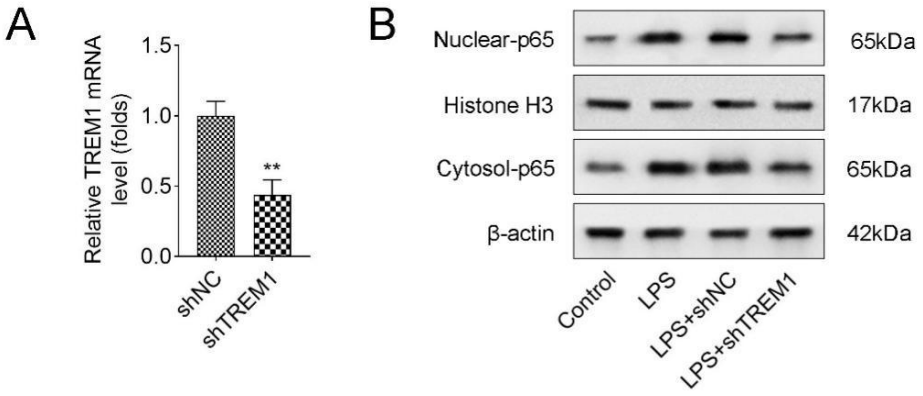



**Figure 1. Increased TREM1 expression after LPS treatment. A, RT-qPCR and (B) Western blot analysis of TREM1 mRNA and protein levels in RAW 264.7 cells treated with LPS for 0, 4, 8, 12, and 24 hours (n = 3). ***$p$<0.001. LPS: lipopolysaccharide; ns: not significant; RT-qPCR: real-time quantitative polymerase chain reaction; TREM1: triggering receptor expressed on myeloid cells 1.**





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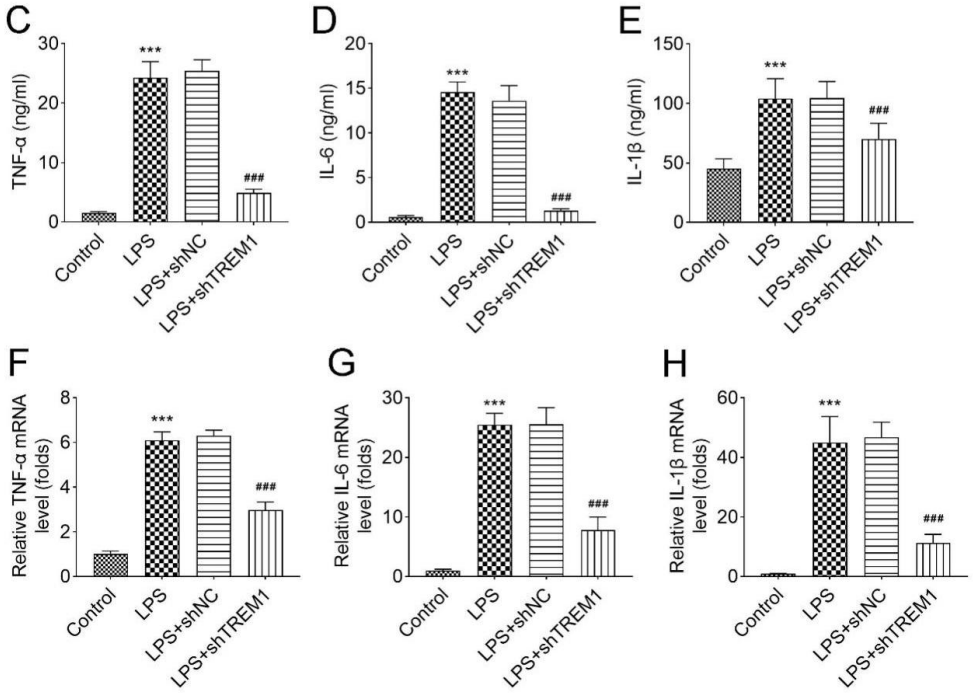


**Figure 2. Suppression of p65 signaling and inflammation by TREM1 inhibition. A, RT-qPCR analysis of *TREM1* expression after TREM1 inhibition in RAW 264.7 cells (n = 3). B, Western blot analysis of nuclear and cytosolic p65 protein levels in each group (n = 3). C-E, ELISA analysis of TNF-α, IL-6, and IL-1β levels in each group (n = 3). F-H, RT-qPCR analysis of *TNF-α*, *IL-6*, and *IL-1β* mRNA levels in each group (n = 3). ***p*<0.01, ****p*<0.001 vs the shNC or control group; ###*p*<0.001 vs the LPS + shNC group. ELISA: enzyme-linked immunosorbent assay; IL: interleukin; LPS: lipopolysaccharide; RT-qPCR: real-time quantitative polymerase chain reaction; shNC: short hairpin negative control; shTREM1: short hairpin triggering receptor expressed on myeloid cells 1; TNF-α: tumor necrosis factor-alpha; TREM1: triggering receptor expressed on myeloid cells 1.**

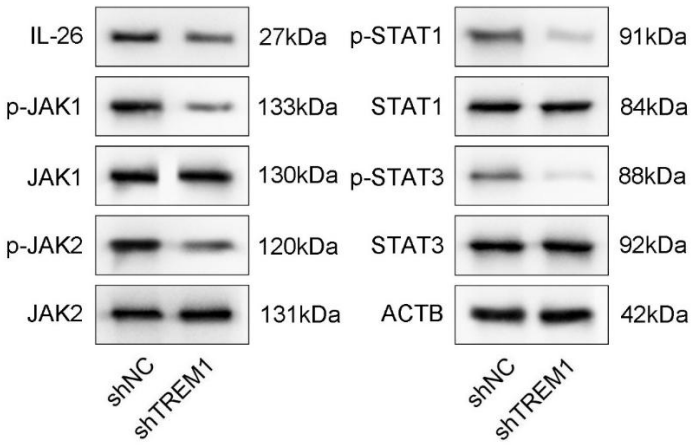


**Figure 3. Association of TREM1 silencing with reduced IL-26 expression and JAK/STAT signaling. Western blot analysis of IL-26, p-JAK1, JAK1, p-JAK2, JAK2, p-STAT1, STAT1, p-STAT3, and STAT3 protein levels in the shNC and shTREM1 groups in RAW 264.7 cells (n = 3). IL: interleukin; JAK1: Janus kinase 1; JAK2: Janus kinase 2; p-: phosphorylated; shNC: short hairpin negative control; shTREM1: short hairpin triggering receptor expressed on myeloid cells 1; STAT1: signal transducer and activator of transcription 1; STAT3: signal transducer and activator of transcription 3; TREM1: triggering receptor expressed on myeloid cells 1.**

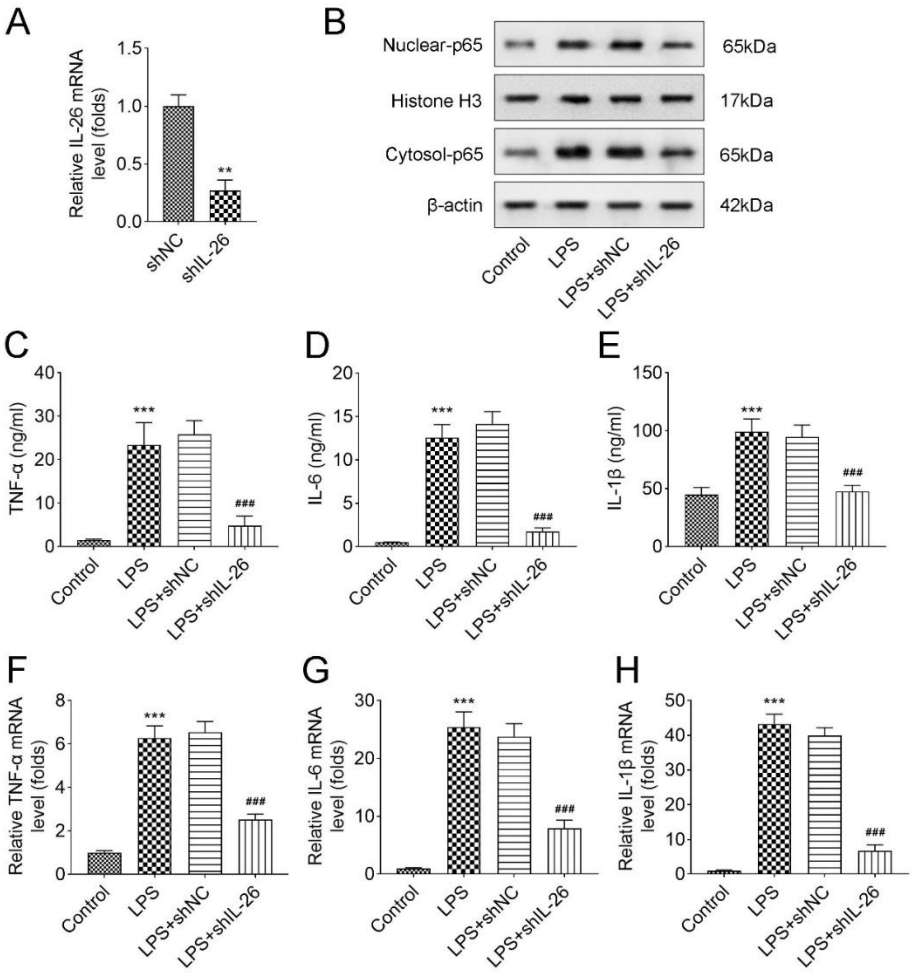


**Figure 4. Suppression of p65 pathway and inflammation by IL-26 inhibition. A. RT-qPCR analysis of *IL26* expression after IL-26 inhibition in RAW 264.7 cells (n = 3). B. Western blot analysis of nuclear and cytosolic p65 protein levels in each group (n = 3). C-E. ELISA analysis of TNF-α, IL-6, and IL-1β levels in each group (n = 3). F-H. RT-qPCR analysis of *TNF-α*, *IL-6*, and *IL-1β* mRNA levels in each group (n = 3). ***p*<0.01, ****p*<0.001 vs the shNC or control group; ###*p*<0.001 vs the LPS + shNC group. ELISA: enzyme-linked immunosorbent assay; IL: interleukin; LPS: lipopolysaccharide; RT-qPCR: real-time quantitative polymerase chain reaction; shIL-26: short hairpin interleukin; shNC: short hairpin negative control; TNF-α: tumor necrosis factor-alpha.**

### Suppression of p65 Pathway and Inflammation by JAK Pathway Inhibition

Treating RAW 264.7 cells with AG490, a JAK2 inhibitor, led to decreased nuclear p65 and cytosolic p65 levels, along with reduced TNF-α, IL-6, and IL-1β concentrations and mRNA levels compared with the LPS-only group. Conversely, adding LPS to the AG490-treated group reversed these effects ($p$<0.01) (Figure 5A–G), confirming that JAK/STAT pathway inhibition suppresses the p65 pathway and associated inflammation.

### Enhancement of Inflammatory Markers by IL-26 Overexpression

Transfection of an *IL26* overexpression vector into RAW 264.7 cells upregulated *IL26* mRNA levels (Figure 6A). Compared with the LPS + shTREM1 + vector group, IL-26 overexpression increased nuclear p65 and cytosolic p65 protein levels, as well as the concentrations and mRNA levels of TNF-α, IL-6, and IL-1β ($p$<0.01), indicating that IL-26 promotes inflammation in these cells.

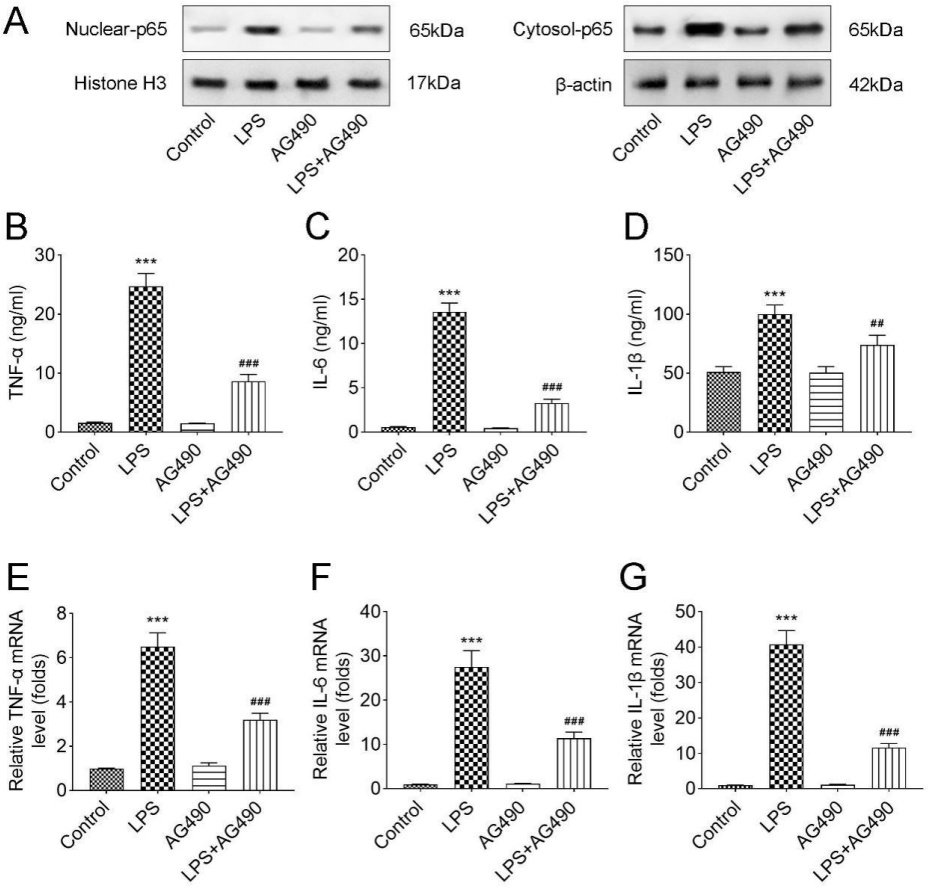


**Figure 5. Suppression of p65 pathway and inflammation by JAK pathway inhibition. A, Western blot analysis of nuclear and cytosolic p65 protein levels in each group (n = 3). B-D, ELISA analysis of TNF-*α*, IL-6, and IL-1β levels in each group (n=3). E-G, RT-qPCR analysis of *TNF-α*, *IL-6*, and *IL-1β* mRNA levels in each group (n=3). ***$p<0.001$ vs the control group; ##$p<0.01$, ###$p<0.001$ vs the AG490 group. ELISA: enzyme-linked immunosorbent assay; IL: interleukin; JAK: Janus kinase; RT-qPCR: real-time quantitative polymerase chain reaction; TNF-α: tumor necrosis factor-alpha.**

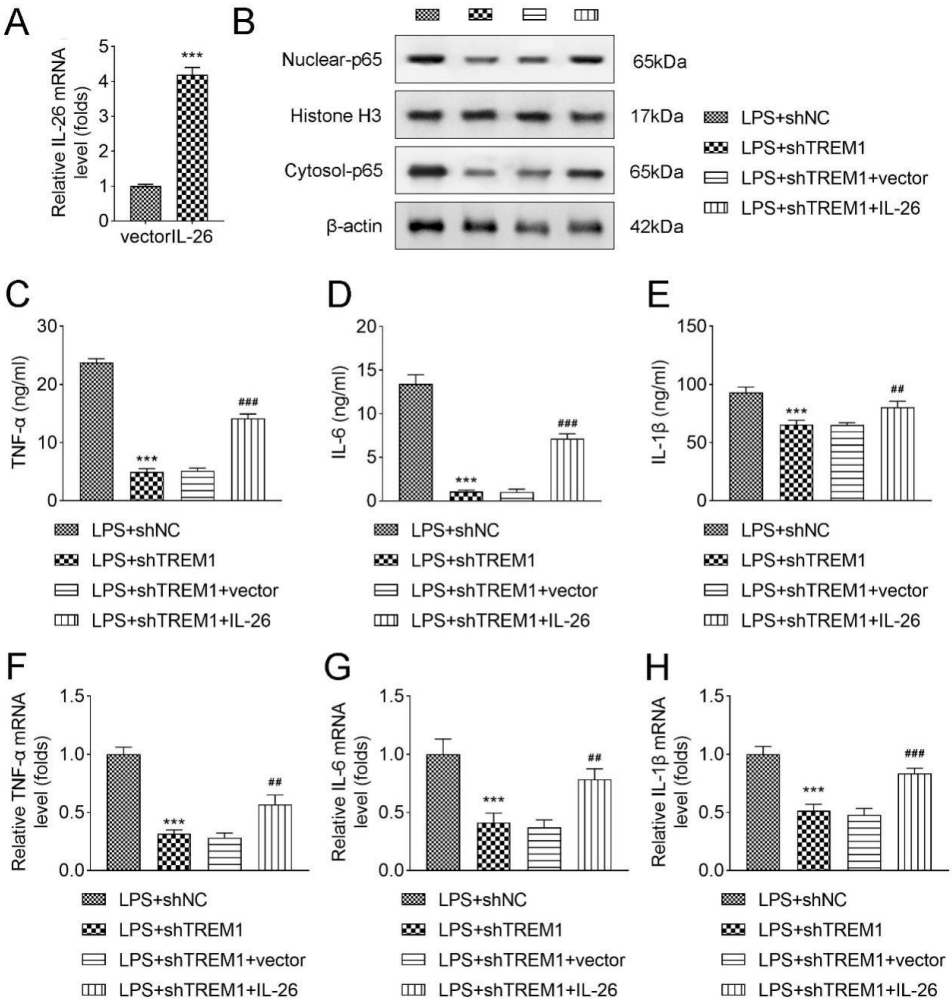


**Figure 6. Enhancement of inflammatory markers by IL-26 overexpression. A, RT-qPCR analysis of *IL26* expression after IL-26 overexpression in RAW 264.7 cells (n = 3). B, Western blot analysis of nuclear and cytosolic p65 protein levels in each group (n = 3). C-E, ELISA analysis of TNF-α, IL-6, and IL-1β levels in each group (n = 3). F-H, RT-qPCR analysis of *TNF-α*, *IL-6*, and *IL-1β* mRNA levels in each group (n = 3). ***p*<.001 vs the vector or LPS + shNC group; ##*p*<.01, ###*p*<.001 vs the LPS + shTREM1 + vector group. ELISA: enzyme-linked immunosorbent assay; IL: interleukin; LPS: lipopolysaccharide; RT-qPCR: real-time quantitative polymerase chain reaction; shNC: short hairpin negative control; shTREM1: short hairpin triggering receptor expressed on myeloid cells 1; TNF-α: tumor necrosis factor-alpha.**

## DISCUSSION

Mononuclear macrophages initiate inflammation by secreting cytokines and recruiting immune cells upon encountering pathogens or tissue damage. This study demonstrated that LPS stimulation significantly upregulated TREM1 expression in RAW 264.7 macrophages, peaking at 12 hours. This finding aligned with a previous study reporting that 12-hour LPS treatment effectively induces inflammatory responses in RAW 264.7 cells in inflammatory bowel disease.[19] However, in some studies, RAW 264.7 cells are often treated with LPS for 24 hours to induce inflammation.[20,21] This discrepancy might be due to differences in experimental purpose, environment, and LPS concentration. Consistent with our results, TREM1 is strongly induced by bacterial components, such as LPS, and amplifies inflammatory responses in myeloid cells.[11,22] Additionally, Dong et al[23] revealed that hypoxia stimulation increased TREM1 expression in tumor-associated macrophages. Our data further revealed that TREM1 knockdown suppressed NF-κB

(p65) activation and reduced the secretion of proinflammatory cytokines. This is consistent with studies indicating that TREM1 enhances TLR4-mediated NF-κB signaling, leading to excessive cytokine production.[24] In addition, Zhong et al[22] reported that blocking TREM1 mitigated NLRP3 inflammasome activation and glycolysis in LPS-induced acute lung injury in mice. Furthermore, an earlier in vivo investigation showed that TREM1 knockout decreased the expression of TLR2/3/4/9, p-IκBα, and p-NF-κB proteins in mice after spinal cord injury.[25]

Notably, we found that TREM1 silencing downregulated IL-26 expression and inhibited JAK/STAT pathway activation. This finding suggested that TREM1 may regulate inflammation partly through IL-26–mediated JAK/STAT signaling.[26,27] IL-26 signals primarily through a heterodimeric receptor complex (IL-10R2/IL-20R1), which triggers JAK1/STAT1/STAT3 activation in immune cells. Although the receptor composition in macrophages needs further validation, our data suggest that TREM1-induced IL-26 engages this canonical pathway to amplify JAK/STAT signaling. Similarly, Rudick et al[28] demonstrated that for sepsis management, partially blocking TREM1 signaling, along with modifying soluble TREM1 (sTREM1) and the JAK/STAT pathway, represents a promising approach to reestablish the host immune response and enhance survival. In addition, a similar interaction was reported in rheumatoid arthritis, where triptolide not only remarkably lowered the levels of TREM1 but also significantly suppressed the activation of JAK2 and STAT3.[12] Interestingly, Xiong et al[29] revealed that hepatocyte-derived HMGB1 activates TREM1/JAK2/STAT3 in Kupffer cells, creating an immunosuppressive tumor microenvironment. However, the specific link between TREM1 and IL-26 in macrophages has not been previously explored. Our findings provide novel evidence that IL-26 acts as a downstream effector of TREM1, bridging its proinflammatory effects to JAK/STAT signaling.

Further supporting this mechanism, IL-26 knockdown or JAK2 inhibition (AG490) attenuated NF-κB activation and cytokine production, mirroring the effects of TREM1 silencing. This aligns with studies showing that JAK/STAT signaling interacts with NF-κB to amplify inflammation.[30] Conversely, IL-26 overexpression reversed the anti-inflammatory effects of TREM1 knockdown, confirming its pivotal role in this regulatory axis. These results are consistent with reports that IL-26 exacerbates inflammation in autoimmune diseases,[31] but our study is the first to link IL-26 to TREM1-driven macrophage activation. Notably, therapeutic targeting of TREM1 for inflammatory diseases requires careful consideration of its dual immunological roles. Although TREM1 exacerbates pathological inflammation in most conditions, it also mediates protective immune responses during bacterial infections. For example, a previous study demonstrated that TREM1 protects human immunodeficiency virus type 1 (HIV-1)–infected macrophages from apoptosis through maintenance of mitochondrial function.[32] This duality implies that systemic TREM1 inhibition could inadvertently compromise innate immunity, necessitating strategies that selectively modulate TREM1 signaling in inflamed tissues rather than globally. Thus, future translational studies should prioritize context-specific approaches to balance anti-inflammatory efficacy with preserved host defense.

In summary, this study showed that TREM1 heightens LPS-induced macrophage inflammation by activating the JAK/STAT and NF-κB pathways through IL-26. These discoveries indicate that blocking TREM1 or IL-26 could be a viable treatment for inflammatory disorders. This study is the first to establish a direct link between TREM1 and IL-26 in macrophages, demonstrating that TREM1 regulates the JAK/STAT pathway via IL-26.

However, this study has several limitations. First, the knockdown efficiency of TREM1 and IL-26 was not validated by Western blot, which could potentially affect the interpretation of gene silencing effects. Second, as an immortalized macrophage cell line, RAW 264.7 cells may not fully recapitulate the complex biological characteristics and regulatory networks of primary macrophages, which might limit the translational relevance of our findings to in vivo physiological and pathological contexts. Third, although AG490 was used at a concentration previously validated for JAK2-selective inhibition, potential off-target effects on JAK1 and JAK3 cannot be completely ruled out, which may influence the specificity of conclusions drawn regarding JAK/STAT pathway–mediated mechanisms. These limitations highlight important directions for future investigations.

## STATEMENT OF ETHICS

Not applicable.

## FUNDING

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## ACKNOWLEDGMENTS

Not applicable.

## DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## AI ASSISTANCE DISCLOSURE

Not applicable.

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