Submitted: 29 November, 2024 Accepted: 05 March, 2025 Published: 08 June, 2025 DOI:10.22514/sv.2025.086

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ORIGINAL RESEARCH

TRIM21 alleviates acute pancreatitis cell injury and promotes efferocytosis by targeting *CD47*

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Abstract

Background: Acute pancreatitis (AP) is a debilitating inflammatory condition of the pancreas that significantly impacts patient health and quality of life. Tripartite motif-containing protein 21 (*TRIM21*), an E3 ubiquitin ligase predominantly expressed in the immune system, owns pivotal role in the pathogenesis of various diseases. Nevertheless the regulatory effects and underlying molecular mechanisms of *TRIM21* in AP remain vague. **Methods**: Cholecystokinin (CCK) was utilized for evoking 266-6 cells to establish AP cell model. The protein expressions were inspected through western blot. The cell apoptosis and phagocytic index (%) were assessed through flow cytometry. **Results**: In this study, we established an AP cell model using 266-6 cells stimulated with cholecystokinin (CCK). Our results showed that *TRIM21* expression was significantly reduced in the AP cell model, and its overexpression mitigated AP-induced cell damage by inhibiting apoptosis. Furthermore, *TRIM21* overexpression enhanced macrophage efferocytosis and increased the efferocytosis rate from 20% to 32%. Mechanistically, we demonstrated that *TRIM21* exerts its effects by downregulating the expression of Cluster of Differentiation 47 (*CD47*), and overexpression of *CD47* partially rescued the regulatory effects of *TRIM21*. **Conclusions**: This study reveal that *TRIM21* alleviates cell injury in AP and accelerates efferocytosis by targeting *CD47*, providing valuable insights into the *TRIM21*/*CD47* axis as a potential therapeutic target in AP progression.

Keywords

Acute pancreatitis; *TRIM21*; *CD47*; Efferocytosis

1. Introduction

Acute pancreatitis (AP) is an inflammatory disease that occurred in pancreas, characterized by the activation of digestive enzymes, inflammatory cell infiltration and tissue necrosis [1], with a global incidence that has been increasing, from 2.30% to 3.84% between 1961 and 2016 [2]. About 5–10% of patients may develop severe acute necrotizing pancreatitis, as well as AP owns a significantly higher morbidity rate (13 to 45 per 100,000) and a mortality rate of approximately 30% [3–5]. Currently, there are no effective pharmacological treatments to prevent or cure AP, making the identification of novel therapeutic targets crucial.

Tripartite motif-containing protein 21 (*TRIM21*) exhibits pivotal roles in regulating various cellular processes, including autophagy, apoptosis and inflammation [6, 7]. It is widely expressed across different tissues and has been manifested in the pathogenesis of several diseases. For instance, in nasopharyngeal carcinoma, *TRIM21* inhibits irradiation-induced mitochondrial DNA release, thereby suppressing anti-tumor immunity [8]. In other contexts, *TRIM21* has been shown to reduce inflammation triggered by *Corynebacterium pseudotuberculosis* and improve survival in mouse models [9]. Additionally, *TRIM21* has been found to alleviate inflammation and metabolic dysfunction associated with obesity [10]. On the other hand, *TRIM21* can also exacerbate disease progression, as evidenced by its role in promoting ferroptosis and worsening acute kidney injury induced by ischemia/reperfusion [11]. Moreover, *TRIM21* has been shown to enhance M1 macrophage polarization, contributing to cardiac injury in myocardial infarction [12]. In colorectal cancer, *TRIM21* facilitates the degradation of c-Myc and enhances the efficacy of regorafenib treatment [13]. Despite these increasing understanding and diverse roles of *TRIM21*, its regulatory effects and underlying molecular mechanisms in AP remain poorly understood.

In this study, results uncover that *TRIM21* alleviates AP-induced cell damage and promotes efferocytosis by targeting *CD47*. These findings suggest that the *TRIM21*/*CD47* axis may represent a helpful therapeutic target for AP.

2. Materials and methods

2.1 Cell culture and treatment

The mouse pancreatic acinar cancer cell line 266-6 was got from the American Type Culture Collection (ATCC, Manassas,

VA, USA), cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and put in a humidified incubator at 37 °C with 5% CO_2.

To establish the AP cell model, 266-6 cells were treated with cholecystokinin (CCK, 10 μM, Sigma-Aldrich, USA) for 6 hours [14]. The cells were authenticated by short tandem repeat (STR) profiling and were confirmed to be free from mycoplasma contamination.

2.2 Cell transfection

TRIM21 overexpression (adTRIM21) and *CD47* overexpression (adCD47) adenoviruses, along with negative control adenovirus (adNC), were transfected into 266-6 cells using Lipofectamine 2000 (11668019, Invitrogen, Carlsbad, CA, USA). The adenoviruses were purchased from Vigene Biosciences (Jinan, China).

2.3 Western blot

Proteins from 266-6 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% gel). After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat dry milk, followed by incubation with primary antibodies. Subsequently, the membranes were incubated with goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:2000; ab7090, Abcam, Shanghai, China). Protein bands were visualized using a chemiluminescence detection kit (89880, Thermo Fisher Scientific, Waltham, MA, USA).

Primary antibodies used were: TRIM21 (1:1000; ab207728), CD47 (1:1000; ab319049), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000; ab8245), all sourced from Abcam (Shanghai, China).

2.4 Immunofluorescence (IF) assay

To assess TRIM21 localization, 266-6 cells (1×10^5 cells) were fixed in 4% paraformaldehyde, followed by permeabilization with 0.2% Triton X-100 and blocking with 5% bovine serum albumin (BSA). The cells were then incubated with the primary antibody against TRIM21 (1:1000; ab207728, Abcam, Shanghai, China), followed by incubation with the secondary antibody (1:1000, ab7149, Abcam, Shanghai, China). The corresponding fluorescent images were captured using an Olympus BX53 microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

2.5 LDH detection

To assess cell damage, 266-6 cells were cultured in a 24-well plate. After centrifugation (500× g for 5 minutes), the supernatant was incubated with 60 μL of lactate dehydrogenase (LDH) working reagent for 30 minutes. The LDH release rate (%) was determined using the LDH assay kit (catalog number: A020-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [15].

2.6 Flow cytometry for cell apoptosis

Cell apoptosis was analyzed using the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (556547, BD Biosciences, Franklin Lakes, NJ, USA). After washing, 266-6 cells were stained with FITC-conjugated Annexin V and propidium iodide (PI) in the absence of light. Apoptosis was determined using a flow cytometer (BD FACSCanto, BD Biosciences, Franklin Lakes, NJ, USA).

2.7 Flow cytometry for phagocytic index

To evaluate efferocytosis, 266-6 cells were treated with CCK for 6 hours, then co-cultured with bone marrow-derived macrophages. The phagocytic index was measured by flow cytometry, as previously described [16].

2.8 Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analysis was done using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Comparisons between groups were conducted using Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1 *TRIM21* expression is downregulated in the AP cell model

The significant reduction in TRIM21 protein expression was displayed after CCK stimulation in the AP cell model (Fig. 1A), which was further confirmed by IF assay (Fig. 1B), indicating that TRIM21 protein expression is downregulated in the AP cell model.

3.2 Overexpression of *TRIM21* alleviated AP-induced cell injury

Following the CCK treatment, we observed reduced TRIM21 protein expression, and this reduction could be reversed by overexpressing TRIM21 (Fig. 2A). Additionally, the release of lactate dehydrogenase (LDH) was significantly elevated after CCK stimulation, and this increase could be attenuated by *TRIM21* overexpression (Fig. 2B). Cell apoptosis was also enhanced upon CCK stimulation, and this was mitigated by *TRIM21* overexpression (Fig. 2C). Furthermore, the levels of cleaved-caspase-3 and high mobility group box-1 protein (HMGB1) were increased following CCK induction, and these effects could be reversed upon TRIM21 overexpression (Fig. 2D). Results indicate that *TRIM21* overexpression alleviates AP-induced cell injury.

3.3 Overexpression of *TRIM21* accelerated macrophage efferocytosis

The phagocytic index was significantly repressed after CCK treatment; however, this decrease was counteracted by *TRIM21* overexpression (Fig. 3A). Moreover, the protein expression of CD47 was lifted after CCK stimulation, and this impact was neutralized following *TRIM21* overexpression

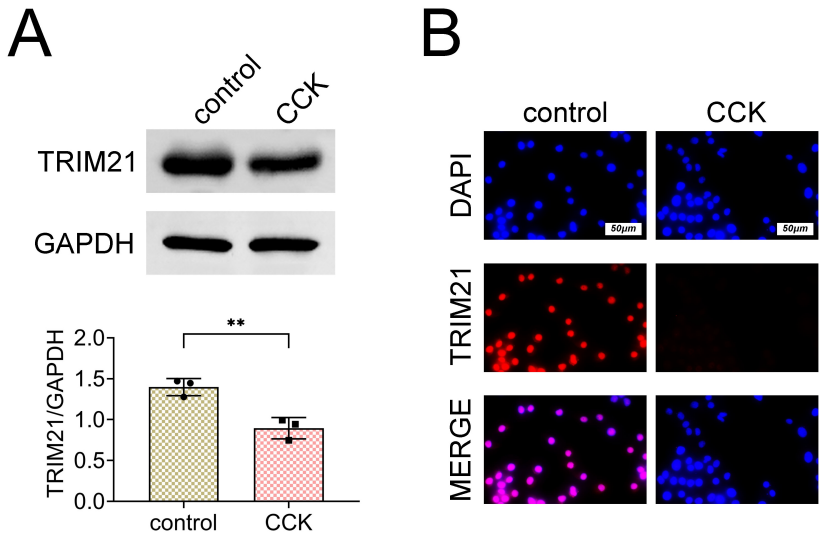

FIGURE 1. TRIM21 protein expression is downregulated in the AP cell model. (A) Western blot analysis confirmed the reduced protein expression of TRIM21 in the CCK-treated group compared to the control group. N = 3. Data are presented as the mean ± SD. $p < 0.01$. (B) Immunofluorescence (IF) assay further verified the downregulation of TRIM21 protein expression following CCK treatment. TRIM21: tripartite motif-containing protein 21; CCK: cholecystokinin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MERGE: merge; DAPI: 4′,6-Diamidino-2-phenylindole. $**p < 0.01$.

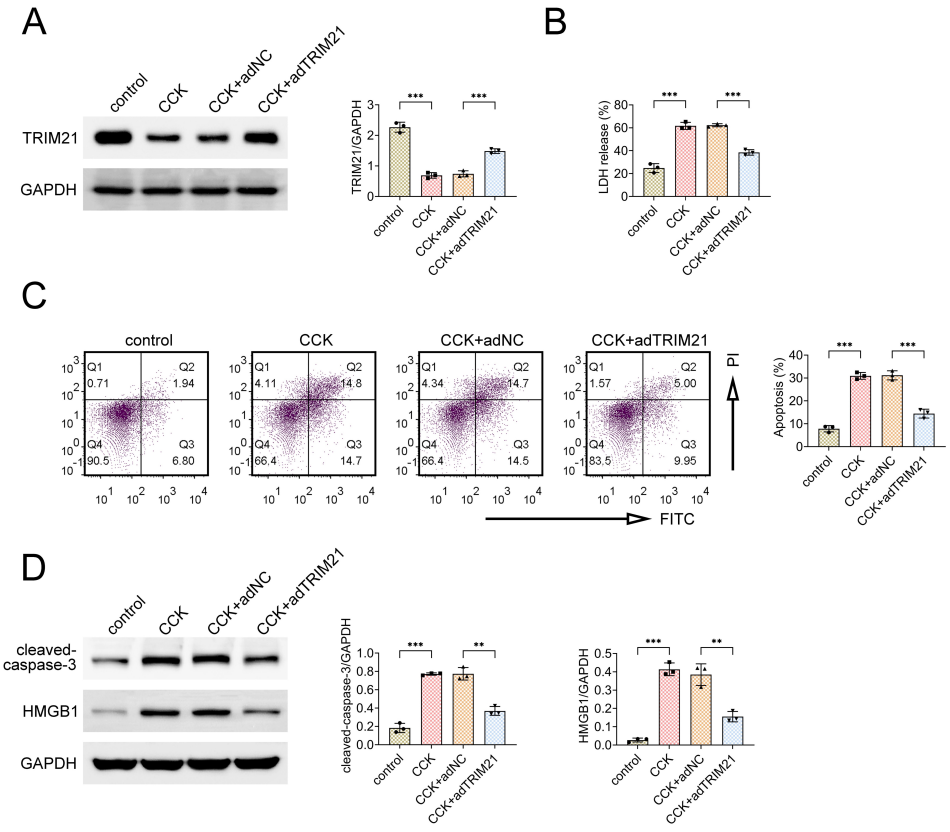

FIGURE 2. Overexpression of *TRIM21* alleviates AP-induced cell injury. Cells were divided into four groups: control, CCK, CCK + adNC and CCK + adTRIM21. (A) Western blot analysis confirmed *TRIM21* overexpression in the CCK + adTRIM21 group. (B) LDH release was measured using an LDH assay kit, showing reduced cell injury in the CCK + adTRIM21 group. (C) Flow cytometry analysis of cell apoptosis revealed a significant reduction in apoptosis following *TRIM21* overexpression. (D) Western blot analysis of cleaved-caspase-3 and HMGB1 expression showed that TRIM21 overexpression attenuated apoptosis-related protein levels. N = 3. Data are presented as the mean ± SD. $**p < 0.01$, $***p < 0.001$. TRIM21: tripartite motif-containing protein 21; CCK: cholecystokinin; adNC: negative control adenovirus; adTRIM21: tripartite motif-containing protein 21 overexpression; LDH: lactate dehydrogenase; PI: propidium iodide; HMGB1: high mobility group box-1 protein; GAPDH: 4′,6-Diamidino-2-phenylindole; FITC: fluorescein isothiocyanate.

(Fig. 3B). Taken together, these findings indicate that *TRIM21* overexpression may accelerate macrophage efferocytosis in the AP cell model.

3.4 Overexpression of *CD47* reversed the regulatory effects of *TRIM21*

In the AP cell model, CD47 protein expression was decreased after *TRIM21* overexpression, but this reduction was offset by *CD47* upregulation (Fig. 4A). The LDH release rate was significantly decreased following *TRIM21* overexpression, but this impact was reversed by *CD47* overexpression (Fig. 4B). Similarly, the phagocytic index was enhanced after *TRIM21* overexpression, but this effect was diminished upon *CD47* upregulation (Fig. 4C). These results indicate that *CD47* overexpression can reverse the regulatory effects of *TRIM21* in the AP cell model.

4. Discussion

Although *TRIM21* has been shown to be involved in the regulation of various diseases [8–13], the precise regulatory roles and molecular mechanisms of TRIM21 in AP remain dimness. In this project, results uncover that TRIM21 protein expression is downregulated in the AP cell model, and also show that its overexpression could alleviate AP-induced cell injury.

Macrophages exhibit considerable plasticity, allowing them to adapt to various microenvironments [17]. During efferocytosis, apoptotic cells release chemokines and lipids that attract efferocytic immune cells [18]. Macrophage efferocytosis has gained increasing attention in the context of pancreatic diseases. For example, in chronic pancreatitis, the acinar ATP8b1/lysophosphatidylcholine (LPC) pathway enhances macrophage efferocytosis and facilitates the resolution of inflammation [19]. Additionally, the *TRIM28*/miR-133a/*CD47* axis has been shown to inhibit efferocytosis, suggesting a potential therapeutic role in pancreatic necrosis [20]. In pancreatic cancer, Annexin A1 has been found to enhance efferocytosis and modulate the immune microenvironment [21].

In this study, we further demonstrate that *TRIM21* overexpression accelerates macrophage efferocytosis in the AP cell model.

CD47 is a key molecule that signals "don't eat me" to phagocytic cells [22, 23]. It has been implicated in the modulation of macrophage phagocytosis across various diseases. For instance, epidermal growth factor receptor (*EGFR*) mutations increase *CD47* expression, impairing macrophage phagocytosis and promoting immune evasion in non-small cell lung cancer (NSCLC) [24]. In gastric cancer, *CD47* interacts with galectin-3 to inhibit phagocytosis and suppress T-cell immunity [25]. Similarly, in colon cancer, Prolyl-4-Hydroxylase Alpha Polypeptide III (*P4HA3*) upregulates *CD47* expression to inhibit macrophage phagocytosis [26]. Moreover, Potassium Channel Tetramerisation Domain-Containing Protein 21-antisense RNA 1 (*KCTD21-AS1*) has been shown to modulate *CD47* to regulate macrophage phagocytosis [27]. Notably, the suppression of *CD47* has been found to enhance macrophage phagocytosis of cancer cells and inhibit tumor growth [28]. Importantly, *TRIM21* has been demonstrated to mediate *CD47* degradation [28], although the interaction between *TRIM21* and *CD47* in the context of AP progression has remained poorly understood. In this study, we show that *TRIM21* negatively regulates *CD47* expression, and overexpression of *CD47* reverses the regulatory effects of *TRIM21* in the AP cell model. These findings suggest that the *TRIM21*/*CD47* axis exhibits

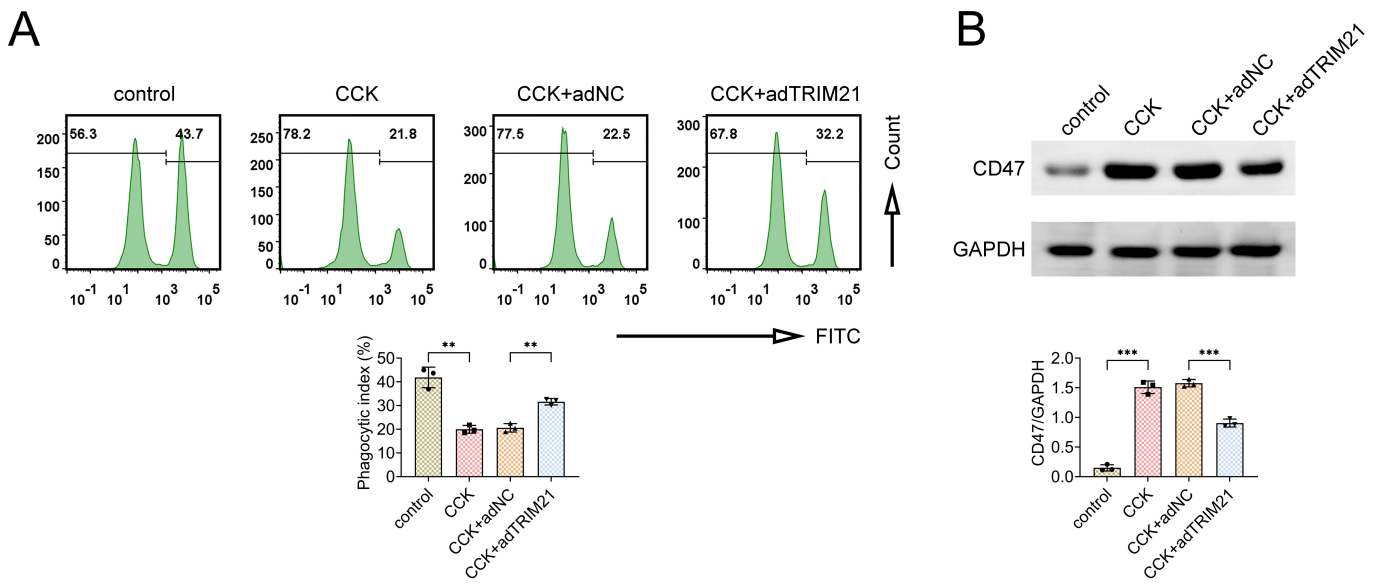

FIGURE 3. Overexpression of *TRIM21* accelerates macrophage efferocytosis. Cells were divided into four groups: control, CCK, CCK + adNC and CCK + adTRIM21. (A) The phagocytic index (%) was assessed by flow cytometry, showing enhanced efferocytosis in the CCK + adTRIM21 group. (B) Western blot analysis demonstrated decreased CD47 expression following *TRIM21* overexpression in the CCK + adTRIM21 group. N = 3. Data are presented as the mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$. CCK: cholecystokinin; adNC: negative control adenovirus; adTRIM21: tripartite motif-containing protein 21 overexpression; GAPDH: 4′,6-Diamidino-2-phenylindole; FITC: fluorescein isothiocyanate; CD47: cluster of differentiation 47.

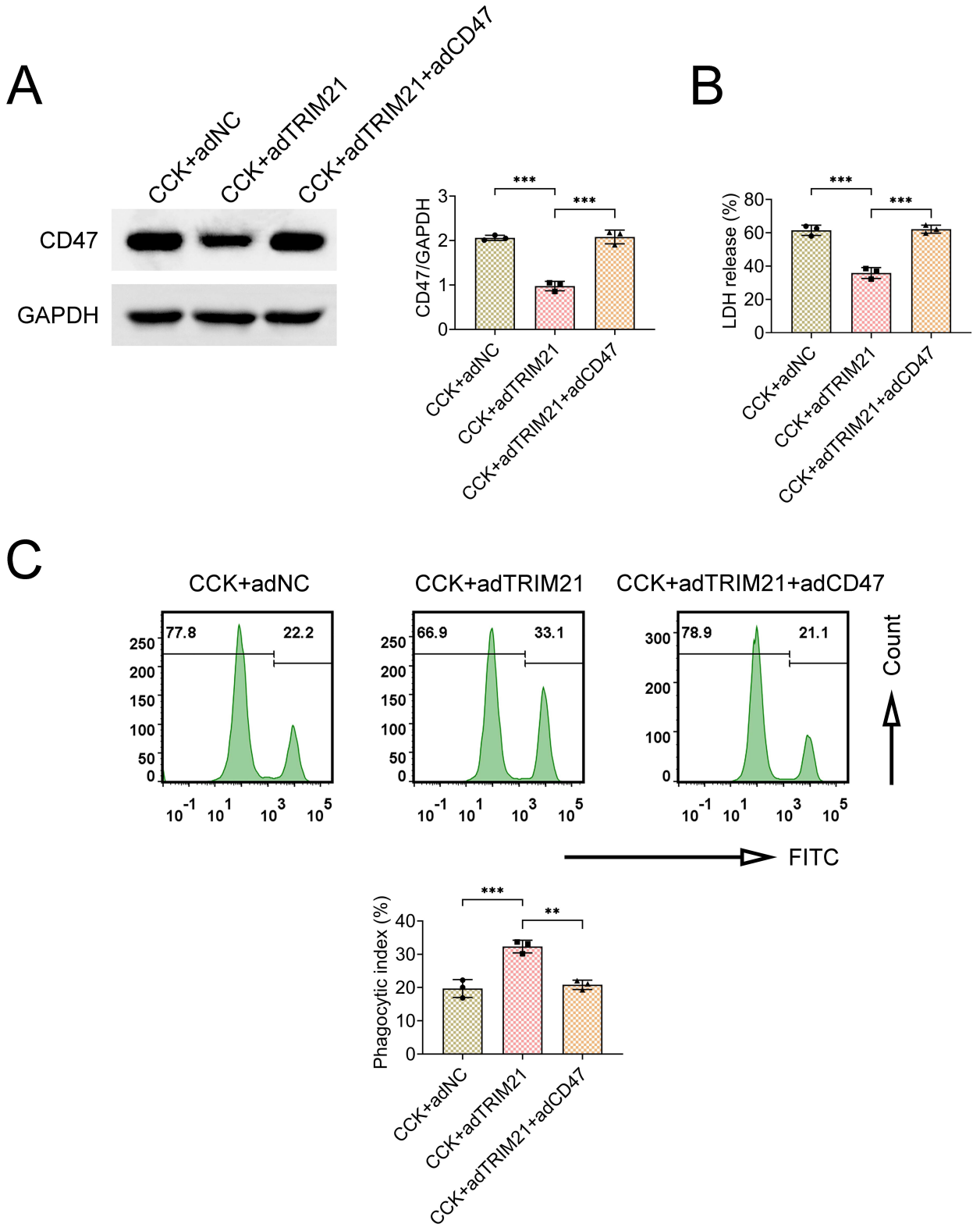

FIGURE 4. Overexpression of *CD47* reverses the regulatory effects of *TRIM21*. Cells were divided into three groups: CCK + adNC, CCK + adTRIM21 and CCK + adTRIM21 + adCD47. (A) Western blot analysis confirmed the successful overexpression of *CD47* in the CCK + adTRIM21 + adCD47 group. (B) LDH release was assessed using an LDH assay kit, demonstrating that *CD47* overexpression reversed the protective effects of *TRIM21* on cell injury. (C) Flow cytometry analysis of the phagocytic index (%) revealed that *CD47* overexpression reduced *TRIM21*-induced acceleration of efferocytosis. N = 3. Data are presented as the mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$. CCK: cholecystokinin; adNC: negative control adenovirus; adTRIM21: tripartite motif-containing protein 21 overexpression; LDH: lactate dehydrogenase; GAPDH: 4′,6-Diamidino-2-phenylindole; FITC: fluorescein isothiocyanate; CD47: cluster of differentiation 47.

critical impacts in the progression of AP.

5. Conclusions

In conclusion, this project presents the novel finding that *TRIM21* mitigates AP-induced cell damage and accelerates efferocytosis by targeting *CD47*. However, some limitations exist in this study, including the lack of *in vivo* animal models and clinical validation. Future studies are made to further explore the therapeutic potential of targeting the *TRIM21*/*CD47* axis in the clinical management of AP.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

TZZ, CMZ—designed the study and carried them out; prepared the manuscript for publication and reviewed the draft of the manuscript. TZZ, JD, SDL, ZJW—supervised the data collection; analyzed the data; interpreted the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

ACKNOWLEDGMENT

Not applicable.

FUNDING

This work was supported by Natural Science General Project of Bengbu Medical University (Grant No. 2024byzd062).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Taizhe Zhang, Jie Du, Sandang Li, Zhenjie Wang, Chuanming Zheng. *TRIM21* alleviates acute pancreatitis cell injury and promotes efferocytosis by targeting *CD47*. Signa Vitae. 2025; 21(6): 86-92. doi: 10.22514/sv.2025.086.