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Tripartite motif protein 32 overexpression promotes inflammation and pyroptosis in INS-1 cells via the NF- κ B/NLRP3 signaling pathway under high glucose conditions

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Abstract

Introduction: β -cell dysfunction and insulin resistance are hallmarks of type 2 diabetes mellitus (T2DM), in which inflammation and pyroptosis are critically involved. Tripartite motif protein 32 (TRIM32) participates in inflammation and programmed cell death, but its role in β -cell dysfunction remains unclear. This study investigated the role of TRIM32 in inflammation and pyroptosis in rat insulinoma (INS-1) cells.

Material and methods: INS-1 cells were cultured under high-glucose conditions. Real-time quantitative polymerase chain reaction (RT-qPCR) was used to determine TRIM32 gene expression. Western blot analysis was performed to examine the expression of proteins associated with inflammation and pyroptosis. TUNEL staining and lactate dehydrogenase (LDH) release assays were performed to quantify cell death. Rescue experiments were conducted to verify the findings.

Results: TRIM32 overexpression promoted inflammation and pyroptosis in INS-1 cells under high-glucose conditions, whereas TRIM32 silencing had the opposite effects. In addition, BAY 11-7082 blocked the NF- κ B/NLRP3 signaling pathway and significantly attenuated inflammation and pyroptosis in INS-1 cells under high-glucose conditions.

Conclusions: TRIM32 overexpression promoted inflammation and pyroptosis in INS-1 cells partly through the NF- κ B/NLRP3 signaling pathway under high-glucose conditions. (Endokrynol Pol 2026; 77 (x): xx-xx)

Keywords: inflammation; pyroptosis; NF- κ B pathway; tripartite motif protein 32; type 2 diabetes mellitus

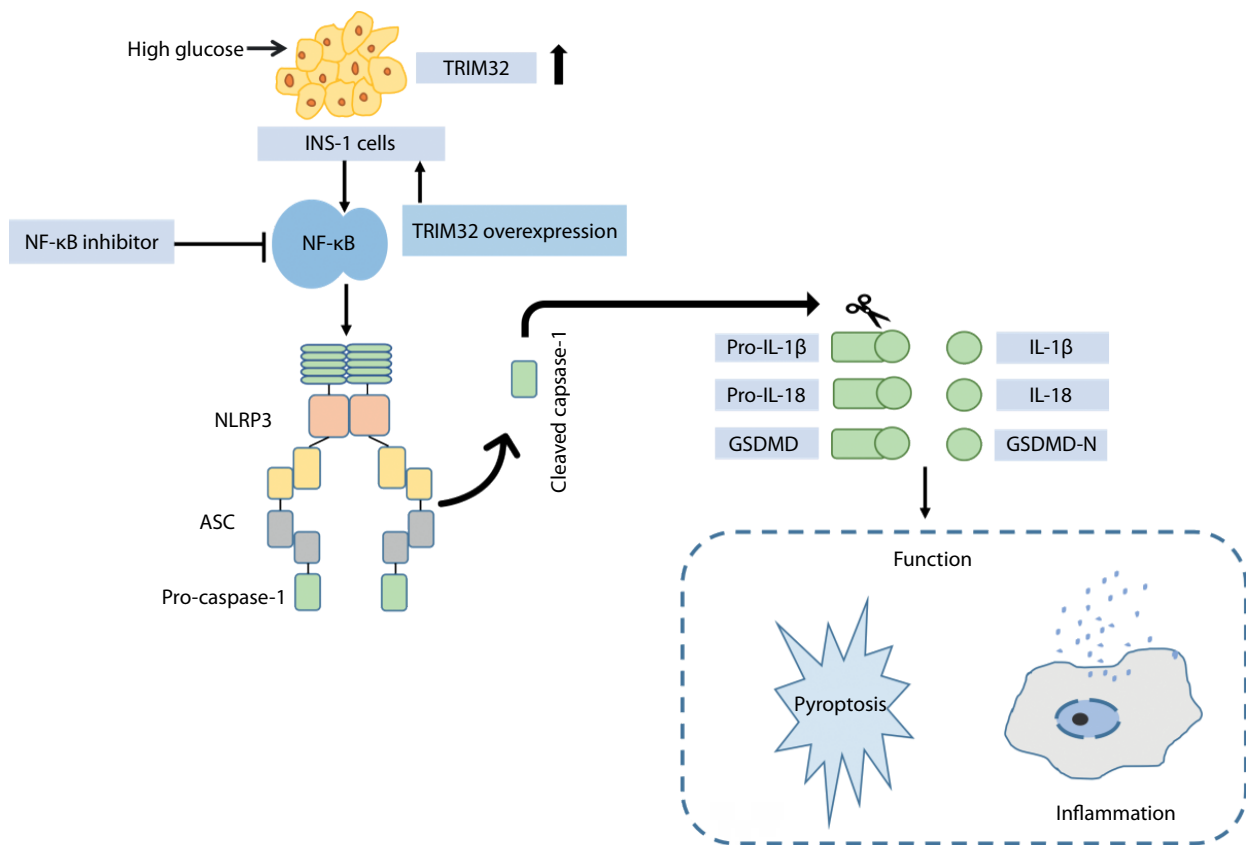
Introduction

Type 2 diabetes mellitus (T2DM) has emerged as a serious public health issue, and its prevalence has increased at an alarming rate worldwide in recent decades. An estimated 529 million people had diabetes worldwide in 2021, and this figure is expected to reach 1.31 billion by 2050 [1]. T2DM is a complex metabolic disorder, and pancreatic β -cell dysfunction and insulin resistance are central to its pathogenesis [2]. Moreover, T2DM is an inflammatory disease [3]. Immune cells, such as macrophages, infiltrate the islets of patients with T2DM; IL-1 β and other proinflammatory cytokines are elevated; and chronic islet inflammation contributes substantially to pancreatic β -cell dysfunction [4, 5]. High glucose increases the production and release of IL-1 β in human islets, followed by NF- κ B activation and impaired β -cell function [4]. Moreover, activated NF- κ B initiates pro-IL-1 β transcription. A recent study also showed that blocking IL-1 β signaling ameliorates glycemia and restores insulin secretion in β -cells [6].

Our previous studies confirmed that autophagy reduces oxidative stress-induced NLRP3 inflammasome activation, thereby protecting pancreatic islet β -cells from inflammatory damage. The NLRP3 inflammasome is a multiprotein complex consisting of NLRP3, ASC, and pro-caspase-1. Pyroptosis is an inflammasome-mediated form of programmed cell death [7]. The NLRP3 inflammasome activates caspase-1. Cleaved caspase-1 then processes pro-IL-1 β and pro-IL-18 into mature IL-1 β and IL-18 and cleaves GSDMD to generate GSDMD-N, which has membrane pore-forming activity. Thus, pyroptosis is characterized by caspase-1-mediated lytic cell death and the release of mature IL-1 β and IL-18 [8, 9]. An increasing number of studies suggest that GSDMD-N-mediated pyroptosis promotes the progression of T2DM [10, 11]. NF- κ B regulates NLRP3 expression, which represents one of the two key checkpoints in NLRP3 inflammasome activation [12]. Therefore, NF- κ B, the NLRP3 inflammasome, GSDMD-N, and IL-1 β play important roles in inflammation and pyroptosis in T2DM.



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Graphical Abstract. The role of TRIM32 in INS-1 cell dysfunction was investigated; this study demonstrated that TRIM32 overexpression promoted inflammation and pyroptosis in INS-1 cells under high-glucose conditions; mechanistic studies suggested that TRIM32 may act partly through the NF-κB/NLRP3 signaling pathway

TRIM family proteins constitute the largest subfamily of RING E3 ubiquitin ligases and play significant roles in cancer, autoimmune diseases, and inflammatory diseases by regulating NF-κB transcriptional activity [13, 14]. NF-κB activation is essential for the transcriptional induction of TRIM37 during hepatitis B virus infection [15], and TRIM14 is involved in psoriasis progression by activating the NF-κB pathway [16]. TRIM46 overexpression also activates the NF-κB pathway in pancreatic cancer cells [17]. The TRIM family member pyrin/TRIM20, a canonical inflammasome component, is implicated in IL-1β and IL-18 production and pyroptosis [18, 19]. Furthermore, TRIM29 promotes podocyte pyroptosis under diabetic conditions by activating the NF-κB/NLRP3 inflammasome pathway [20]. TRIM32 is involved in intestinal inflammation through robust activation of NF-κB signaling [21] and activates NF-κB signaling and NLRP3 in rheumatoid arthritis [22]. Another study indicated that TRIM32 may promote NF-κB signaling activation [23]. Furthermore, TRIM32 contributes to diet-induced hepatic insulin resistance by targeting the insulin receptor for degradation [24]. Chen et al. showed that TRIM32 expression is increased in the glomeruli of diabetic mice and that TRIM32 regulates high glucose-induced apoptosis, oxidative stress, and inflammatory injury in

podocytes [25]. These findings led us to postulate that TRIM32 participates in β-cell inflammation and pyroptosis via the NF-κB/NLRP3 signaling pathway.

The results of this study demonstrated that TRIM32 overexpression aggravated high glucose (HG)-induced inflammation and pyroptosis in INS-1 cells, whereas TRIM32 silencing had the opposite effects. Blockade of the NF-κB/NLRP3 pathway significantly attenuated HG-induced inflammation and pyroptosis in INS-1 cells. Our study provides new insight into the role of TRIM32 in inflammation and pyroptosis in INS-1 cells and supports its potential value as a future therapeutic target for T2DM (Graphical abstract).

Material and methods

Cell culture

The rat insulinoma cell line INS-1 used in this study was provided by the Department of Endocrinology, The Second Affiliated Hospital of Harbin Medical University (Heilongjiang, China). INS-1 cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA). RPMI-1640 medium containing 11.1 mmol/L glucose was used as the standard culture condition for INS-1 cells. Therefore, 11.1 mmol/L glucose served as the control condition in this study. The culture medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO₂.

Table 1. Antibodies used for Western blot analysis

Proteins	Dilution	Cat. no
Primary antibodies		
TRIM32	1:1000	BAS-bs-0964R
NLRP3	1:1000	WL02635j
NF- κ B p65	1:800	WL01273bj
Phospho-NF- κ B p65	1:800	WL02169j
GSDMD	1:1000	A10164
Cleaved caspase-1	1:800	WL02996aj
ASC	1:500	WL02462j
IL-18	1:1000	WL01127j
IL-1 β	1:500	WL00891j
β -actin	1:1000	WL01372j
Secondary antibody		
HRP-goat anti-rabbit IgG	1:5000	S0001

Reagents and antibodies

BAY 11-7082, biconchonic acid (BCA) kit, and penicillin/streptomycin were purchased from Beyotime (Shanghai, China). TRIzol reagent and DAPI were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cDNA First-Strand Synthesis Kit and SYBR Green Master Mix were purchased from Roche (Basel, Switzerland). LipoFiter 3.0 was obtained from Hanbio Biotechnology (Shanghai, China), and FBS was obtained from Sijiqing Biological Engineering Materials Co., Ltd (Hangzhou, China). Antibodies against TRIM32 were purchased from Bioss (Beijing, China). NLRP3, NF- κ B p65, phospho-NF- κ B p65, cleaved caspase-1, ASC, IL-18, and IL-1 β were purchased from Wanleibio (Shenyang, Liaoning, China). GSDMD-N and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies were purchased from Abclonal (Wuhan, China).

Total RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was isolated from cell samples using TRIzol reagent. A First-Strand cDNA Synthesis Kit was used to synthesize cDNA according to the manufacturer's instructions. Quantitative PCR was performed using SYBR Green Master Mix on a CFX96 Real-Time System. The primer sequences were as follows: rat TRIM32, forward: 5'-CCGGGAAGTGCTAGAATGTC-3' and reverse: 5'-CAGCGGACACCATGATGCT-3'; rat GAPDH, forward: 5'-ATGCCGCCTGGAGAAACC-3' and reverse: 5'-GCATCAAAGGTGGAAGATGG-3'. GAPDH mRNA was used as the endogenous control, and relative gene expression was calculated using the comparative Ct method ($2^{-\Delta\Delta C_t}$).

Western blot analysis

Proteins were extracted from cells using radioimmunoprecipitation assay (RIPA) lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined using a BCA kit. Equal amounts of protein were separated by gel electrophoresis and then transferred to polyvinylidene difluoride membranes. Sodium dodecyl sulfate-polyacrylamide gels (10% and 12%) were used. Membranes were blocked with 5% skim milk for 2 h and then incubated with primary antibodies overnight at 4°C. Subsequently, HRP-conjugated secondary antibodies were added for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system ((Pierce; Thermo Fisher Scientific, Rockford, IL, USA)) according to the supplier's instructions. Primary antibodies against the following proteins were used: TRIM32, NLRP3, NF- κ B p65, phospho-NF- κ B p65, GSDMD, cleaved

caspase-1, ASC, IL-18, IL-1 β , and β -actin. Detailed information on the antibodies is provided in Table 1.

Plasmid construction and cell transfection

For TRIM32 overexpression in INS-1 cells, full-length TRIM32 was inserted into the pcDNA3.1-CMV-EF1-ZsGreen-T2A-puro vector. Short hairpin RNA (shRNA) sequences targeting mouse Trim32 were inserted into the pcDNA3.1-U6-MCS-CMV-ZsGreen-T2A-puro vector. The shRNA sequence was designed based on the literature [26] and was as follows (5' to 3'): GATCTTCAGGCAAGGTATA. Cell transfection was performed according to the manufacturer's protocol (Hanbio Biotechnology, Shanghai, China), and transfection efficiency was subsequently assessed. Images were acquired using a Leica DM 4000B fluorescence microscope.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity in the cell supernatant was determined using an LDH cytotoxicity assay kit (Elabscience, Wuhan, China) according to the manufacturer's protocol and was used to quantify cell death. Briefly, cell supernatant (50 μ L) and reaction solution (50 μ L) were added to the kit reagent and incubated for 10 min at 37°C, after which stop solution was added. Absorbance was measured at 450 and 600 nm using a microplate reader (Tecan, Männedorf, Switzerland).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay

Cell death was evaluated using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) detection kit (Yuhengbio, Suzhou, Jiangsu, China). Briefly, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 for 10 min. A TUNEL reaction mixture (50 μ L) was then added to the cells, which were incubated in the dark for 1 h. DAPI was used to counterstain the nuclei. Images were acquired using fluorescence microscopy.

Statistical analyses

All data are presented as the mean $\pm$ standard deviation (SD) from three independent experiments. Statistical analyses were performed using SPSS v. 25.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism v. 8.0 (GraphPad Software, San Diego, CA, USA). Data were analyzed using one-way analysis of variance (ANOVA) or Student's t test. $P < 0.05$ was considered statistically significant.

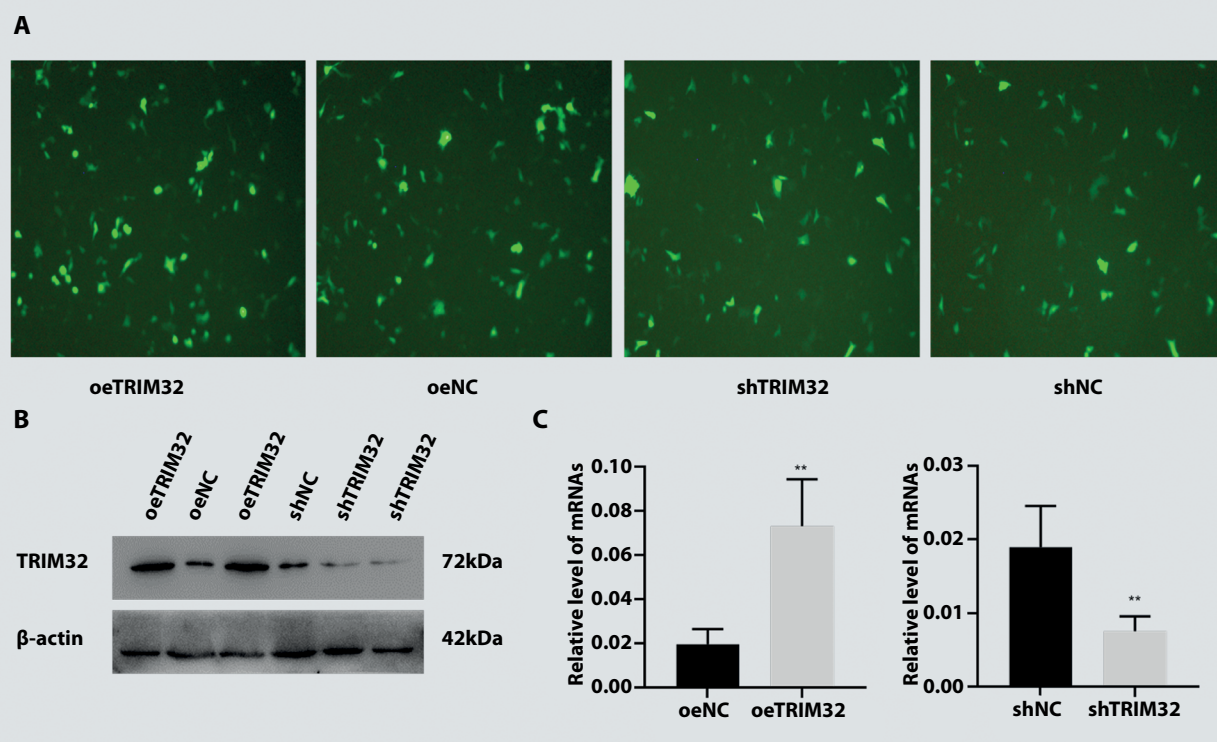


Figure 1. Construction of INS-1 cells with TRIM32 overexpression or silencing; **A.** Transfection efficiency was assessed by fluorescence microscopy; **B.** TRIM32 protein levels were measured by western blotting; **C.** TRIM32 mRNA levels were measured by real-time quantitative polymerase chain reaction; * $p < 0.05$; ** $p < 0.01$; all experiments were repeated at least three times, and data are presented as the mean $\pm$ standard deviation; oe — overexpression; sh — silencing

Results

Construction of TRIM32-overexpressing and TRIM32-silenced INS-1 cells

Our previous studies demonstrated that TRIM32 expression was upregulated in the peripheral blood of patients with T2DM and that high glucose induced TRIM32 expression in INS-1 cells in a concentration- and time-dependent manner [27]. To examine the role of TRIM32 in HG-induced inflammatory damage and pyroptosis, we generated INS-1 cells overexpressing TRIM32 (oeTRIM32) or with TRIM32 silenced (shTRIM32). Transfection efficiency was assessed using fluorescence microscopy, western blotting, and real-time quantitative polymerase chain reaction (RT-qPCR) (Fig. 1).

TRIM32 overexpression promoted the inflammatory response in INS-1 cells under HG conditions

Previous studies have demonstrated that activation of NF- κ B and the NLRP3 inflammasome contributes to pancreatic islet β -cell dysfunction [28]. To evaluate the effect of TRIM32 on HG-induced inflammatory damage, INS-1 cells were induced to overexpress or silence TRIM32 and exposed to HG for 48 h. The protein expression levels of NF- κ B and NLRP3 inflammasome

components were then assessed by western blotting. The expression levels of phosphorylated NF- κ B p65, NLRP3, cleaved caspase-1, ASC, IL-1 β , and IL-18 were higher in the HG-treated TRIM32-overexpression group than in the HG group. TRIM32 silencing had the opposite effects (Fig. 2). These western blotting results indicated that TRIM32 overexpression exacerbated HG-induced inflammation.

TRIM32 overexpression promoted HG-induced pyroptosis in INS-1 cells

TRIM32 overexpression increased the protein expression levels of NLRP3 inflammasome components, cleaved caspase-1, IL-1 β , and IL-18 in INS-1 cells. We then determined the effect of TRIM32 on pyroptosis. As shown in Figure 3, GSDMD-N protein expression, LDH activity, and the number of TUNEL-positive cells were assessed to determine the role of TRIM32 in pyroptosis in INS-1 cells. GSDMD-N protein expression was significantly increased in HG-treated TRIM32-overexpressing INS-1 cells (Fig. 3A). Lactate dehydrogenase activity was measured in cell culture supernatants at the indicated time points. Lactate dehydrogenase release from TRIM32-overexpressing cells cultured under HG conditions was significantly higher than that from cells in the HG group (Fig. 3B). Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining showed that the number of TUNEL-positive cells was significantly

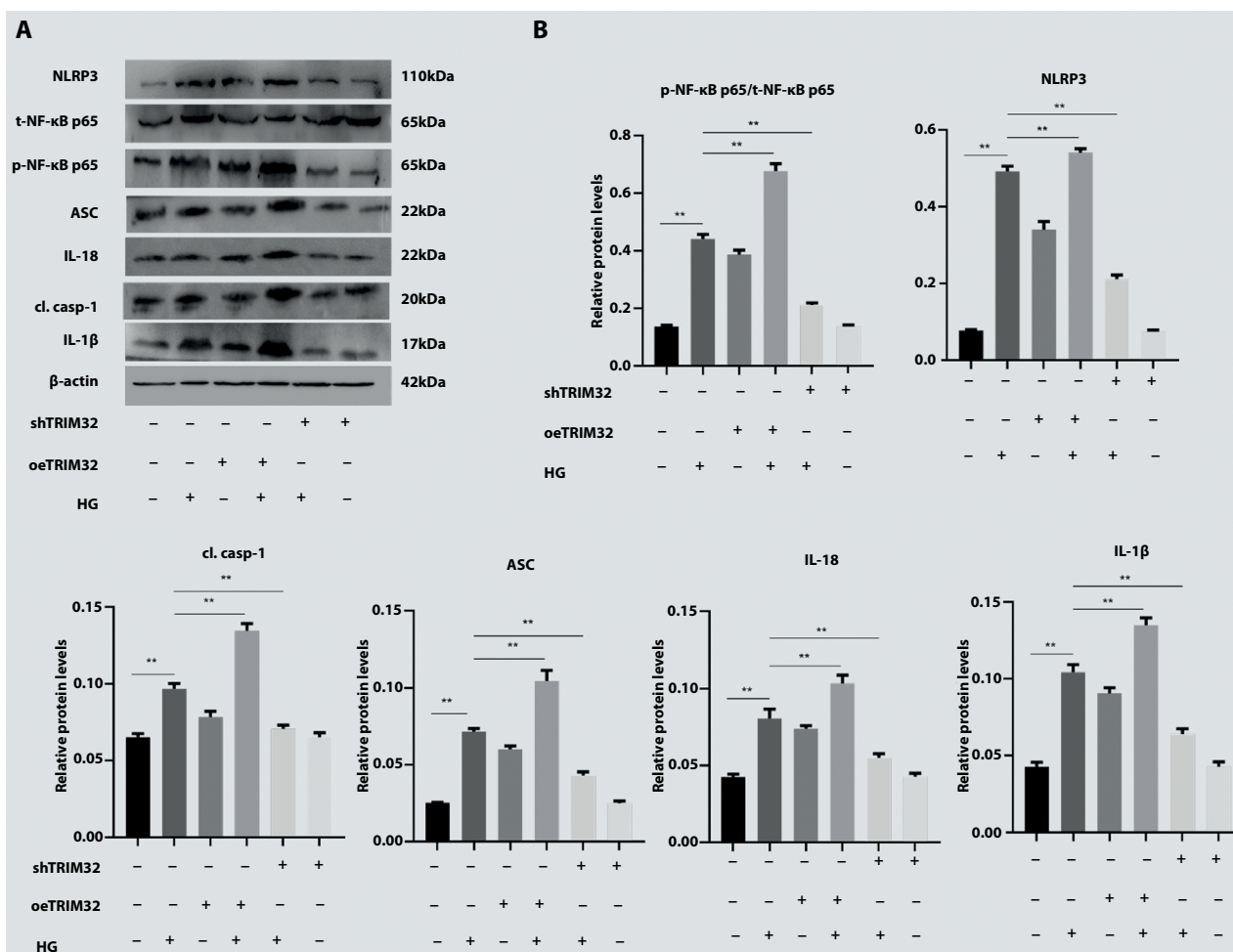


Figure 2. Effect of TRIM32 overexpression on the inflammatory response in high-glucose (HG)-treated INS-1 cells; protein levels of phosphorylated NF-κB p65 (p-NF-κB p65), NLRP3, ASC, cleaved caspase-1 (cl. casp-1), IL-1β, and IL-18 were detected by western blot; all experiments were repeated at least three times, and the data are presented as the mean ± standard deviation; * $p < 0.05$; ** $p < 0.01$; oe — overexpression; sh — silencing; t-NF-κB p65 — total NF-κB p65;

higher in the HG-treated TRIM32-overexpression group than in the HG group (Fig. 3C). However, TRIM32 silencing in INS-1 cells had the opposite effects.

TRIM32 overexpression promoted HG-induced inflammation and pyroptosis through the NF-κB/NLRP3 axis in INS-1 cells

To further determine the relationship between TRIM32 and NF-κB/NLRP3-mediated inflammation and pyroptosis, TRIM32-overexpressing or TRIM32-silenced INS-1 cells were cultured under HG conditions with BAY 11-7082. Lactate dehydrogenase release and the number of TUNEL-positive cells were markedly decreased in the BAY 11-7082-treated group (Fig. 3B and Fig. 4A). NF-κB blockade reduced GSDMD-N protein expression (Fig. 4B). The protein expression levels of NLRP3, cleaved caspase-1, ASC, IL-1β, and IL-18 were also significantly reduced by BAY 11-7082 (Fig. 4CD). Collectively, these results demonstrated that TRIM32 overexpression promoted HG-induced inflammation and pyroptosis by regulating the NF-κB/NLRP3 signaling pathway.

Discussion

This study demonstrated that TRIM32 played a critical role in HG-induced damage in INS-1 cells. TRIM32 expression was elevated in the peripheral blood of patients with T2DM, and HG stimulated TRIM32 expression in INS-1 cells [27]. TRIM32 overexpression promoted HG-induced inflammatory injury and pyroptosis, partly through the NF-κB/NLRP3 signaling pathway.

As the prevalence of T2DM continues to rise, the number of people with diabetic complications is expected to reach an unprecedented level [1]. Thus, new therapeutic targets are urgently needed. Inflammation, oxidative stress, autophagy, apoptosis, and pyroptosis play essential roles in the pathogenesis of T2DM and its complications [29–33]. Reducing inflammation and pyroptosis may represent a therapeutic strategy for T2DM and its complications. TRIM32, a member of the TRIM protein family, has been reported to contribute to insulin resistance in various metabolic conditions, including

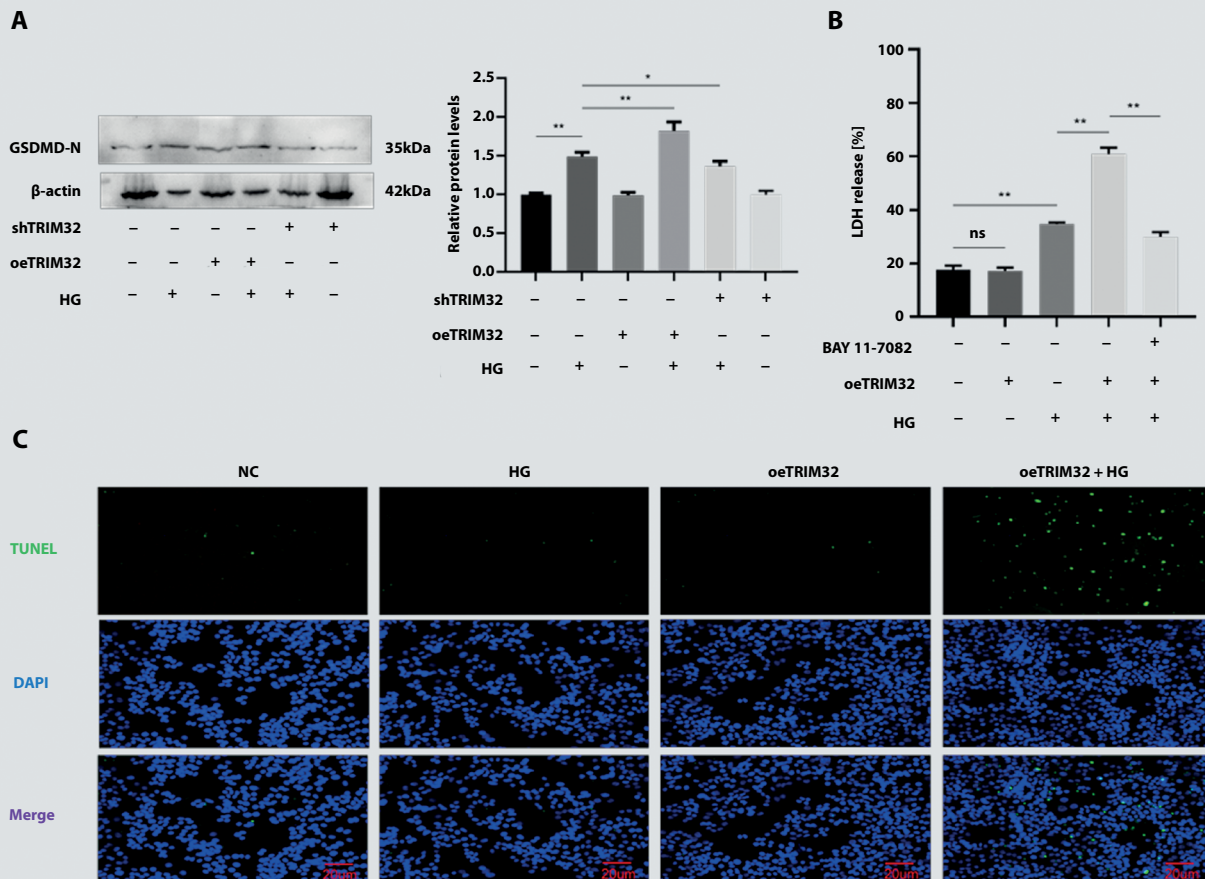


Figure 3. TRIM32 overexpression promoted pyroptosis in high-glucose (HG)-treated INS-1 cells; **A**, The protein level of GSDMD-N was measured by western blotting; the cell death of INS-1 cells was measured by lactate dehydrogenase (LDH) release (**B**) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (**C**); all experiments were repeated at least three times, and the data are presented as the mean $\pm$ standard deviation; * $p < 0.05$; ** $p < 0.01$; ns — nonsignificant; oe — overexpression; sh — silencing

diabetes mellitus (DM) and metabolic syndrome [24]. In our previous study, we demonstrated that TRIM32 modulates autophagic cell death in pancreatic β -cells through the Akt/mTOR pathway [27]. However, the effect of TRIM32 on pancreatic β -cell inflammation and pyroptosis has not been reported. Our previous results indicated that TRIM32 expression is elevated in the peripheral blood of patients with T2DM, but the underlying mechanism remains unclear. The effect of TRIM32 upregulation in the peripheral blood of patients with T2DM requires further investigation. We therefore examined the effect of HG on TRIM32 expression in INS-1 cells and found that TRIM32 was highly expressed in HG-treated INS-1 cells. Recent studies have demonstrated that TRIM32 is involved in inflammation through the regulation of NF- κ B signaling in rheumatoid arthritis [22] and plays a critical role in HG-induced inflammatory injury and oxidative stress in podocytes [25]. However, whether TRIM32 is involved in inflammation in INS-1 cells remains unknown.

Inflammation is implicated in the development of T2DM and its complications. Inflammatory markers are

often elevated in patients with T2DM, and inhibition of inflammatory signaling pathways has been shown to reduce insulin resistance and improve hyperglycemia in animal models [34]. The NLRP3 inflammasome has been one of the most extensively studied inflammasomes over the past decade and is considered a therapeutic target for various diseases, including T2DM [35–37]. The NF- κ B/NLRP3 signaling pathway is involved in the pathogenesis of multiple inflammatory diseases [38–40]. The present study demonstrated that TRIM32 silencing reduced the HG-induced inflammatory response and cell death. However, studies on the mechanisms by which TRIM32 modulates inflammation in INS-1 cells are lacking.

Pyroptosis (“fiery death”) is a form of inflammatory caspase-mediated programmed cell death. The canonical inflammasome pathway activates the inflammatory caspase-1, which promotes inflammatory cytokine release and pyroptotic cell death [41]. The activated NLRP3 inflammasome converts pro-caspase-1 into cleaved caspase-1, which then

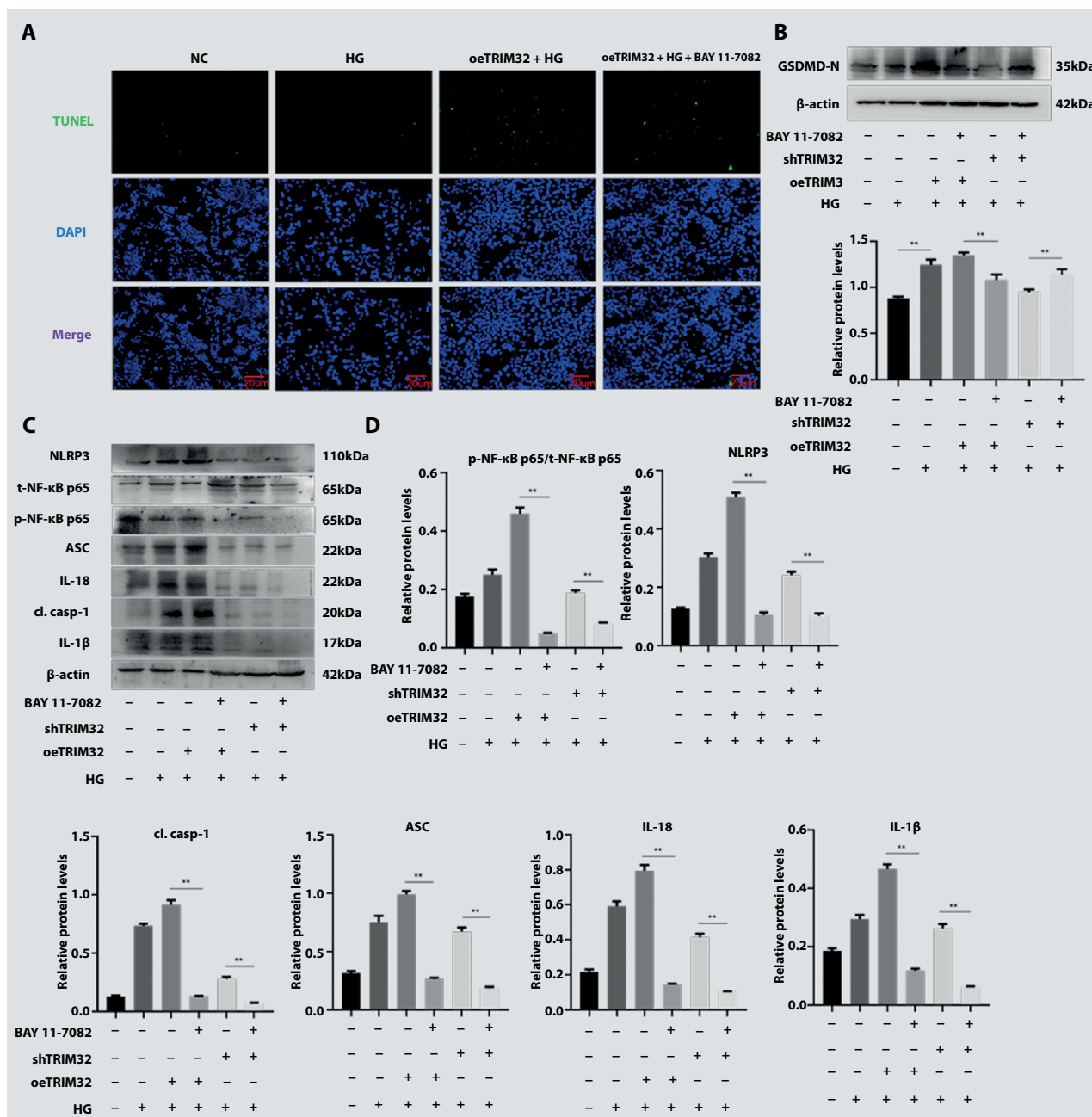


Figure 4. BAY 11-7082 reversed the effects of TRIM32 overexpression on inflammatory response and pyroptosis in INS-1 cells under high-glucose (HG) conditions; **A.** The cell death of INS-1 cells was measured by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining; **B.** The protein level of GSDMD-N was measured by western blotting; **CD.** Protein levels of phosphorylation of NF-κB p65 (p-NF-κB p65), NLRP3, ASC, cleaved caspase-1 (cl. casp-1), IL-1β, and IL-18 were detected by western blotting; all experiments were repeated at least three times, and the data are presented as the mean ± standard deviation; **p* < 0.05; ***p* < 0.01; oe — overexpression; sh — silencing; t-NF-κB p65 — total NF-κB p65

cleaves GSDMD and proinflammatory cytokines to generate pore-forming GSDMD-N and mature cytokines (IL-1β and IL-18). Subsequently, pores form in the cell membrane, resulting in the release of IL-1β and IL-18 and pyroptotic cell death [42]. In this study, the protein levels of IL-1β, IL-18, and GSDMD-N were significantly elevated in the HG-treated TRIM32-overexpressing INS-1 cell group. When HG-treated cells were cocultured with BAY 11-7082,

NF-κB was inhibited in TRIM32-overexpressing INS-1 cells, and the inflammatory response and pyroptosis were attenuated (Fig. 4). These findings demonstrated that TRIM32 regulated inflammation and pyroptosis in INS-1 cells partly via the NF-κB/NLRP3 signaling pathway.

As discussed above, TRIM32 plays multiple roles in diverse physiological processes, including apoptosis, inflammation, oxidative stress, autophagy, neuronal

development and differentiation, and cell proliferation. The present study indicated that TRIM32 regulates inflammation and pyroptosis in INS-1 cells partly via the NF- κ B/NLRP3 signaling pathway. However, the regulatory effects of TRIM32 on apoptosis and oxidative stress in INS-1 cells have not been studied and require further investigation. Furthermore, TRIM32 inhibition attenuates HG-induced apoptosis, oxidative stress, and inflammatory injury in podocytes, and TRIM32 may also play an important role in the pathogenesis of diabetic nephropathy by mediating podocyte injury [25]. Several TRIM proteins are also involved in the regulation of diabetic complications. For example, TRIM46 contributes to the progression of diabetic retinopathy by promoting GPX4 ubiquitination, which in turn exacerbates high glucose-induced ferroptosis in human retinal capillary endothelial cells (HRECEs) [43]. TRIM63 is associated with the pathogenesis of diabetic nephropathy [44] and diabetes-associated muscular dystrophy [45]. In addition, TRIM32 plays a protective role against aortic banding-induced cardiac hypertrophy [46]. Moreover, mutations in TRIM32 cause limb-girdle muscular dystrophy 2H (LGMD2H), and TRIM32 inhibition leads to defective autophagy accompanied by elevated levels of reactive oxygen species [47]. A complex consisting of the ubiquitin ligase TRIM32 and the deubiquitinating enzyme USP7 balances c-Myc ubiquitination and determines neural stem cell fate [48]. However, the effects of TRIM32 on diabetic cardiomyopathy, diabetic retinopathy, diabetes-related muscular atrophy, and diabetic neuropathy have not been investigated. Further research may establish TRIM32 as a promising therapeutic target for T2DM and its complications.

Conclusions

The results of this study indicate that TRIM32 regulates inflammation and pyroptosis in INS-1 cells partly via the NF- κ B/NLRP3 signaling pathway. Our findings suggest that TRIM32 may be a novel target for improving pancreatic β -cell function in T2DM. However, the precise role of TRIM32 in pancreatic β -cell dysfunction requires further investigation in animal models *in vivo*. The effects of TRIM32 on apoptosis and oxidative stress in pancreatic β -cells also require further study.

Data availability statement

Data used and/or analyzed during the study are available from the corresponding author upon reasonable request.

Role of artificial intelligence in creating content

No generative artificial intelligence tools were used in the preparation of this manuscript.

Ethics statement

Not required.

Author contributions

Y.L. and T.W.: designed the studies; T.W., H.W., Ch.W. and Y.W.: performed the experiments; T.W., H.W., Ch.W. and Y.W. and X.L.: analyzed the data; T.W.: the first draft of the manuscript. All authors revised the manuscript, read and approved the final manuscript.

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Acknowledgments

None.

Conflict of interest

All authors have no competing interests to declare that are relevant to the content of this article.

Supplementary material

None.

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