

# Tumor necrosis factor $\alpha$ -induced protein-3 protects zinc transporter 8 against proinflammatory cytokine-induced downregulation

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**Abstract.** Zinc transporter 8 (ZnT8) is exclusively expressed in the pancreatic islet and is essential for insulin crystallization, hexamerization and secretion. Tumor necrosis factor  $\alpha$ -induced protein-3 (TNFAIP3) is a zinc finger protein that serves a major role in the negative feedback regulation of NF- $\kappa$ B signaling in response to multiple stimuli, and is a central regulator of immunopathology. Although the role of TNFAIP3 in diabetes has been extensively studied, its effect on ZnT8 has not been fully elucidated. The present study aimed to verify whether proinflammatory cytokines, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ), are able to affect ZnT8 expression in islet cells. In addition, the study aimed to determine the effect of TNFAIP3 overexpression on cytokine-altered ZnT8 activity, considering its effect on NF- $\kappa$ B signaling. Cell-based studies using NIT-1 cells overexpressing TNFAIP3 were used to assess the effect of cytokines on ZnT8 and NF- $\kappa$ B activation, as well as the effect of TNFAIP3 on ZnT8 expression. Western blot analysis and immunofluorescence staining were employed to determine the protein expression and NF- $\kappa$ B activation, respectively. The results indicated that cytokine stimulation led to TNFAIP3 upregulation, ZnT8 downregulation and NF- $\kappa$ B activation. Furthermore, TNFAIP3 overexpression protected ZnT8 from cytokine-induced downregulation. In conclusion, the current results suggest that inflammation or

TNFAIP3 dysfunction may be involved in the pathogenesis of diabetes via ZnT8 expression, besides from islet cell apoptosis. In addition, restricting inflammation and enhancing TNFAIP3 expression may exert a positive effect in diabetes prevention, treatment and pancreatic cell transplantation.

## Introduction

Diabetes, a class of metabolic diseases that are prevalent worldwide affecting 422 million people in 2014 according to the World Health Organization (1), is characterized by hyperglycemia and can cause severe damage to different organs, particularly the eyes, kidneys, nerves, heart and blood vessels. According to the American Diabetes Association, the disease can be classified into two main types, including type 1 and type 2 diabetes (2). Type 1 diabetes results from a cell-mediated autoimmune attack on  $\beta$  cells, whereas type 2 diabetes is a low-grade, chronic inflammatory disease that shares a common final pathway with type 1 diabetes, in which activation of the nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling pathway causes a reduction in pancreatic  $\beta$  cells (3-10).

Zinc participates in numerous biological processes, and is essential for the correct processing, storage, secretion, and function of insulin in pancreatic  $\beta$  cells (11-13). Zinc flux is controlled by two types of transporters: The zinc transporter (ZnT; also known as SLC30A) family, and the Zrt- and Irt-like protein (ZIP) family. Typically, the ZnT family regulates Zn<sup>2+</sup> efflux out of the cytoplasm, while the ZIP family regulates Zn<sup>2+</sup> influx into the cytoplasm. Although 10 ZnTs and 14 ZIPs have been identified in mammals, ZnT8 is exclusively expressed in the pancreatic islet and serves a major role in Zn<sup>2+</sup> efflux from cytoplasm to insulin secretory granules, which is indispensable for insulin crystallization and hexamerization (14-16).

Alterations to ZnT8 expression may affect diabetes pathogenesis. For instance, ZnT8 deletion leads to impaired insulin secretion from pancreatic  $\beta$  cells (17,18). In addition, the expression of ZnT8 has been demonstrated to be decreased in the  $\beta$  cells of diabetic mice (19). A ZnT8 (or SLC30A8) gene polymorphism has been identified, and is associated with type 2 diabetes (20-22). Furthermore, an autoantibody to ZnT8 is widely considered as a biomarker of autoimmune diabetes (23,24). There is also evidence that zinc has an anti-inflammatory effect,

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*Abbreviations:* ZnT8, zinc transporter 8; TNFAIP3, tumor necrosis factor  $\alpha$ -induced protein-3; GAPDH, glyceraldehyde phosphate dehydrogenase; ZIP, Zrt- and Irt-like protein; TNF, tumor necrosis factor; NF- $\kappa$ B, nuclear factor  $\kappa$ B; IL-1 $\beta$ , interleukin-1 $\beta$ ; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum

*Key words:* zinc transporter 8, tumor necrosis factor  $\alpha$ -induced protein-3, diabetes, nuclear factor- $\kappa$ B

and that ZnT8 expression can be downregulated by proinflammatory cytokines, which are expressed at high levels in type 2 diabetes (25-27).

Tumor necrosis factor  $\alpha$ -induced protein-3 (TNFAIP3) is a zinc finger and ubiquitin-editing protein that is able to inhibit or promote the NF- $\kappa$ B signaling cascade by attaching to the K48-linked or K63-linked polyubiquitin chains, respectively (28,29). Although TNFAIP3 was originally characterized as an inhibitor of tumor necrosis factor (TNF)-induced apoptosis, it is best characterized as an inhibitor of NF- $\kappa$ B activation (30). Notably, a number of studies have shown the association of TNFAIP3 with common inflammatory and immune diseases, including type 1 diabetes (31-36). Thus, it is possible that TNFAIP3 participates in the pathogenesis of type 1 and type 2 diabetes. Indeed, there is certain evidence indicating this role of TNFAIP3, since TNFAIP3 expression has been found to inhibit hallmarks of diabetes in a number of models, including transplanted islets (37,38), endothelial cells exposed to high glucose (39) and streptozotocin-induced diabetes mice (40).

Based on the aforementioned previous findings, we conclude that cytokines may alter insulin secretion through the induction of apoptosis in pancreatic  $\beta$  cells and through the inhibition of insulin secretion via alterations to ZnT8 expression. Therefore, the aim of the present study was to assess whether the expression of TNFAIP3 is altered in patients with diabetes. Furthermore, the study investigated whether proinflammatory cytokines are able to alter the expression of ZnT8 in islet cells, and whether the expression of TNFAIP3 can protect against cytokine-induced ZnT8 alterations. The observations of the present study may reveal a novel view on how proinflammatory cytokines inhibit insulin secretion besides apoptosis of  $\beta$  cells, and how TNFAIP3 exerts a beneficial effect on insulin secretion besides its anti-apoptosis effect, which will also provide a potential conception in the prevention and therapy of diabetes.

## Materials and methods

**NIT-1 cell culture.** NIT-1 cells (murine) were purchased from Focusbio (Guangzhou, China) and were cultured in a 5% CO<sub>2</sub> atmosphere at 37°C in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 g/ml streptomycin. All the aforementioned reagents mentioned were purchased from GE Healthcare Life Sciences (Shanghai, China).

**Cytokine stimulation.** For stimulation assays, cells were plated (10<sup>6</sup> cells/well) in 6-well plates (Sigma-Aldrich, St. Louis, MO, USA). After 48 h of culturing, the culture medium was changed to serum-reduced culture medium (DMEM and 1% FBS), containing 1% FBS supplemented with no cytokines (control), with 10 ng/ml recombinant murine TNF- $\alpha$  or with 5 ng/ml recombinant murine interleukin-1 $\beta$  (IL-1 $\beta$ ; ProSpec, Ness Ziona, Israel). The cells were stimulated with cytokines for 6, 24 or 48 h, and then collected by centrifugation at 200 x g for 5 min at 4°C after digestion with pancreatin digestion (Sigma-Aldrich). Each assay was performed in triplicate.

**Adenoviral transfection of NIT-1 cells.** The adenovirus expressing human TNFAIP3 and enhanced green fluorescent protein (Ad-TNFAIP3-EGFP; cat. no. V494-20), and the control

adenovirus expressing EGFP (Ad-EGFP; cat. no. V493-20) were purchased from Wuhan GenSil Biotechnology (Wuhan, China). Adenovirus-mediated gene transfection was performed following the manufacturer's protocol. Briefly, NIT-1 cells were plated in 6-well plates (10<sup>6</sup> cells/well). At 1 day after cell adhesion, 1 ml DMEM with Ad-EGFP or Ad-TNFAIP3-EGFP virus [multiplicity of infection (MOI), 200] was added to each well, and the cells were infected for 24 h. The MOI was selected based on the lowest toxicity by viral infection. Following infection, the cells were cultured for an additional 24 h before being used in further experiments. TNFAIP3 and indicator protein EGFP were co-expressed, and the latter was verified using fluorescence microscopy, while transfection was detected by western blot analysis.

**Determination of NF- $\kappa$ B activation.** NF- $\kappa$ B activity was assessed using an NF- $\kappa$ B Activation and Nuclear Translocation Assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Briefly, the NF- $\kappa$ B major subunit, p65, was labeled with Cy3 and the nucleus was stained with DAPI. After washing and fixing, cytokine-treated NIT-1 cells were incubated with blocking buffer to block non-specific epitopes. Subsequently, cells were incubated with rabbit anti-mouse NF- $\kappa$ B p65 antibody at 4°C overnight, followed by incubation with a Cy3-conjugated secondary antibody for 1 h at room temperature and DAPI for 5 min prior to fluorescence microscopy. All the reagents used in this experiment were included in the kit.

**Western blot analysis.** Expression of proteins in cells was determined by western blot analysis. GAPDH was used as an internal control. Three rabbit anti-mouse/human polyclonal antibodies for GAPDH (cat. no. sc-25778; 1:400 dilution), TNFAIP3 (cat. no. 23456-1-AP; 1:1,000 dilution) and ZnT8 (cat. no. R12-3525; 1:1,000 dilution) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), Proteintech Group Inc. (Wuhan, China) and Assay Biotechnology Company, Inc. (Sunnyvale, CA, USA), respectively. Polyvinylidene difluoride (PVDF) membranes were purchased from EMD Millipore (Billerica, MA, USA). All other reagents were purchased from Beyotime Institute of Biotechnology. Briefly, harvested NIT-1 cells were lysed and extracted by a standard protocol with cell lysis buffer, and the protein levels were quantified using an Enhanced BCA Protein Assay kit. Proteins were separated by 10% SDS-PAGE and then transferred to PVDF membranes. Subsequent to blocking with QuickBlock blocking buffer for 10 min at 25°C followed by three washes for 10 min with western wash buffer, the membranes were incubated overnight at 4°C with polyclonal rabbit anti-TNFAIP3, anti-ZnT8 or anti-GAPDH antibodies. Subsequent to washing three times for 10 min with western wash buffer, the membranes were incubated with goat anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (cat. no. A0208; 1:1,000 dilution), and an enhanced chemiluminescence detection system (BeyoECL Plus; cat. no. P0018; Beyotime Institute of Biotechnology) were used for detection.

**Statistical analysis.** Data are presented as the raw values or as the mean  $\pm$  standard deviation. IBM SPSS software (version 19.0; IBM Corp., Armonk, NY, USA) was used to perform statistical

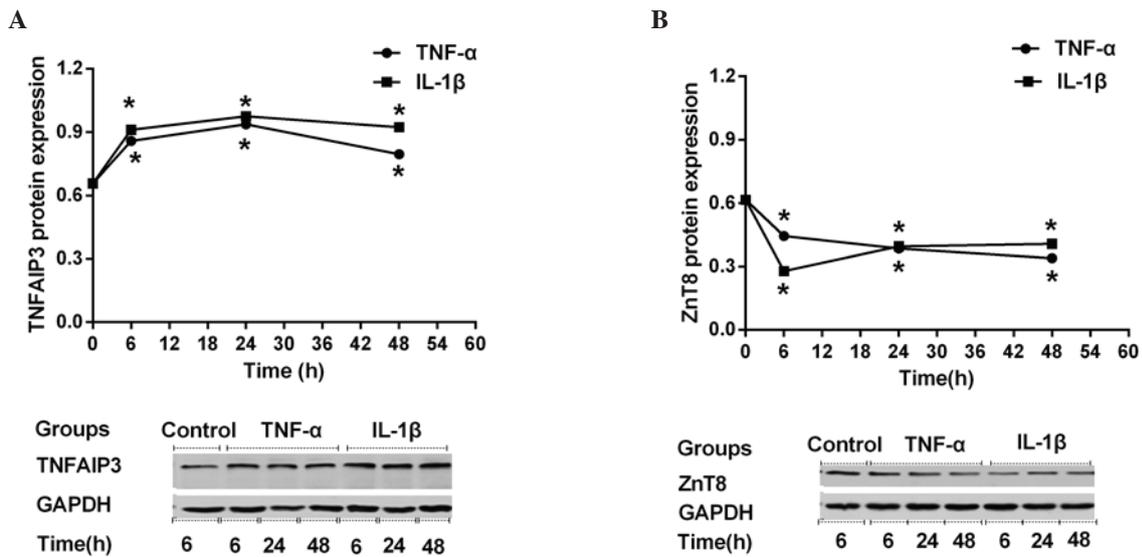


Figure 1. Expression levels of (A) TNFAIP3 and (B) ZnT8 following TNF- $\alpha$  and IL-1 $\beta$  stimulation. NIT-1 cells were treated with no cytokines, with 5 ng/ml murine IL-1 $\beta$  or with 10 ng/ml murine TNF- $\alpha$  for 6, 24 or 48 h. \*P<0.05 vs. non-stimulated control TNFAIP3, TNF  $\alpha$ -induced protein-3; ZnT8, zinc transporter 8; TNF, tumor necrosis factor; IL, interleukin.

analyses using one-way analysis of variance, Kruskal-Wallis test or Mann-Whitney U test in order to assess differences, as appropriate. A P-value (two-tailed) of  $\leq 0.05$  was considered to indicate a statistically significant difference.

**Results**

*Cytokine-induced alterations in TNFAIP3 and ZnT8 expression and NF- $\kappa$ B activation.* Previous studies have demonstrated the expression of TNFAIP3 (41,42) and ZnT8 (43,44) in several pancreatic  $\beta$  cell lines or primary islet cells; however, to date, no such reports concerning their expression in insulin-producing NIT-1 cells exist. Therefore, the present study investigated whether TNFAIP3 and ZnT8 were expressed in NIT-1 cells, and then examined whether their expression was altered by stimulation with TNF- $\alpha$  or IL-1 $\beta$ . As shown in Fig. 1, TNFAIP3 (Fig. 1A) and ZnT8 (Fig. 1B) were both expressed in NIT-1 cells, as assessed by western blot analysis. NIT-1 cells demonstrated similar TNFAIP3 expression-time curves (0, 6, 24 or 48 h) when stimulated by TNF- $\alpha$  and IL-1 $\beta$ . During the first 6 h of cytokine stimulation (0-6 h), TNFAIP3 expression increased sharply. During the subsequent 18 h (6-24 h), the upregulation slowed, while TNFAIP3 expression began to slowly decline during the subsequent 24 h (24-48 h), despite still being higher compared with prior to stimulation (Fig. 1A). Stimulated TNFAIP3 expression levels at all time points (6, 24 and 48 h) were significantly altered compared with the control (all P<0.001). In contrast to TNFAIP3 expression, ZnT8 expression declined rapidly during the first 6 h of cytokine stimulation. During the subsequent 42 h, ZnT8 expression decreased or recovered slowly in the TNF- $\alpha$  or IL-1 $\beta$  groups, respectively (Fig. 1B). In conclusion, stimulated ZnT8 expression levels at all time points (6, 24 and 48 h) were significantly altered compared with the control (all P<0.001).

Furthermore, the present study examined the NF- $\kappa$ B activation in NIT-1 cells subsequent TNF- $\alpha$  and IL-1 $\beta$

stimulation (Fig. 2). In resting cells, NF- $\kappa$ B is known to be inactivated through its interaction with I $\kappa$ B. After cells are stimulated, NF- $\kappa$ B is released and translocates into the nucleus (45). In order to assess NF- $\kappa$ B activation, an NF- $\kappa$ B activation and Nuclear Translocation Assay kit was used in the current study. The cellular p65 subunit of NF- $\kappa$ B was identified using anti-p65 antibodies, which were then labeled by Cy3-coupled secondary antibodies (red fluorescence). Nuclear DNA of the same group was stained with DAPI (blue fluorescence) as an indicator. Merged images demonstrated the positional association between p65 and the nucleus. Compared with the control (Fig. 2A), the TNF- $\alpha$  (Fig. 2B) and IL-1 $\beta$  (Fig. 2C) stimulation resulted in assembly of the p65 subunit in and around the nucleus, which indicated NF- $\kappa$ B activation.

*TNFAIP3 overexpression protects ZnT8 from cytokine-induced downregulation.* As previously discussed, stimulation with the TNF- $\alpha$  and IL-1 $\beta$  inflammatory cytokines resulted in NF- $\kappa$ B activation and ZnT8 downregulation. It was therefore hypothesized that TNFAIP3, an inhibitor of NF- $\kappa$ B activation (30), may reverse the low expression of ZnT8 in NIT-1 cells exposed to TNF- $\alpha$  and IL-1 $\beta$ . Therefore, the influence of TNFAIP3 overexpression (using a TNFAIP3 overexpressing adenovirus) on the suppression of ZnT8 expression resulting from cytokine intervention was investigated. As shown in Fig. 3, when stimulated by the same cytokine, the experimental group (transfected with an Ad-TNFAIP3-EGFP adenovirus) presented higher expression of TNFAIP3 compared with the control group (transfected with an Ad-EGFP adenovirus), indicating successful transfection (P<0.001 for TNF- $\alpha$  and IL-1 $\beta$ ). Similarly, the experimental group with high TNFAIP3 expression was shown to express a greater level of ZnT8 when compared with the control group (P<0.001 for TNF- $\alpha$  and IL-1 $\beta$ ). These findings suggest that TNFAIP3 overexpression significantly suppresses cytokine-induced ZnT8 downregulation.

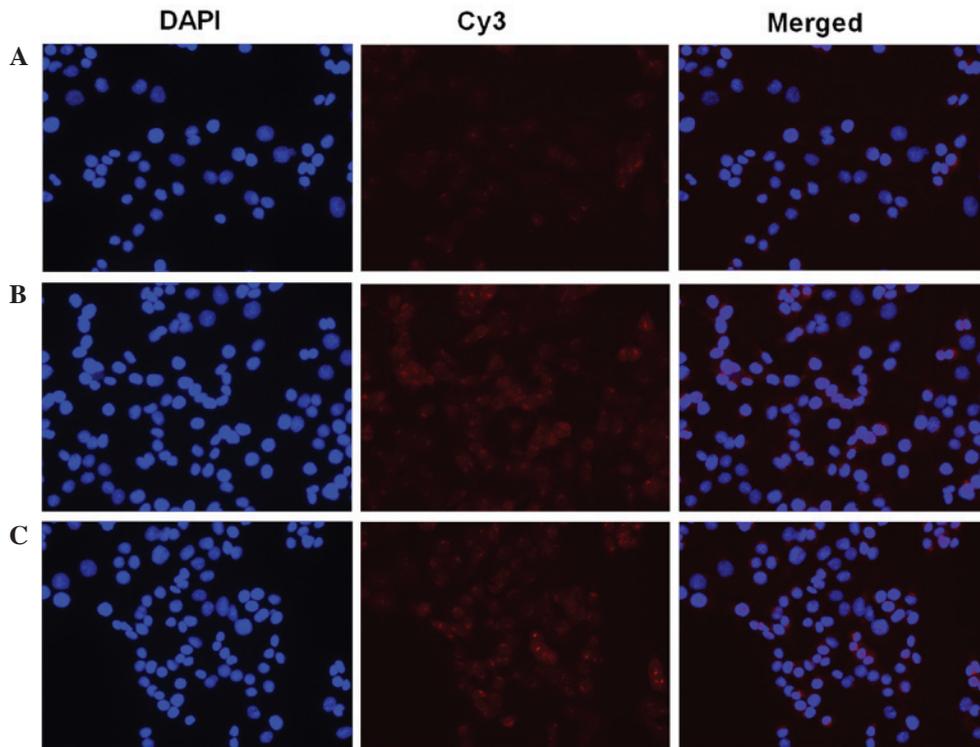


Figure 2. Effects of TNF- $\alpha$  and IL-1 $\beta$  stimulation on NF- $\kappa$ B activation and nuclear translocation. NIT-1 cells were cultured in (A) serum-reduced culture medium containing 1% fetal bovine serum and Dulbecco's modified Eagle's medium, or exposed to the serum-reduced culture medium supplemented with (B) 10 ng/ml murine TNF- $\alpha$  for 6 h or (C) 5 ng/ml murine IL-1 $\beta$  for 6 h. Cells were labeled with Cy3 (red) and DAPI (blue), and were imaged simultaneously. TNF, tumor necrosis factor; IL, interleukin.

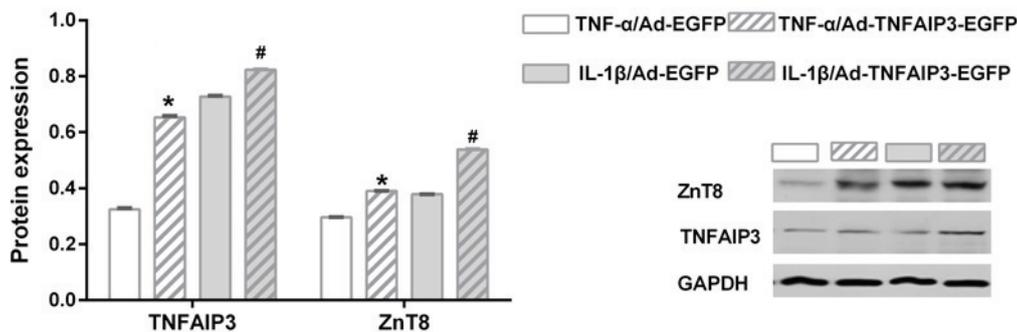


Figure 3. Expression levels of TNFAIP3 and ZnT8 subsequent to TNF- $\alpha$  and IL-1 $\beta$  stimulation in NIT-1 cells that were transfected with Ad-EGFP or Ad-TNFAIP3-EGFP. NIT-1 cells were treated with 5 ng/ml murine IL-1 $\beta$  or 10 ng/ml murine TNF- $\alpha$  for 6 h after transfection. TNF- $\alpha$ -stimulated expression: \* $P$ <0.05 vs. Ad-EGFP adenovirus transfected control; IL-1 $\beta$ -stimulated expression: # $P$ <0.05 vs. Ad-EGFP adenovirus transfected control. TNFAIP3, TNF  $\alpha$ -induced protein-3; ZnT8, zinc transporter 8; TNF, tumor necrosis factor; IL, interleukin; Ad-EGFP, adenovirus expressing EGFP (control); Ad-TNFAIP3-EGFP, adenovirus expressing TNFAIP3 and EGFP; EGFP, enhanced green fluorescent protein.

## Discussion

The results of the present study, in agreement with the findings of previous studies (27,46), indicated that insulin secretion can be inhibited by proinflammatory cytokines causing dysregulation of  $\beta$  cells via ZnT8 downregulation, and a decrease in  $\beta$  cell number by affecting apoptosis. The current study showed that proinflammatory cytokines, TNF- $\alpha$  and IL-1 $\beta$ , downregulate ZnT8 protein expression in NIT-1 cells. This effect was previously observed in MIN6 cells (27), indicating that decreased ZnT8 expression may contribute to impaired  $\beta$  cell function and

insulin secretion. ZnT8 is predominantly expressed in pancreatic islets, and is responsible for transporting zinc from the cytoplasm into insulin secretory vesicles, where insulin is stored as a solid hexamer bound with two Zn<sup>2+</sup> ions (15). Thus, Zn<sup>2+</sup> is essential for the storage, crystallization and secretion of insulin. In addition, a previous study observed that TNF- $\alpha$  and IL-1 $\beta$  were higher in the plasma of diabetes patients (47). It is well established that numerous cytokines, including TNF- $\alpha$ , IL-1 $\beta$  and interferon- $\gamma$ , contribute to  $\beta$  cell dysfunction and death, via activation of the NF- $\kappa$ B signaling pathway (48-50), thus promoting the immune system activity and the transcription of

proinflammatory and cell survival genes (51). This mechanism has been previously verified using therapeutic drugs (52) and using a mouse allogeneic pancreatic islet graft experiment (53). Furthermore, studies of the human genome have identified numerous genes associated with the pathophysiology of autoimmune and inflammatory diseases, of which numerous are involved in the NF- $\kappa$ B signaling pathway (33,35). Therefore, NF- $\kappa$ B serves a major role in cytokine-dependent  $\beta$  cell dysfunction, and may represent a target for therapeutics to improve islet function during inflammatory insults.

Inappropriate activation of NF- $\kappa$ B signaling pathway is a key event in the progressive loss of  $\beta$  cells in autoimmune diabetes and type 2 diabetes (54-56). TNFAIP3, an immediate early response gene, serves a critical role in the negative regulation of the NF- $\kappa$ B signaling pathway, as well as in  $\beta$  cell protection, through its actions as a dual ubiquitin-editing protein (49,57). The mechanism through which TNFAIP3 turns off inflammation signaling has previously been described as the disruption of ubiquitin enzyme complexes (58); in cells expressing deficient or mutant TNFAIP3 protein, there is defective removal of Lys63-linked ubiquitin from ubiquitin enzyme complexes following stimulation with TNF (59). Our previous study found that the plasma levels of TNF- $\alpha$  and IL-1 $\beta$  were significantly increased, and TNFAIP3 mRNA and protein expression levels were significantly decreased in the peripheral blood mononuclear cells of diabetes patients (60). The decrease in TNFAIP3 mRNA and protein expression levels is consistent with other inflammatory diseases. For instance, TNFAIP3 expression is decreased in systemic lupus erythematosus patients, and the expression level of TNFAIP3 negatively correlates with the systemic lupus erythematosus disease activity index (61).

TNFAIP3 is known for its potent anti-apoptotic activity, particularly in  $\beta$  cells. Apoptosis is the main form of  $\beta$ -cell death in the two forms of diabetes. Previous research suggested that the mechanisms leading to nutrient- and cytokine-induced  $\beta$  cell death in type 2 and type 1 diabetes, respectively, share the activation of a final common pathway involving IL-1 $\beta$ , NF- $\kappa$ B and Fas (50). TNFAIP3 is one of the anti-apoptotic genes induced by cytokines through NF- $\kappa$ B in cultured human islets (62) and INS-1E cells (42); induction of protective genes, such as TNFAIP3, leads to islet survival and function (37). The results of the present study provide a novel role for TNFAIP3 in the protection of normal  $\beta$  cell functions via ZnT8 downregulation. This was supported by the data obtained through TNFAIP3 overexpression *in vitro* in islet (NIT-1) cells. Furthermore, inflammatory cytokines were found to downregulate ZnT8 protein expression in NIT-1 cells. Notably, TNFAIP3 overexpression protected against cytokine-induced downregulation of ZnT8. This role is in addition to the anti-apoptotic activity of TNFAIP3.

To the best of our knowledge, this is the first study to examine the influence of TNFAIP3 overexpression on cytokine-induced ZnT8 inhibition. One limitation of the current study is that single nucleotide polymorphism of TNFAIP3 was not considered, which may influence its affinity with targets. Future studies will further analyze whether single nucleotide polymorphisms of TNFAIP3 alter the effect of TNFAIP3 on ZnT8 expression, and a gene knockout model will be investigated.

In conclusion, the results of the present study revealed that TNFAIP3 overexpression was able to protect ZnT8 from

cytokine-induced downregulation. Thus, it is hypothesized that cytokines may not only lead to the death of  $\beta$  cells, but also alter their normal function. Notably, TNFAIP3 serves a positive role through its anti-apoptotic effects and its ability to maintain normal  $\beta$  cell function. Therefore, TNFAIP3 may serve a pivotal role in the pathogenesis of diabetes and may offer a novel therapeutic target for the prevention and treatment of diabetes.

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