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Original Research

TRIM24 Regulates NLRP3 Transcription via RARα to Promote Inflammasome Activation and IL-1β-Mediated Inflammation

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Abstract

Background: NLRP3 participates in initiating and amplifying inflammatory responses. TRIM24 is a transcriptional regulator and has been implicated in tumorigenesis and metabolic pathways. This study aimed to systematically investigate the function of TRIM24 in inflammation and elucidate its molecular regulation of NLRP3. **Methods:** An inflammatory model was established by stimulating THP-1 cells with LPS/ATP. TRIM24 was overexpressed or silenced to evaluate its impact on pro-inflammatory cytokine expression. A THP-1–HepG2 co-culture system with fatty acid treatment was used to assess TRIM24 regulation of NLRP3 expression. U937 cells were used for independent validation of the TRIM24/RARα/NLRP3 regulatory axis. Techniques including qRT-PCR, Western blotting, immunofluorescence, ELISA, CO-IP, dual-luciferase reporter assays, ChIP-PCR, and flow cytometry were employed to analyze inflammasome activation, pyroptosis, and transcriptional regulation. **Results:** TRIM24 positively regulated LPS/ATP-induced TNF-α, IL-6, and IL-1β expression. In the THP-1–HepG2 co-culture system, TRIM24 upregulated NLRP3 transcription and protein expression. Mechanistically, TRIM24 cooperated with the nuclear receptor RARα to enhance NLRP3 promoter activity by binding to the RARE region and promoting transcriptional activation. Additionally, TRIM24-mediated inflammasome activation and pyroptosis were dependent on NLRP3. RARα inhibition attenuated, whereas RARα activation rescued, TRIM24-mediated regulation of NLRP3 inflammasome-associated responses. Independent validation in U937 cells further confirmed the regulatory role of the TRIM24/RARα/NLRP3 axis in inflammasome activation. **Conclusion:** This study identifies a novel role for TRIM24 in activating the NLRP3 inflammasome via RARα-dependent transcriptional regulation, providing new insights into inflammasome-mediated inflammation and potential therapeutic targets.

Keywords: TRIM24; NLRP3; inflammasome; pyroptosis

1. Introduction

Inflammation represents an essential biological response that enables the host to sense and eliminate infectious agents, repair damaged tissues, and adapt to internal stress signals. This process involves coordinated interactions among immune cell activation, inflammatory mediator production, and complex regulatory networks that collectively preserve tissue equilibrium [1,2,3]. In physiological settings, acute inflammatory responses are precisely controlled and transient, facilitating pathogen clearance while promoting subsequent tissue restoration [4]. However, in certain severe infectious diseases, such as acute peritonitis, a clinical emergency characterized by intra-abdominal infection and intense immune activation, the inflammatory response often manifests as rapid onset, excessive intensity, and progressive amplification. When dysregulated, this process can result in overwhelming inflammation, local and distant organ injury, and even progression to inflammatory response syndrome and organ dysfunction [5,6,7]. Therefore, elucidating the mechanisms underlying the abnormal amplification and sustained activation of inflammation in severe infectious diseases such as acute peritonitis is of great importance for understanding their pathophysiology.

The NLRP3 inflammasome serves as a central intracellular immune sensor that integrates microbial cues and cellular stress-associated signals. Upon activation, this multiprotein complex promotes Caspase-1 processing, thereby driving the conversion and release of the inflammatory cytokines IL-1β and IL-18 [8,9]. As a critical complex for initiating inflammatory immune responses, NLRP3 is important for a variety of acute inflammatory diseases, including acute peritonitis [10]. A previous study has demonstrated elevated levels of NLRP3 inflammasome-associated proteins in mouse models of acute peritonitis [11]. Moreover, accumulating evidence indicates that inhibition of NLRP3 effectively attenuates LPS/ATP-induced inflammatory responses in acute peritonitis models [11,12,13]. Thus, in-depth investigation of the role of the NLRP3 inflammasome and its fine-tuned regulatory mechanisms in acute

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peritonitis may provide novel insights into disease progression.

TRIM24, transcriptional intermediary factor 1-alpha (TIF1α), is a multifunctional regulatory protein with E3 ubiquitin ligase activity. TRIM24 participates in transcriptional regulation by interacting with DNA-binding proteins such as nuclear receptors or by directly recognizing specific chromatin modification states through its PHD-bromodomain [14,15]. TRIM24 regulates gene expression involved in cell proliferation, metabolic homeostasis, and immune responses [16,17,18]. In recent years, increasing evidence has implicated TRIM24 in inflammatory signaling pathways and the fine regulation of inflammatory responses [18,19,20]. However, the precise role of TRIM24 in infectious inflammatory settings, particularly in inflammasome regulation and sustained inflammatory activation during acute peritonitis, remains poorly understood and lacks systematic investigation.

Therefore, we employed *in vitro* inflammatory models to systematically investigate the role of TRIM24 in pro-inflammatory responses and NLRP3 inflammasome activation, as well as the underlying molecular mechanisms. This study is expected to deepen our understanding of TRIM24-mediated inflammatory regulation, provide new insights into the pathogenesis of severe infectious inflammatory progression, and offer novel perspectives for the development of inflammasome-targeted therapeutic strategies and potential treatment targets.

2. Methods

2.1 Cell Culture

THP-1 human monocytic leukemia cells (#CL-0233) and U937 cells (#CL-0239) were obtained from Wuhan Procell Life Science & Technology Co., Ltd. (Wuhan, China) and cultured in RPMI-1640 medium supplemented with 10% FBS (cat. no 164210, Procell, Wuhan, China), 0.05 mM β-mercaptoethanol (cat. no M31355, Abmole, Shanghai, China), and 1% penicillin–streptomycin (P/S, cat. no PB180120, Procell, Wuhan, China). HepG2 cells (#CL-0103, Procell) were cultured in MEM medium, supplemented with 10% FBS, along with 1% non-essential amino acids (NEAA), 2 mM L-glutamine and 1% P/S. All cells were cultured at 37 °C in a humidified incubator with 5% CO_2. All the cell lines used in this study have been authenticated for identity and tested for mycoplasma contamination. The cell identity was verified by short tandem repeat (STR) typing, and the obtained STR profiles were compared with the reference profiles in the ATCC cell bank to confirm that there was no cross-contamination and that the identity was correct. Mycoplasma detection was performed using PCR, and nucleic acid amplification tests for mycoplasma were conducted on the supernatants of the cultured cells on a regular basis. The results of mycoplasma detection for all batches of cells were negative. All the cells used in this study were subjected to functional experiments within the passage range of P3 to P8, and cells beyond this passage range were not used for formal experiments.

2.2 Cell Transfection

Genetic manipulation of THP-1 or U937 cells was performed using jetPRIME transfection reagent (#101000046, Polyplus, Illkirch-Graffenstaden, France). TRIM24 knockdown was achieved by transfection with pLKO.1-sh-TRIM24 plasmid (400 ng) or a negative control shRNA, using the same constructs and transfection conditions in both cell lines; no siRNA targeting TRIM24 was used in this study. TRIM24 overexpression was performed using the pCDNA3.1(–)-TRIM24-FLAG plasmid (2 μg; NheI/KpnI digestion, 3183 bp) or the empty pCDNA3.1(–) vector. NLRP3 knockdown was carried out using pLKO.1-sh-NLRP3 plasmid (400 ng), and RARα silencing was achieved by transfection with si-RARα (50 nM). For RARα overexpression, cells were transfected with the pCDNA3.1(–)-RARα-FLAG plasmid.

For promoter reporter assays, luciferase reporter plasmids (pGL3-WT or pGL3-ΔRARE), the pRL-TK Renilla luciferase internal control plasmid, and the pCDNA3.1(–)-TRIM24-FLAG expression plasmid were co-transfected.

2.3 Cell Treatment and Co-Culture

For NLRP3 inflammasome activation, transfected THP-1 cells were exposed to LPS (100 ng/mL; #HY-D1056, MCE, NJ, USA) for 4 h, followed by ATP stimulation (5 mM; #HY-B2176, MCE) for 30 min. After stimulation, both cell lysates and culture supernatants were harvested for subsequent analyses. HepG2 cells were challenged with 1.2 mmol/L free fatty acids (palmitic acid:oleic acid= 1:2) for 48 h. Subsequently, treated THP-1 cells were directly co-cultured with HepG2 cells to evaluate the potential interaction between TRIM24 and NLRP3 signaling. In parallel, U937 cells were differentiated using PMA (MedChemExpress (MCE), HY-18739, Monmouth Junction, NJ, USA, 100 ng/mL) and subsequently exposed to LPS combined with nigericin (MCE, HY-100381, Monmouth Junction, NJ, USA) to induce NLRP3 inflammasome activation. To modulate RARα activity, cells were incubated with the selective RARα agonist AM580 (MCE, HY-10475, Monmouth Junction, NJ, USA, 1 μM) for 24 h.

2.4 Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from cells using 600 μL Buffer RLT Plus and the RNeasy Plus Mini Kit (#74134, QIAGEN, Germany). RNA quantity and purity were determined with a NanoDrop spectrophotometer (Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, China). For cDNA synthesis, 1 μg RNA was processed with the QuantiTect Reverse Transcription Kit (#205311, QIAGEN). qPCR amplification was conducted in a 20 μL reaction volume containing 10 μL Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix (#11184ES08, YEASEN, Shanghai, China), 0.4 μL each of forward and reverse primers (10

μM), 1 μL cDNA, and 8.2 μL nuclease-free water (#R1600, Solarbio, Beijing, China). The amplification program consisted of an initial denaturation step at 95 °C for 30 s and 40 cycles of 95 °C for 3 s and 60 °C for 20 s. β-actin served as the reference gene, and relative transcript abundance was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were TRIM24 (5′–3′): forward-GCT GGC ACT TGT TCT GGA AA, reverse-GCA TGG TCG CAC ACT ACT TT; TNF-α (5′–3′): forward-CTC ATG CAC CAC CAT CAA GG, reverse, ACC TGA CCA CTC TCC CTT TG; IL-6 (5′–3′): forward-AGT GGC TAA GGA CCA AGA CC, reverse-ACC ACA GTG AGG AAT GTC CA; IL-1β (5′–3′): forward-AGC TTC AAA TCT CGC AGC AG, reverse-TCT CCA CAG CCA CAA TGA GT; NLRP3 (5′–3′): forward-CAG AGC CTA CAG TTG GGT GA, reverse-TTC CAC GCC TAC CAG GAA AT; β-actin (5′–3′): forward-CCT CTA TGC CAA CAC AGT GC, reverse-GCT AGG AGC CAG AGC AGT AA.

2.5 Western Blotting

Cells were harvested and lysed in ice-cold lysis buffer (#HKW2012, HaoKebio, Hangzhou, China) containing PMSF (#HKW2017, HaoKebio) and phosphatase inhibitor cocktail (#HKW2018, HaoKebio) for 30 min. Following centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatants were collected, and protein levels were quantified using a BCA assay kit (#HKW2019, HaoKebio). Equal amounts of protein (20 μg) were prepared with 5× SDS loading buffer (#HKW2020, HaoKebio), and resolved by SDS-PAGE on 10% precast fast gels. Separated proteins were transferred to PVDF membranes (#36125ES, YEASEN), which were blocked with 5% non-fat milk for 1 h at room temperature. The membranes were then incubated with corresponding primary antibodies at 4 °C overnight. After three washes with TBST (#HK0003, HaoKebio), HRP-linked secondary antibodies (#RGAR001, Proteintech, Wuhan, China; 1:5000) were applied for 1 h at room temperature. Immunoreactive signals were detected using an enhanced chemiluminescence reagent (#HKW2095, HaoKebio) and captured with a chemiluminescence imaging system (Qinxiang Scientific Instruments Co., Ltd., Shanghai, China). The primary antibodies used in this study were as follows: TRIM24 (1:1000, #HA721052, Huabio, Hangzhou, China), TNF-α (1:1000, #SJ-AB6395, Sanjing Bio, Hangzhou, China), IL-6 (1:1000, #SJ-AB4630, Sanjing Bio), IL-1β (1:1000, #SJ-AB0622, Sanjing Bio), NLRP3 (1:1000, #SJ-AB21427, Sanjing Bio), cleaved-Caspase-1 (1:1000, #SJ-AB3932, Sanjing Bio), cleaved-GSDMD (1:1000, #SJ-AB56467, Sanjing Bio), GSDMD (1:1000, #SJ-AB48183, Sanjing Bio), Caspase-1 (1:2000, #SJ-AB5733, Sanjing Bio), ASC (1:5000, #HA723917, Huabio) and RARα (1:1000, #ER1706-81, Huabio). β-actin (1:1000, #SJ-AB5351, Sanjing Bio) or GAPDH (1:5000, #10494-1-AP, Proteintech) was used as the loading control.

2.6 Immunofluorescence Staining

Cells cultured in 6-well plates were fixed with 4% fixative (#P1110, Solarbio, Beijing, China) for 20 min and permeabilized using immunostaining permeabilization buffer (#PR30007, Proteintech) for 15 min. After blocking with TBST blocking solution for 1 h, samples were incubated with primary antibodies targeting IL-1β, NLRP3, TRIM24, or ASC (1:100) for 1 h. Fluorescent secondary antibodies were subsequently applied for 1 h at room temperature under light-protected conditions, including Multi-rAb™ CoraLite® Plus 488-conjugated goat anti-rabbit IgG (1:500, #RGAR002, Proteintech), Multi-rAb™ CoraLite® Plus 594-conjugated goat anti-mouse IgG (1:500, #RGAM004, Proteintech), and AMCA-conjugated goat anti-rabbit IgG (1:500, #SA00010-2, Proteintech). Nuclear staining was performed with DAPI (#E607303-0020, Sangon Biotech, Shanghai, China) for 3 min. Following three PBS washes, fluorescence images were acquired using a fluorescence microscope. ImageJ was used for subsequent fluorescence colocalization analysis.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of secreted IL-1β and IL-18 in culture supernatants were quantified using human IL-1β (#JN19669, Jining Bio, Shanghai, China) and IL-18 ELISA kit (#JN7044, Jining Bio), respectively. Optical density was recorded at 450 nm with a microplate reader. Cytokine concentrations were determined based on the corresponding standard curves.

2.8 Co-Immunoprecipitation (Co-IP)

Cells were lysed on ice for 30 min with lysis buffer (#B605116, Sangon Biotech) supplemented with protease inhibitors (100×, #B605116, Sangon Biotech), with gentle mixing every 5 min. Cell lysates were further subjected to sonication (power 20%, 3 s on/3 s off, total 5 min) and centrifuged at 12,000 rpm for 10 min to collect the supernatants. An aliquot of 100 μL supernatant was reserved as input and stored at –20 °C. For immunoprecipitation, 3–5 μg of species-matched IgG or the indicated primary antibody was added to the lysates for the IgG control and IP groups, respectively. The volume was adjusted to 800 μL with incubation buffer, followed by gentle mixing overnight. Protein A/G magnetic beads (#B605116, Sangon Biotech) were washed three times and then incubated with the antibody–lysate mixtures for 2 h. After magnetic separation and three washes with wash buffer, the immune complexes were eluted with 100 μL elution buffer by boiling for 10 min. The eluates were mixed with 20 μL 6× SDS loading buffer and boiled for 10 min. For nuclear Co-IP analysis, nuclear fractions were prepared before immunoprecipitation. The nuclear lysates were subjected to the same immunoprecipitation procedure described above using antibodies against TRIM24 or RARα, with species-matched IgG as the negative control.

2.9 Chromatin Immunoprecipitation (ChIP)–PCR

Approximately 1×10^7 cells were harvested and subjected to chromatin cross-linking with 1% formaldehyde prepared in PBS at room temperature for 5 min. The reaction was terminated by glycine buffer (#JKR23002A, Jinkairui, Wuhan, China) treatment for 5 min. After two washes with cold PBS, cells were lysed in lysis buffer containing protease inhibitors on ice. The extracted chromatin was fragmented by sonication to generate DNA fragments ranging from 200 to 500 bp. After centrifugation at 10,000 ×g for 10 min at 4 °C, the clarified chromatin fractions were collected. A portion of each chromatin sample (50 µL) was retained as input control, while the remaining lysates were subjected to immunoprecipitation with antibodies against TRIM24 or RARα (3–5 µg per reaction). Corresponding species-matched IgG antibodies were included as negative controls. Following overnight incubation with antibodies at 4 °C under rotation, Protein A/G magnetic beads were added and further incubated for 2 h. The bead-bound complexes were sequentially rinsed using wash buffers 1–4 and subsequently released with elution buffer at room temperature for 15 min. The recovered chromatin complexes were treated with RNase A at 37 °C for 5 min and Proteinase K at 65 °C for 3 h to remove RNA and reverse protein-DNA interactions. Purified DNA was obtained using a DNA purification kit (Active Motif, cat#58002, Carlsbad, CA, USA) and analyzed by qPCR. The binding enrichment of TRIM24 and RARα at the RARE-containing region of the NLRP3 promoter was determined using the following primer pair: forward, 5′-CCA GAT GTC TCA GGT CAG TG-3′; reverse, 5′-AGC TGA CAC TGG CTA TGA CC-3′. The relative occupancy was calculated based on normalization to input DNA. For re-ChIP experiments, chromatin immunoprecipitated with the first antibody was eluted under conditions compatible with subsequent immunoprecipitation and subjected to a second immunoprecipitation using the indicated antibody. The resulting DNA was purified and analyzed by qPCR using the same NLRP3 promoter RARE-region and distal-region primers. Species-matched IgG controls were included where appropriate.

2.10 Dual-Luciferase Reporter Assay

The pGL3-WT reporter plasmid containing the wild-type NLRP3 promoter region (–1500 to +100 bp) with an intact RARE motif, the pGL3-ΔRARE reporter plasmid carrying a mutation in the RARE core sequence, and the pGL3-SV40 plasmid (negative control) were constructed. Cells were introduced with the indicated reporter constructs together with the pRL-TK Renilla plasmid as an internal normalization control and the TRIM24 overexpression vector. After 48 h of incubation, transfected cells were collected and disrupted using cell lysis buffer. Luciferase signals were subsequently determined using a dual-luciferase reporter assay kit (#JKR23008, Jinkairui) following the provided protocol. Briefly, aliquots of cell lysates (10–20 µL) were transferred into 96-well plates, followed by addition of 100 µL luciferase substrate reagent to quantify firefly luciferase activity. Renilla luciferase activity was then detected after supplementation with 100 µL of the corresponding reaction reagent II. Luminescence intensity was recorded using a multimode microplate reader, and promoter activity was represented as normalized firefly luciferase activity relative to Renilla luciferase activity.

2.11 Flow Cytometric Analysis of Pyroptosis

Pyroptotic cell death was evaluated using a FAM-YVAD-FMK/PI double-staining assay. Briefly, cells were harvested after EDTA-free trypsinization (#PB180225, Procell) and collected by centrifugation at 300 ×g for 5 min at 4 °C. Following two PBS washes, cell suspensions were incubated with FAM-YVAD-FMK probe (#13473, AAT Bioquest, CA, USA; 100 µg/mL) at 37 °C for 1 h in darkness to detect activated Caspase-1. The labeling reaction was subsequently stopped by placing samples on ice. After additional PBS washing, cells were stained with propidium iodide (PI; #40710ES, YEASEN; 1 mg/mL) to identify cells with compromised plasma membranes. Flow cytometry was performed by measuring FAM-YVAD-FMK fluorescence using the FL1 channel (excitation 488 nm, emission 530 nm) and PI fluorescence using the FL3 channel (excitation 488 nm, emission 620 nm). Data were processed and quantified using FlowJo software (version 10.8.1, BD Biosciences, Ashland, OR, USA). Cells were first gated on FSC-A versus SSC-A to exclude debris, and single cells were then selected on FSC-H versus FSC-A to exclude doublets. Compensation for FL1/FL3 spillover was calculated from single-stained controls, and quadrant boundaries, set using unstained and single-stained controls, were applied identically to all samples. Singlets were displayed as FAM-YVAD-FMK (x-axis) versus PI (y-axis), and the four quadrants were defined as follows: lower left (Q1-LL, FAM^-PI^-), viable cells; lower right (Q1-LR, FAM^+PI^-), Caspase-1-active cells with intact membranes (early pyroptosis); upper right (Q1-UR, FAM^+PI^+), Caspase-1-active cells with ruptured membranes (late pyroptosis); and upper left (Q1-UL, FAM^-PI^+), Caspase-1-independent membrane damage, which was not counted as pyroptosis. Because Caspase-1 activation is the defining event of canonical pyroptosis and precedes PI uptake, pyroptotic cells were defined as the total FAM-YVAD-FMK-positive population and calculated as: pyroptotic cells (%) = Q1-LR (%) + Q1-UR (%), expressed as a percentage of singlets.

2.12 ASC Oligomerization Assay

ASC oligomerization was assessed to evaluate NLRP3 inflammasome assembly. Briefly, cells were collected after the indicated treatments and lysed on ice for 30 min in ASC oligomerization assay buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, and protease inhibitor cocktail). The cell lysates were cen-

trifuged at 6000 ×g for 15 min at 4 °C to separate detergent-soluble and detergent-insoluble fractions. The insoluble pellets containing ASC aggregates were washed twice with ice-cold PBS, resuspended in 500 µL PBS, and cross-linked with 2 mM disuccinimidyl suberate (DSS; #HY-W019543, MCE, NJ, USA; freshly prepared in DMSO) at 37 °C for 30 min with gentle rotation. After centrifugation at 6000 ×g for 15 min at 4 °C and washing with PBS, the cross-linked products were resuspended in SDS loading buffer and boiled at 100 °C for 10 min. ASC monomers (~22 kDa) and oligomerized ASC complexes (dimers and higher-order oligomers) were detected by immunoblotting, and band intensities were quantified using ImageJ software (Version 1.43, National Institutes of Health, Bethesda, MD, USA). ASC oligomerization was expressed as the ratio of the summed intensity of the dimer and higher-order oligomer bands to that of the monomer band in the same lane, normalized to the control group.

2.13 Lactate Dehydrogenase (LDH) Release Assay

The extent of plasma membrane disruption was determined by quantifying LDH released into the culture medium using an LDH cytotoxicity detection kit (YEASEN, cat#40209ES76, Shanghai, China). Following the indicated treatments, culture supernatants were collected and centrifuged to eliminate residual cell debris. The clarified samples were mixed with LDH reaction reagents, and absorbance values were recorded at 490 nm using a microplate reader. LDH release levels were calculated based on the calculation method provided with the assay kit.

2.14 Statistical Analysis

Each experiment was independently performed with a minimum of three biological replicates; technical replicates are specified in individual figure legends. Quantitative data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 10.1.3 (GraphPad Software, San Diego, CA, USA). Two-group comparisons were conducted using a two-sided Student's *t*-test. For multi-group comparisons, Levene's test was used to check variance homogeneity. One-way ANOVA with Tukey's HSD post-hoc test was used for equal variance data; Welch's ANOVA with Games–Howell post-hoc test was used for data with unequal variance. One-way ANOVA was appropriate for these analyses because the experimental design manipulated only one primary independent variable, with all other experimental conditions standardized across groups. Exact p values for all pairwise comparisons are provided in figure legends. Statistical significance was defined as $p < 0.05$. The original, uncropped Western blot (WB) images are provided in the **Supplementary Material**.

3. Results

3.1 TRIM24 Positively Regulated LPS/ATP-Induced Pro-Inflammatory Cytokine Expression

First, an *in vitro* inflammatory model was established, and TRIM24 overexpression (oe-TRIM24) and knockdown (sh-TRIM24) cell lines were generated. As shown in Fig. 1A,B, compared with the control group, the mRNA and protein levels of *TRIM24* were significantly increased in the oe-TRIM24 group, while they were markedly decreased in the sh-TRIM24 group ($p < 0.01$), indicating efficient transfection. Further detection of pro-inflammatory cytokine expression revealed that TRIM24 overexpression significantly upregulated the levels of TNF-α, IL-6, and IL-1β, whereas TRIM24 knockdown significantly suppressed the expression of these cytokines ($p < 0.01$, Fig. 1A,B). Moreover, immunofluorescence results further confirmed that under LPS/ATP stimulation, the intracellular IL-1β fluorescence signal was markedly enhanced in the oe-TRIM24 group and significantly reduced in the sh-TRIM24 group (Fig. 1C). These results indicated that TRIM24 positively regulated LPS/ATP-induced pro-inflammatory cytokine expression and significantly enhanced the IL-1β-mediated inflammatory response.

3.2 TRIM24 Promoted NLRP3 Expression and Interacted With It in the THP-1–HepG2 Co-Culture System

To further explore the role of TRIM24 in intercellular inflammatory responses, THP-1 cells with TRIM24 overexpression or knockdown were treated with LPS/ATP and co-cultured with fatty acid-treated HepG2 cells. Compared with the corresponding control groups, TRIM24 overexpression upregulated the mRNA levels of TRIM24 and NLRP3 in the co-culture system ($p < 0.001$), while TRIM24 knockdown markedly suppressed NLRP3 transcription ($p < 0.001$, Fig. 2A). Western blotting results were consistent with the transcriptional changes. Compared with the control group, protein levels of TRIM24 and NLRP3 were markedly increased in the oe-TRIM24+Hep group, whereas NLRP3 protein expression was significantly reduced in the sh-TRIM24+Hep group ($p < 0.01$, Fig. 2B), indicating that TRIM24 positively regulated NLRP3 protein expression in the co-culture system. To clarify the subcellular localization relationship between TRIM24 and NLRP3, immunofluorescence double staining was performed. The results showed that TRIM24 and NLRP3 displayed obvious co-localization signals in the cytoplasm (Fig. 2C). Under TRIM24 overexpression, the co-localization fluorescence intensity was markedly enhanced, whereas TRIM24 knockdown significantly weakened this co-localization ($p < 0.001$, Fig. 2C).

3.3 TRIM24 Cooperates With RARα to Activate NLRP3 Transcription Through the Promoter RARE

Given that TRIM24 interacts with nuclear receptors and participates in transcriptional regulation, and that

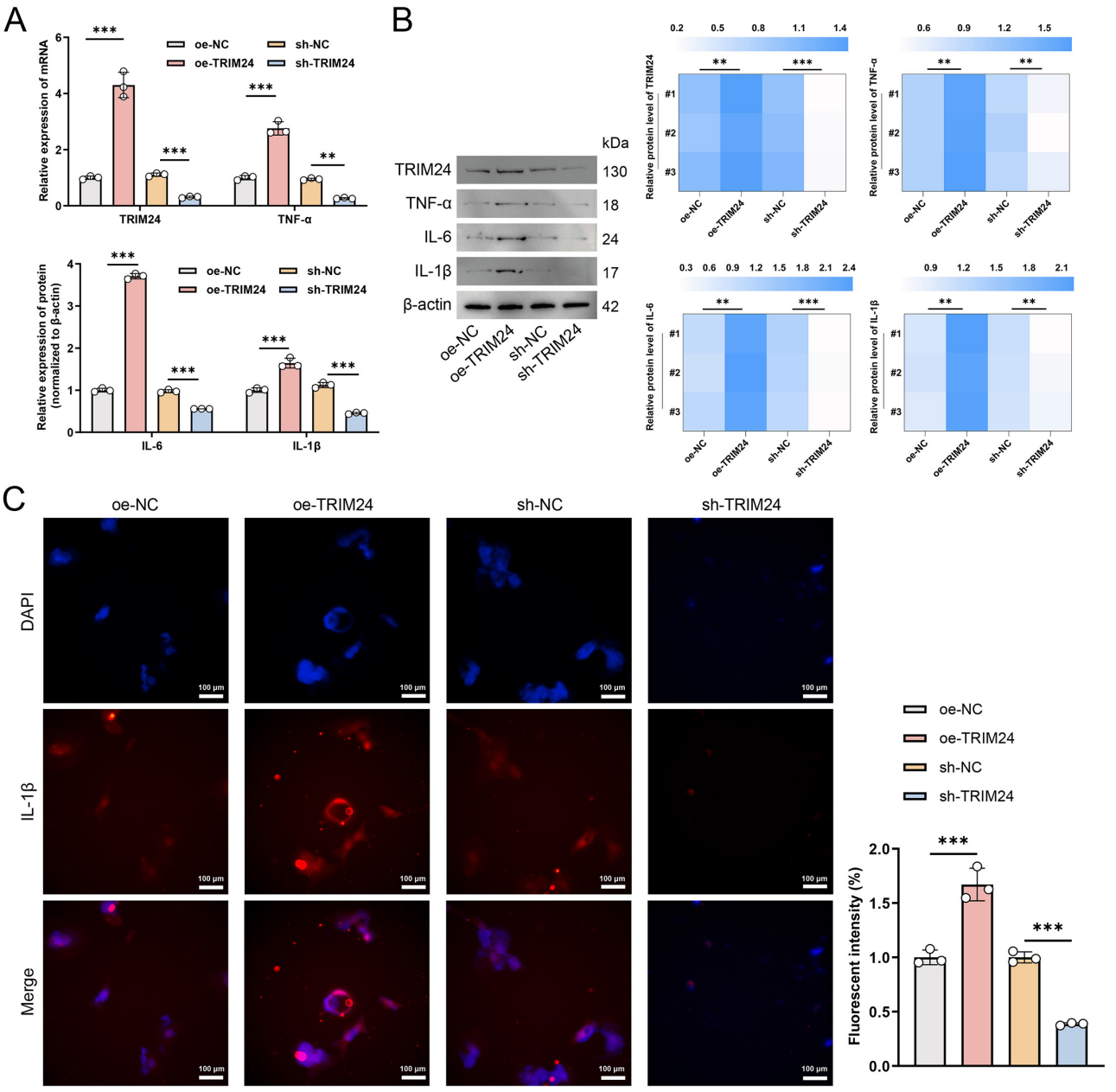


Fig. 1. TRIM24 positively regulates LPS/ATP-induced pro-inflammatory cytokine expression (n = 3). (A) RT-qPCR detected the mRNA expression of TRIM24 and the pro-inflammatory cytokine TNF-α (upper panel), and the relative protein expression of IL-6 and IL-1β normalized to β-actin is shown in the lower panel. (B) Western blotting determined the protein expression of TRIM24, TNF-α, IL-6, and IL-1β. (C) Immunofluorescence showed enhanced intracellular IL-1β in oe-TRIM24 cells and reduced IL-1β in sh-TRIM24 cells under LPS/ATP stimulation; scale bar = 100 μm. $^{**}p < 0.01$, $^{***}p < 0.001$.

RARα, as a classical nuclear receptor transcription factor, can regulate target gene transcription by binding to RAREs, we further investigated whether TRIM24 regulates NLRP3 transcription through RARα. As shown in Fig. 3A, TRIM24 overexpression significantly increased TRIM24 protein levels, whereas TRIM24 knockdown markedly decreased TRIM24 protein expression ($p < 0.001$); under siRNA-mediated RARα knockdown conditions, RARα protein levels were also significantly reduced ($p < 0.001$). Notably, TRIM24 overexpression or knockdown did not significantly affect RARα protein expression. The subcellular distribution of TRIM24, RARα, and NLRP3 was then examined. As shown in Fig. 3B, both TRIM24 and RARα were predominantly distributed in the nucleus, whereas NLRP3 was mainly present in the cytoplasm. Further co-IP analysis of nuclear fractions confirmed the interaction between TRIM24 and RARα, supporting their potential cooperation in transcriptional regulation (Fig. 3C). Subse-

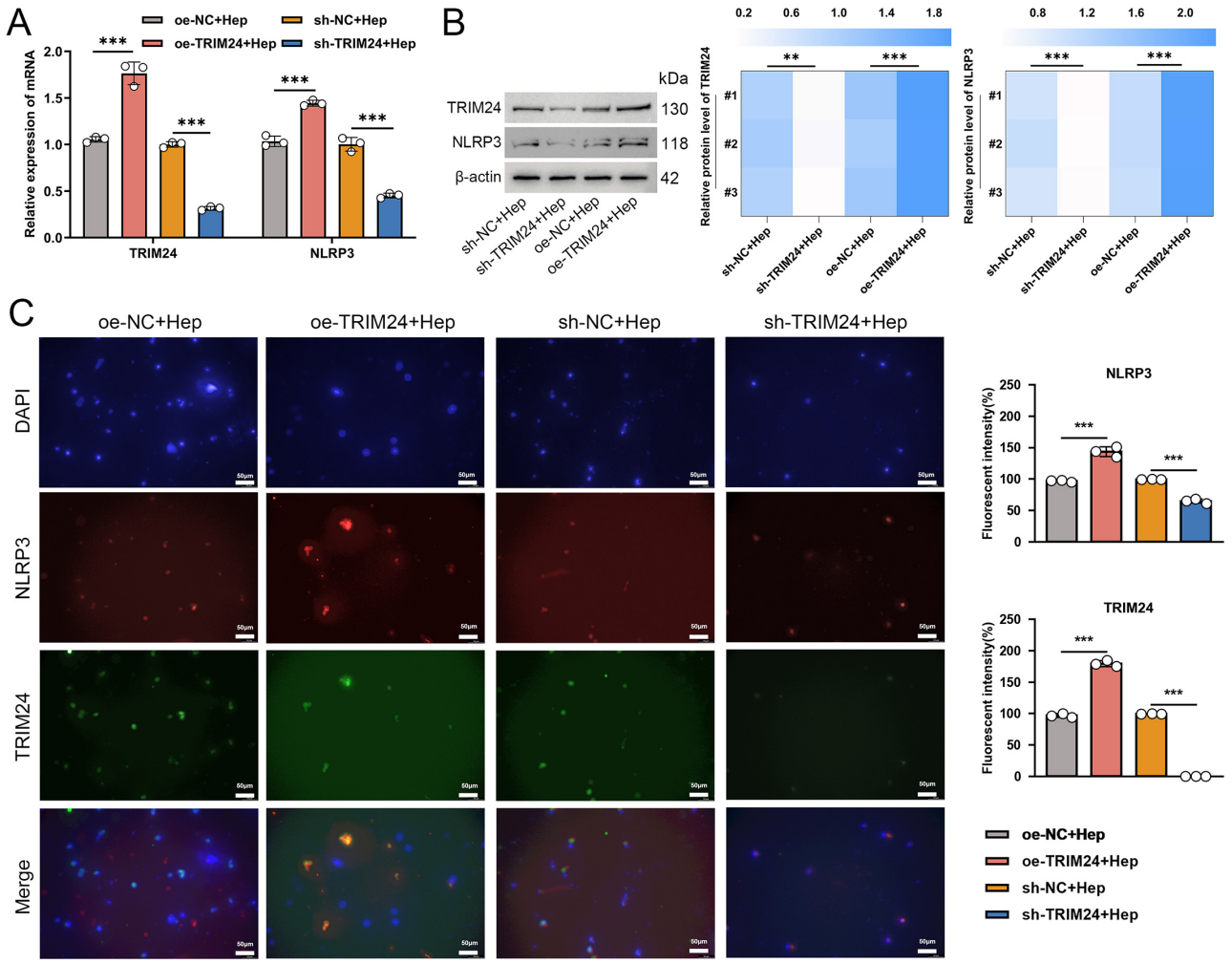


Fig. 2. TRIM24 positively regulates NLRP3 expression and interacts with NLRP3 in a THP-1/HepG2 co-culture system. (A) RT-qPCR detected the mRNA expression of TRIM24 and NLRP3. (B) Western blotting determined the protein expression of TRIM24 and NLRP3. (C) Immunofluorescence double staining reveals cytoplasmic co-localization of TRIM24 and NLRP3; scale bar = 50 µm. The right panels show the quantification of NLRP3 and TRIM24 fluorescence intensity, plotted separately on continuous linear axes starting from zero (mean ± SD, n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$.

quently, whether TRIM24 and RARα cooperatively regulate NLRP3 promoter activity was investigated. As shown in Fig. 3D, overexpression of either TRIM24 or RARα alone significantly increased the luciferase activity of the wild-type NLRP3 promoter, whereas co-overexpression of TRIM24 and RARα further significantly increased NLRP3 promoter activity compared with overexpression of either factor alone ($p < 0.001$). Notably, when the RARE motif in the NLRP3 promoter was mutated, the promoter activation mediated by TRIM24 and RARα was largely abolished, with no statistically significant differences among the treatment groups. Additionally, both TRIM24 and RARα were significantly enriched at the RARE region of the NLRP3 promoter, accompanied by marked enrichment of RNA polymerase II (RNA Pol II) in this region ($p < 0.001$, Fig. 3E). In contrast, no significant enrichment of TRIM24, RARα, or RNA Pol II was observed at the distal control region. Further re-ChIP experiments revealed significant co-occupancy of TRIM24 and RARα at the RARE region of the NLRP3 promoter ($p < 0.001$), whereas no obvious enrichment was observed at the distal region (Fig. 3F). These results suggest that TRIM24 cooperates with RARα to co-occupy the RARE region of the NLRP3 promoter and promote RNA Pol II recruitment and NLRP3 promoter transcriptional activation.

3.4 TRIM24 and RARα Promote NLRP3 Inflammasome Activation

The study subsequently determine whether this transcriptional regulation could be translated into functional activation of the NLRP3 inflammasome. As shown in Fig. 4A, compared with the sh-NC group, TRIM24 knockdown markedly reduced NLRP3 protein expression, whereas RARα overexpression significantly increased NLRP3 pro-

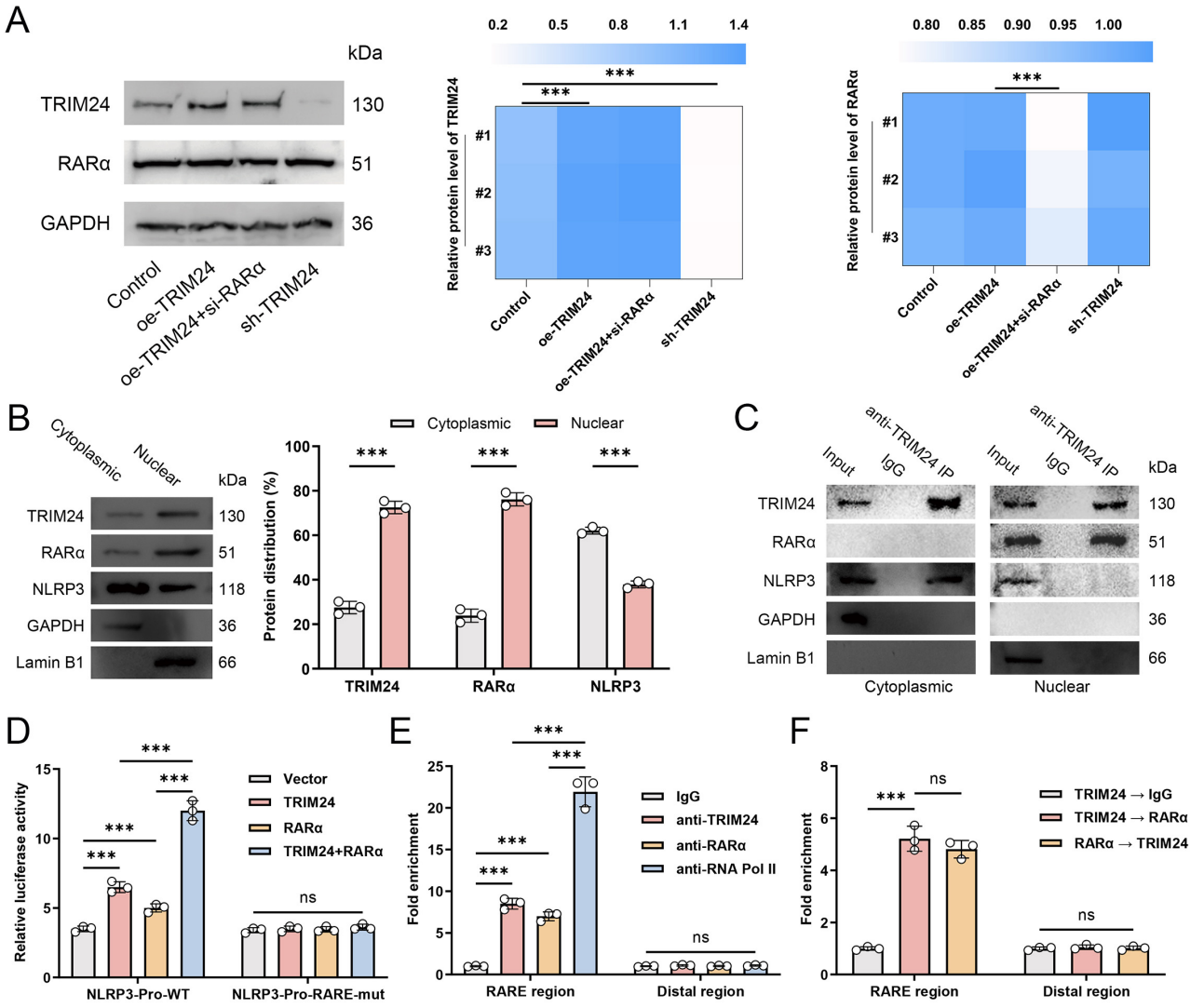


Fig. 3. TRIM24 cooperates with RARα to activate NLRP3 transcription through the promoter RARE. (A) Western blot analysis of TRIM24 and RARα protein expression levels. (B) Western blot analysis of the distribution of TRIM24, RARα, and NLRP3 in cytoplasmic and nuclear fractions. (C) Co-immunoprecipitation analysis of the interactions of TRIM24 with RARα and NLRP3 in cytoplasmic and nuclear fractions. (D) Dual-luciferase reporter assay assessing the effects of TRIM24 and/or RARα overexpression on the transcriptional activity of the wild-type NLRP3 promoter and the RARE-mutant NLRP3 promoter. (E) Chromatin immunoprecipitation (ChIP) assay assessing the enrichment of TRIM24, RARα, and RNA Pol II at the RARE region and distal control region of the NLRP3 promoter. (F) Sequential ChIP (re-ChIP) assay assessing the co-occupancy of TRIM24 and RARα at the RARE region and distal control region of the NLRP3 promoter. THP-1 cells were stimulated with LPS/ATP. ***$p < 0.001$; ns, not significant.

tein levels ($p < 0.05$). We then further assessed NLRP3 inflammasome assembly. Western blot analysis showed that TRIM24 knockdown significantly decreased ASC oligomerization, whereas RARα overexpression markedly enhanced ASC oligomerization ($p < 0.01$, Fig. 4B). Consistently, immunofluorescence staining further demonstrated that ASC aggregation was markedly reduced in the sh-TRIM24 group, whereas stronger ASC aggregation signals were observed in the oe-RARα group ($p < 0.01$, Fig. 4C). These findings indicate that TRIM24 and RARα not only regulate NLRP3 protein expression but also modulate ASC-mediated inflammasome assembly. At the functional level, TRIM24 knockdown significantly decreased IL-1β secretion, whereas RARα overexpression markedly promoted IL-1β release ($p < 0.001$, Fig. 4D). In addition, TRIM24 knockdown significantly reduced LDH release, whereas RARα overexpression substantially increased LDH release ($p < 0.001$, Fig. 4E). Together with the preceding findings, these results further suggest that TRIM24/RARα-mediated transcriptional activation of NLRP3 can be functionally translated into activation of the NLRP3 inflammasome.

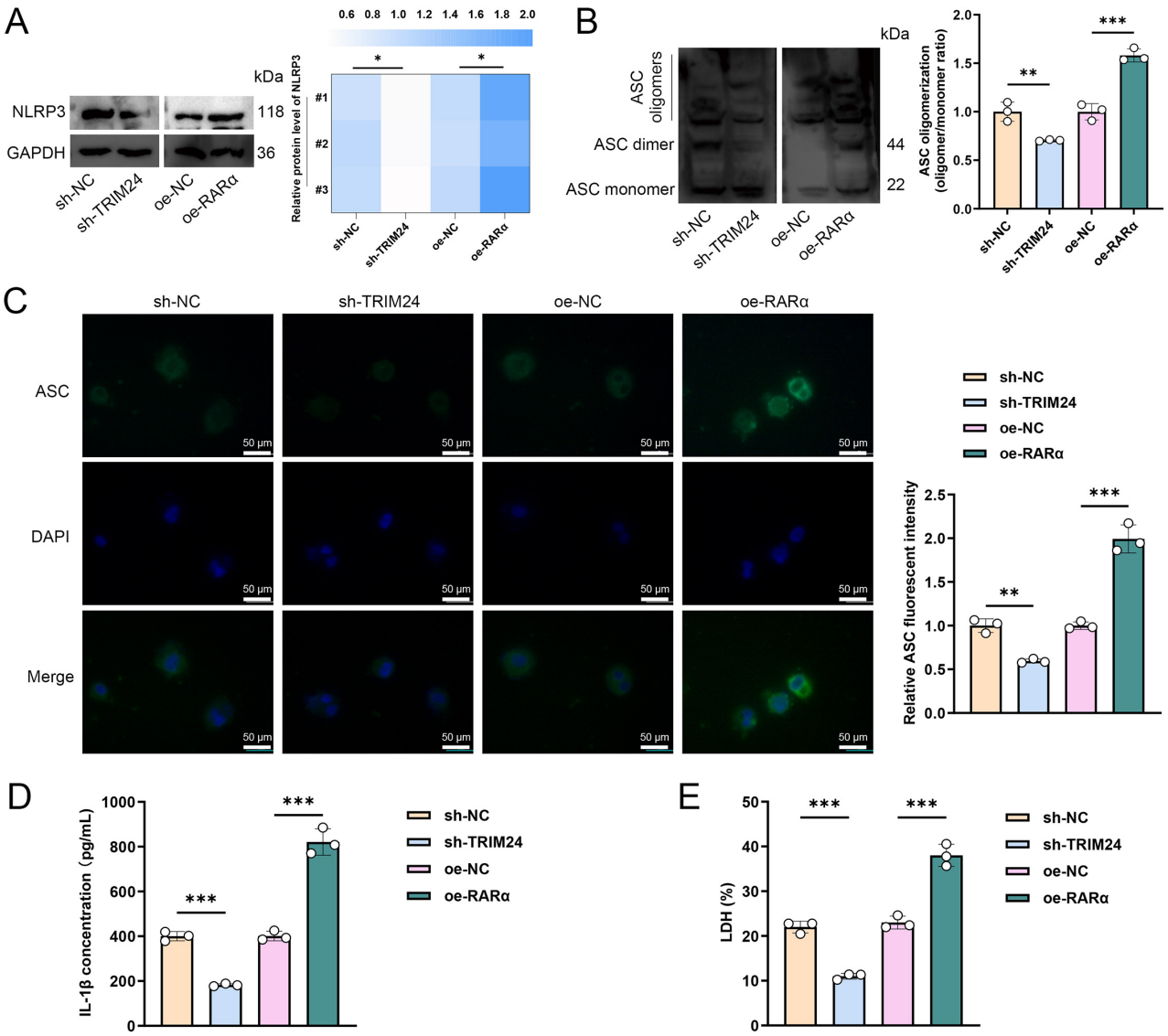


Fig. 4. TRIM24 and RARα promote NLRP3 inflammasome activation and pyroptosis-related inflammatory responses. (A) Western blot analysis of NLRP3 protein expression levels. (B) Western blot analysis of ASC oligomerization levels. (C) Immunofluorescence analysis of ASC aggregation. Scale bar = 50 μm. (D) ELISA measurement of IL-1β secretion levels in the culture supernatants of the indicated groups. (E) Measurement of LDH release in the indicated groups. THP-1 cells were stimulated with LPS/ATP. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.5 TRIM24-Mediated Inflammasome Activation and Pyroptosis Were Dependent on NLRP3

To further verify whether TRIM24-mediated inflammasome activation and pyroptosis were involved in NLRP3, NLRP3 expression was specifically knocked down using shRNA in TRIM24-overexpressing THP-1 cells. Western blotting analysis showed that compared with the TRIM24+sh-NC group, NLRP3 was significantly decreased in the TRIM24+sh-NLRP3 group ($p < 0.001$, Fig. 5A), indicating efficient knockdown of NLRP3. Following NLRP3 knockdown, the protein levels of NLRP3 inflammasome-related molecules, including Caspase-1, GSDMD, and their activated forms, cleaved-Caspase-1 and cleaved-GSDMD, were markedly reduced ($p < 0.01$, Fig. 5A). Further ELISA analysis revealed that the secretion levels of IL-1β and IL-18 were significantly decreased in the TRIM24+sh-NLRP3 group compared with the TRIM24+sh-NC group ($p < 0.001$, Fig. 5B). Additionally, flow cytometry results showed that the proportion of pyroptotic cells was markedly reduced following NLRP3 knockdown ($p < 0.001$, Fig. 5C). These results indicated that TRIM24-mediated inflammasome activation, pro-inflammatory cytokine release, and pyroptosis were all dependent on NLRP3.

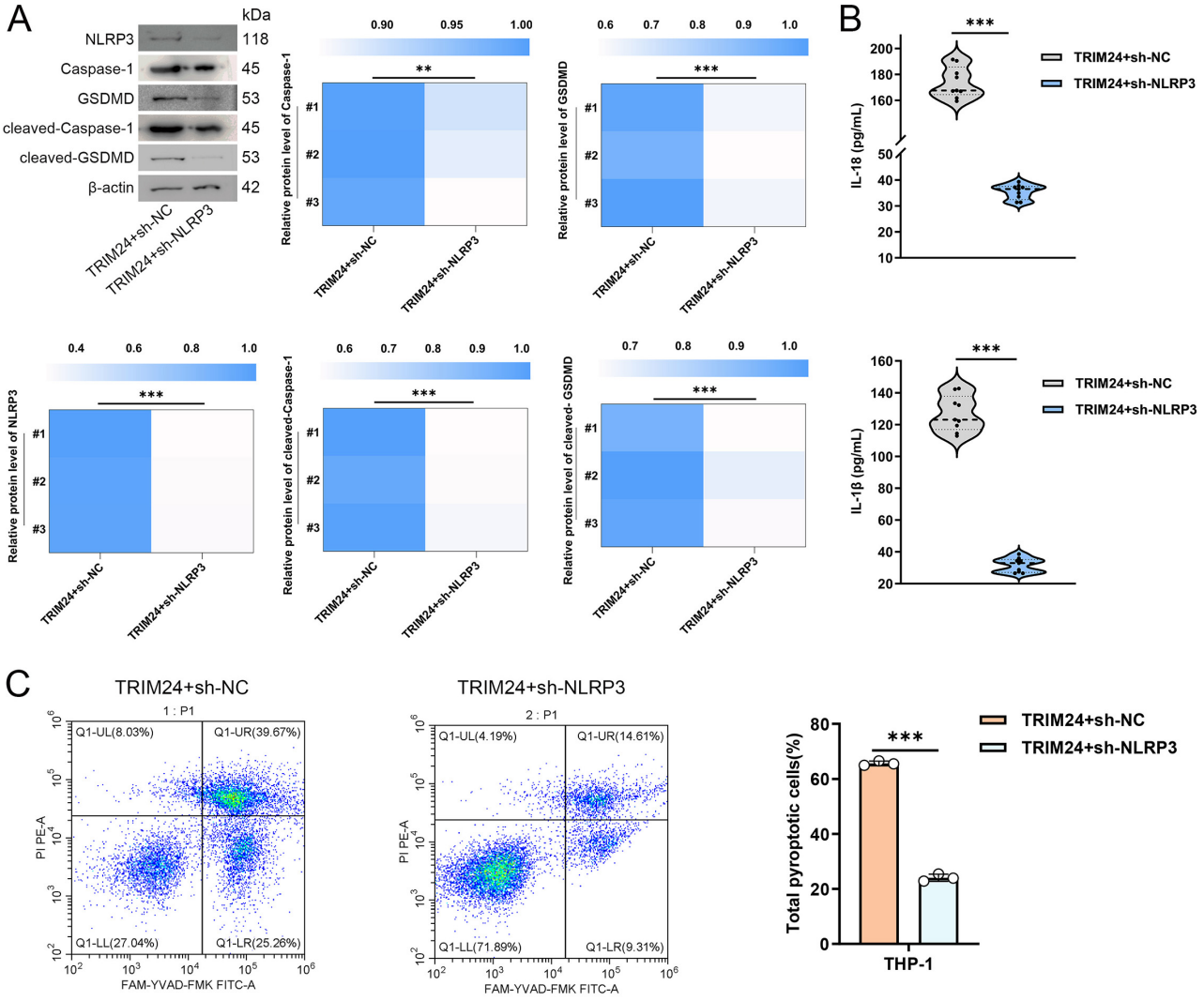


Fig. 5. TRIM24-mediated inflammasome activation and pyroptosis depend on NLRP3. (A) Western blotting detected the protein levels of NLRP3, Caspase-1, GSDMD, cleaved-Caspase-1, and cleaved-GSDMD. (B) ELISA detected the secretion of IL-1β and IL-18. (C) Flow cytometry showed a marked reduction in pyroptotic cell proportion following NLRP3 knockdown. THP-1 cells were stimulated with LPS/ATP. $^{**}p < 0.01$, $^{***}p < 0.001$.

3.6 TRIM24 Promotes NLRP3 Inflammasome Activation and Pyroptosis Through RARα

Subsequently, we explored whether the regulatory effects of TRIM24 on NLRP3 inflammasome activation and pyroptosis depend on RARα. TRIM24 overexpression significantly increased NLRP3 protein expression, whereas either RARα silencing or TRIM24 knockdown markedly attenuated this increase ($p < 0.001$, Fig. 6A). In addition, TRIM24 overexpression markedly increased the levels of cleaved-Caspase-1 and cleaved-GSDMD, whereas RARα silencing or TRIM24 knockdown substantially suppressed the activation of both proteins ($p < 0.001$, Fig. 6B). ELISA analysis showed that TRIM24 overexpression-induced IL-1β secretion was significantly reversed after RARα interference ($p < 0.001$, Fig. 6C). Collectively, these results demonstrate that RARα is involved in TRIM24-mediated activation of the NLRP3 inflammasome and downstream pyroptosis-related inflammatory responses, further supporting the functional role of the TRIM24/RARα regulatory axis in NLRP3 activation.

3.7 Validation of the TRIM24/RARα/NLRP3 Regulatory Axis in U937 Cells

To further validate the reproducibility of the previously identified TRIM24/RARα/NLRP3 regulatory relationship in a different cellular model, we performed independent validation experiments using U937 cells. U937 cells were differentiated with PMA and subsequently stimulated with LPS and Nigericin to induce NLRP3 inflammasome activation. Immunofluorescence analysis showed that, compared with the Control group, sh-TRIM24 significantly decreased ASC fluorescence intensity, whereas

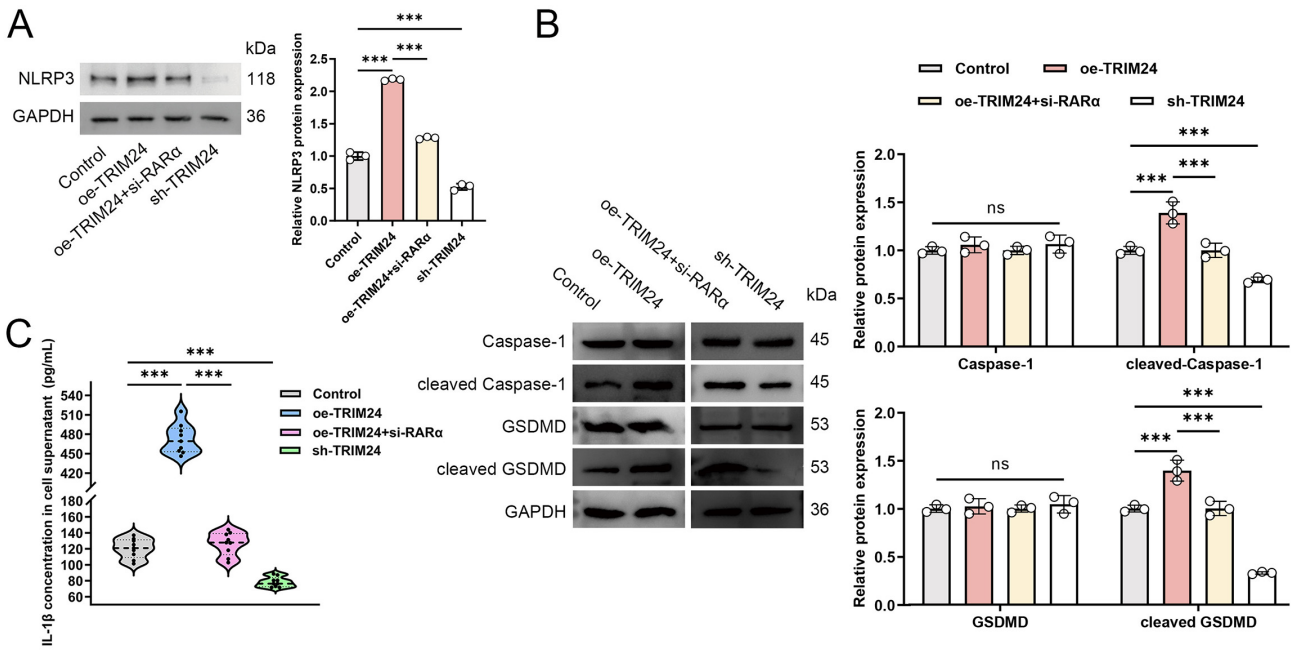


Fig. 6. TRIM24 promotes NLRP3 inflammasome activation and pyroptosis through RARα. (A) Western blot analysis and quantification of NLRP3. (B) Western blot analysis and quantification of total Caspase-1, cleaved Caspase-1, total GSDMD and cleaved GSDMD. Total Caspase-1 and total GSDMD did not differ significantly among the four groups, whereas cleaved Caspase-1 and cleaved GSDMD were increased by TRIM24 overexpression, restored by RARα knockdown and decreased by TRIM24 knockdown. GAPDH was used as the loading control, and protein levels are expressed relative to the Control group (n = 3). (C) IL-1β levels in the cell culture supernatant measured by ELISA. ***$p < 0.001$; ns, not significant.

treatment with the RARα agonist AM580 substantially restored the ASC fluorescence signal in sh-TRIM24-treated cells. AM580 treatment alone also increased ASC fluorescence intensity ($p < 0.001$, Fig. 7A). In addition, sh-TRIM24 markedly reduced NLRP3 protein expression, whereas AM580 reversed the reduction in NLRP3 expression induced by TRIM24 silencing; AM580 alone also increased NLRP3 protein expression ($p < 0.01$, Fig. 7B). Further analysis of downstream effects of NLRP3 inflammasome activation revealed that sh-TRIM24 significantly reduced IL-1β levels in the culture supernatants and LDH release, whereas AM580 substantially restored the decreases in IL-1β and LDH release induced by TRIM24 silencing. AM580 treatment alone also increased IL-1β levels and LDH release ($p < 0.001$, Fig. 7C,D). These findings support the involvement of RARα signaling in TRIM24-mediated regulation of the NLRP3 inflammasome.

4. Discussion

A shared pathological hallmark of severe infection-associated inflammatory diseases, such as acute peritonitis, is the aberrant amplification and sustained activation of inflammatory responses [21,22]. The NLRP3 inflammasome is widely recognized as a key regulator of acute inflammatory injury [23], and inhibition of inflammasome signaling has been proposed as an important therapeutic strategy for preventing pathogenic inflammation [24]. However, most existing studies were focused on the downstream effects of inflammasome activation, while the transcriptional regulatory mechanisms underlying its persistent activation under infectious inflammatory conditions remain poorly understood. In this study, our findings demonstrate that TRIM24 cooperates with the nuclear receptor RARα to bind the RARE region within the NLRP3 promoter, enhance transcriptional activity, and subsequently promote NLRP3 inflammasome activation and pyroptosis.

Under conditions of robust innate immune activation induced by LPS/ATP, our findings reveal that TRIM24 directly regulates the inflammatory signal. LPS initiates the transcriptional program of inflammatory genes through activation of the TLR4 signaling pathway, while ATP, as a prototypical danger-associated molecular pattern, further amplifies inflammatory responses [25,26,27]. This widely used *in vitro* model provides a controlled platform for investigating mechanisms underlying inflammasome-associated inflammatory responses. Previous studies have largely characterized TRIM24 as a nuclear receptor-associated transcriptional regulator primarily involved in tumorigenesis and progression, as well as the regulation of metabolic homeostasis [16,18,28,29]. Emerging evidence suggests that TRIM24 also exerts immunomodulatory functions [30]. In a cell model of acute lung injury induced by LPS

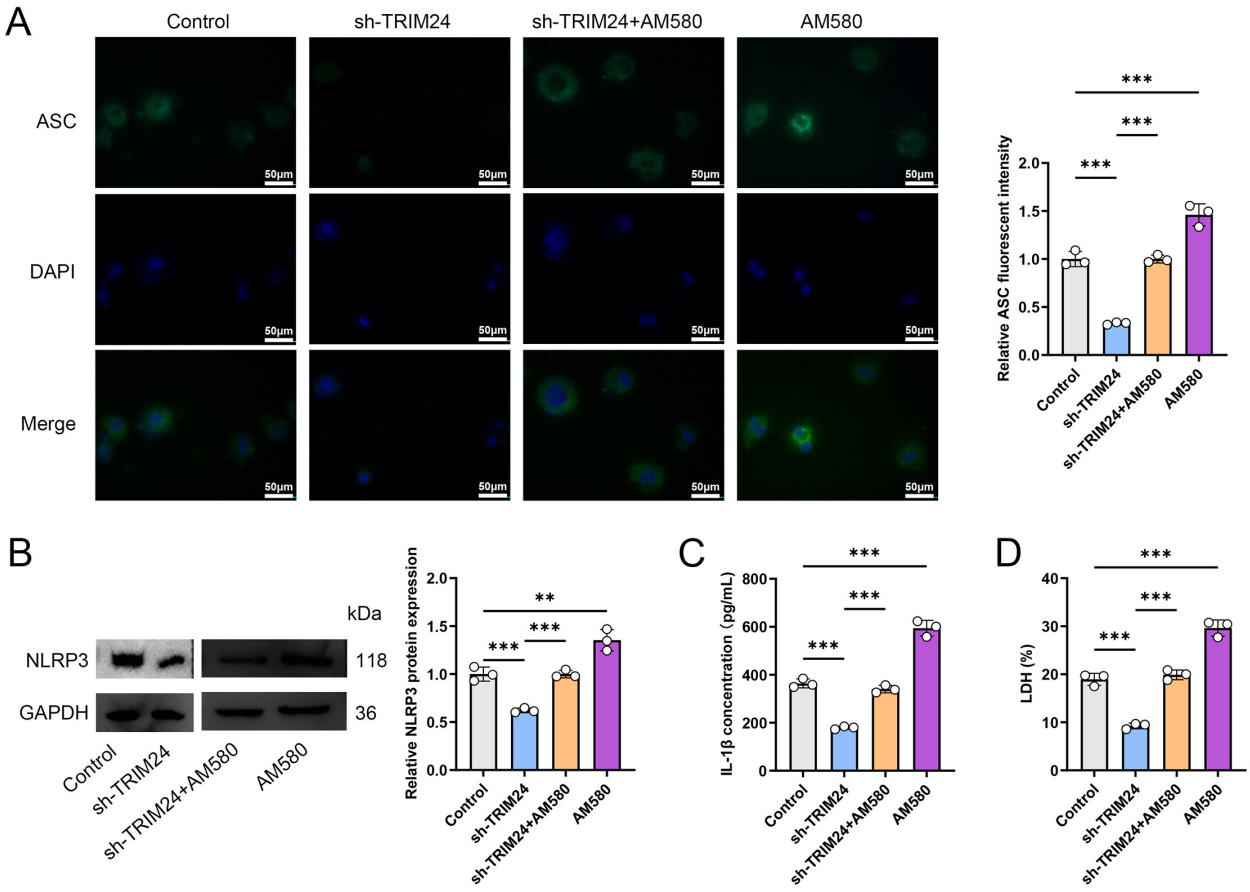


Fig. 7. Validation of the TRIM24/RARα/NLRP3 regulatory axis in U937 cells. (A) Immunofluorescence analysis of ASC fluorescence signals. Scale bar = 50 μm. (B) Western blot analysis of NLRP3 protein expression. (C) ELISA measurement of IL-1β levels in the culture supernatants. (D) Quantification of LDH release. U937 cells were differentiated with PMA (100 ng/mL) for 48 h and subsequently stimulated with LPS and Nigericin to induce NLRP3 inflammasome activation. $^{**}p < 0.01$, $^{***}p < 0.001$.

and IFN-γ, downregulation of TRIM24 expression promoted the expression of M2-like macrophage polarization-related genes while suppressing M1-like macrophage polarization-associated genes [31]. Another study reported that TRIM24 may influence immune-related signaling pathways in breast cancer [32]. In this study, TRIM24 positively regulated the expression of multiple classical pro-inflammatory cytokines in LPS/ATP-stimulated THP-1 cells. TNF-α and IL-6 are broadly involved in the transcriptional activation of inflammation-related genes, regulation of immune cell functions, and shaping of the inflammatory microenvironment, whereas IL-1β represents a central effector cytokine released during NLRP3 inflammasome activation [33,34,35]. The coordinated regulation of these cytokines suggests that TRIM24 contributes to amplification of inflammatory signaling rather than merely acting as an upstream initiator.

Further analyses revealed that the pro-inflammatory effects of TRIM24 are closely associated with the NLRP3 activation. According to the classical paradigm, NLRP3 activation follows a two-step model. The priming step is mainly mediated by signaling pathways such as NF-κB, which induce the expression of NLRP3 and pro-inflammatory cytokine precursors [36,37,38]. However, accumulating evidence indicates that in multiple disease-associated inflammatory settings, the sustained overexpression and aberrant activation of NLRP3 cannot be fully explained by persistent NF-κB activation alone. For example, histone H4K12 lactylation has been shown to directly activate NLRP3 transcription, leading to autophagy dysfunction in microglia [39], and the transcription factor HSF2 has been reported to regulate NLRP3 activation and IL-1β secretion in ulcerative colitis [40]. The present study further identifies TRIM24 as a transcription-associated regulatory protein that directly participates in NLRP3 regulation under infection-related inflammatory conditions. In an LPS/ATP-induced THP-1 and HepG2 co-culture system, TRIM24 overexpression markedly increased NLRP3 expression, whereas TRIM24 knockdown produced the opposite effects. These findings suggest that TRIM24 regulates inflammasome activity primarily through transcriptional control of NLRP3 expression. Supporting this no-

tion, a recent study reported that in a rat model of pulmonary arterial hypertension, TRIM24 indirectly activated the NLRP3 inflammasome by promoting SIRT1 protein degradation and suppressing autophagy [41]. This underscores TRIM24 as an important regulator of inflammasome activity across distinct disease contexts.

Our findings further demonstrated that TRIM24 directly participates in NLRP3 transcriptional regulation. TRIM24 and RARα were specifically enriched at the RARE-containing region of the NLRP3 promoter, and mutation of the RARE motif abolished their transcriptional activation effect, indicating that this regulatory process depends on promoter-specific RARα recognition. Importantly, RARα silencing markedly attenuated TRIM24-induced NLRP3 expression and inflammasome activation, whereas pharmacological activation of RARα restored TRIM24 deficiency-associated suppression of NLRP3-related responses. Together with the U937 validation experiments, these findings establish the TRIM24/RARα axis as a functional transcriptional module controlling NLRP3 inflammasome activation. Unlike several TRIM family members that predominantly regulate inflammasome activity through post-translational modifications such as ubiquitination [42,43], TRIM24 preferentially modulates core inflammasome components at the transcriptional level, thereby exerting its effects during the sustained activation phase of inflammatory responses.

Given that the transcriptional regulatory functions of TRIM24 typically depend on its ability to form transcriptional complexes with nuclear receptors or to recognize specific chromatin modification states via its PHD–bromodomain module [44], we further explored the molecular mechanisms by which TRIM24 achieves fine-tuned transcriptional control of NLRP3. Previous evidence has shown that TRIM24 regulates gene transcription through interaction with nuclear receptors such as RARα [45]. Consistent with these observations, our study demonstrates that RARα functions as an essential transcriptional partner of TRIM24 in regulating NLRP3 expression. RARα participates in the regulation of multiple transcriptional programs associated with immune activation and immune tolerance [46]. Our findings further suggest that, under specific inflammatory stimuli, RARα may function as a transcriptional regulatory platform whose biological outcome is determined by the repertoire of recruited co-regulators. When recruited by TRIM24, RARα may provide promoter-specific DNA recognition capacity and facilitate transcriptional activation of NLRP3 under inflammatory conditions.

5. Limitations

Several limitations should be acknowledged. First, the findings were mainly obtained from *in vitro* THP-1 and U937 cell models, and further validation in animal models and human inflammatory tissues is required to confirm the physiological relevance of the TRIM24/RARα/NLRP3 axis. Second, although LPS/ATP and LPS/nigericin stimulation are widely used to investigate NLRP3 inflammasome activation, these models cannot fully reproduce the complexity of inflammatory environments *in vivo*.

6. Conclusion

This study identifies TRIM24 as a positive transcriptional regulator of NLRP3 inflammasome activation. Under inflammatory stimulation, TRIM24 cooperates with the nuclear receptor RARα to occupy the RARE-containing region of the NLRP3 promoter, enhancing NLRP3 transcriptional activation and promoting downstream inflammasome assembly, cytokine release, and pyroptosis. The identification of the TRIM24–RARα–NLRP3 transcriptional regulatory axis expands the current understanding of inflammasome regulation and provides new insights into the molecular mechanisms underlying inflammatory responses, with potential therapeutic implications for inflammasome-associated diseases.

Availability of Data and Materials

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

JH and NY contributed to the conception and design. JH, WC and MG contributed to the collection and assembly of data. NY, HL, ZJ and ZY analyzed and interpreted the data. JH, NY and MG were responsible for writing the original draft. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL54285.

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