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Inherent ER stress in Pancreatic Islet β cells Causes Self-Recognition by Autoreactive T Cells in Type 1 Diabetes

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease characterized by pancreatic β cell destruction induced by islet reactive T cells that have escaped central tolerance. Many physiological and environmental triggers associated with T1D result in β cell endoplasmic reticulum (ER) stress and dysfunction, increasing the potential for abnormal post-translational modification (PTM) of proteins. We hypothesized that β cell ER stress induced by environmental and physiological conditions generates abnormally-modified proteins for the T1D autoimmune response. To test this hypothesis we exposed the murine $CD4^+$ diabetogenic BDC2.5 T cell clone to murine islets in which ER stress had been induced chemically (Thapsigargin). The BDC2.5 T cell IFNγ response to these cells was significantly increased compared to non-treated islets. This β cell ER stress increased activity of the calcium (Ca^{2+})-dependent PTM enzyme tissue transglutaminase 2 (Tgase2), which was necessary for full stress-dependent immunogenicity. Indeed, BDC2.5 T cells responded more strongly to their antigen after its modification by Tgase2. Finally, exposure of non-antigenic murine insulinomas to chemical ER stress *in vitro* or physiological ER stress *in vivo* caused increased ER stress and Tgase2 activity, culminating in higher BDC2.5 responses. Thus, β cell ER stress induced by chemical and physiological triggers leads to β cell immunogenicity through Ca^{2+}-dependent PTM. These findings elucidate a mechanism of how β cell proteins are modified and become immunogenic, and reveal a novel opportunity for preventing β cell recognition by autoreactive T cells.

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Keywords

Type 1 diabetes; β cell; ER stress; post-translational modification; autoimmunity

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease in which pancreatic islet β cells are targeted and destroyed by the immune system, with autoreactive T cells largely mediating pathogenesis. In individuals genetically predisposed to autoimmunity, autoreactive T cells enter the periphery as a result of failed central tolerance and recognize β cell autoantigens [1, 2]. As disease progresses, additional β cell proteins become immunogenic due to failures in peripheral tolerance [3, 4]. While the identification of β cell autoantigens is of great importance, elucidating how physiological mechanisms lead to defective peripheral tolerance is crucial to designing therapeutic intervention to reduce β cell immunogenicity and prevent T1D.

Islet β cells, like all professional secretory cells, contain a more fully developed endoplasmic reticulum (ER) than nonsecretory cells and are consequently more susceptible to ER stress due to their normal physiology and insulin biosynthesis [5–14]. β cells increase production of preproinsulin by 50-fold in response to heightened blood glucose concentrations, ultimately reaching a translation rate of 1 million molecules per minute [15]. These 1 million molecules of preproinsulin enter the ER lumen for folding and disulfide bond formation, heavily burdening the protein folding machinery of the ER and causing tremendous ER stress. This heightened ER stress due to insulin secretion is detected in the murine pancreas as early as 3 weeks of age [16]. In addition, ER stress is further increased by many physiological and environmental triggers associated with T1D, including viral infection [17–19], exposure to chemicals [20–23] or reactive oxygen species [24–26], dysglycemia [15], and pancreatic inflammation [27, 28]. Therefore, exposure to these known triggers may increase ER stress to levels above those inherent to the physiology of the secretory β cell. Thus, heightened ER stress may be an important factor in early T1D pathogenesis.

ER stress activates the unfolded protein response (UPR), which functions to restore ER homeostasis [29]. The UPR includes three signaling cascades that are initiated by protein sensors of ER stress, protein kinase RNA (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring protein 1 (IRE1). These cascades initiate new chaperone synthesis to aid with folding of accumulated misfolded proteins [30, 31] and attenuate non-chaperone mRNA translation to reduce the protein burden in the ER [32, 33]. If ER stress is too great or too prolonged, the cytoprotective mechanisms of the UPR fail, and pro-apoptotic signaling pathways become activated [34–36]. However, even transient ER stress and UPR activation have significant consequences for the cell. For example, ER stress leads to the release of calcium (Ca^{2+}) from the ER lumen into the cytosol. This efflux of Ca^{2+} negatively affects protein folding in the ER since many chaperones require Ca^{2+} [37–39] and also affects the function of Ca^{2+}-dependent cytosolic enzymes. This Ca^{2+} flux is observed in β cells during insulin production and secretion [40–43], confirming the occurrence of ER stress during glucose-stimulated insulin production.

ER stress has been implicated in a number of autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus [15, 44–51]. In many such diseases, ER stress and dysfunction increase the potential for abnormal post-translational modification (PTM) of proteins. These modified proteins are processed and presented by antigen presenting cells (APC) in draining lymph nodes and, in the context of genetic susceptibility to autoimmunity, activate autoreactive T cells that have escaped central and peripheral tolerance to initiate or exacerbate pathology [52–55]. However, the mechanisms by which ER stress leads to abnormal PTM in these disease models have not been elucidated.

The release of Ca^{2+} from the ER lumen during ER stress increases the activity of cytosolic Ca^{2+}-dependent enzymes. Tissue transglutaminase 2 (Tgase2) is a ubiquitously expressed cytosolic Ca^{2+}-dependent PTM enzyme [56] that is activated during ER stress [50, 57–60]. Active Tgase2 translocates to the ER [50, 59, 61] and to secretory granules [62] to modify proteins through the formation of ε(γ-glutamyl) isopeptide bonds between glutamine and lysine residues that crosslink and aggregate proteins, and through the deamidation of glutamine to glutamic acid [63]. Tgase2 is involved in normal cellular functions including cell growth and inhibition of apoptosis [64, 65]. However, Tgase2 modifications are also associated with breaks in peripheral tolerance in several autoimmune disorders [44, 50, 51, 66, 67], although the mechanisms by which Tgase2 becomes activated in these models has not been explored.

The diabetogenic $CD4^+$ T cell clone BDC2.5 recognizes WE14, a naturally occurring cleavage product of Chromogranin A (CHgA) [68]. However, WE14 is significantly less immunogenic than more physiologically relevant forms of antigen, such as whole β cells [68], suggesting that physiological expression of CHgA in β cells is necessary for immunogenicity. WE14 was later shown to elicit stronger BDC2.5 T cell responses after *in vitro* modification by Tgase2 [69]. However, whether Tgase2 is active in β cells, or whether this activity is relevant for β cell immunogenicity, was not explored. Indeed, although there is precedent to suggest that many murine and human β cell peptides, in addition to WE14, elicit stronger T cell responses after PTM [69–75], these studies have not explored or addressed the cellular and physiological processes that lead to PTM.

We hypothesized that β cell ER stress induced by physiological conditions generates abnormally-modified proteins that activate the autoimmune response in T1D. Here, we demonstrate that β cell ER stress induced by a chemical trigger *in vitro* or by physiological triggers *in vivo* increased the recognition of these β cells by diabetogenic BDC2.5 T cells. Furthermore, ER stress increased cytosolic Ca^{2+} concentrations and Tgase2 activity, both of which were crucial for ER stress-dependent β cell immunogenicity. Together, these results demonstrate that exposure to triggers of ER stress increases the activity of Ca^{2+}-dependent PTM enzymes (such as Tgase2) in β cells. These findings also provide a mechanism to explain how and why the β cell peptides described by others [69–75] may undergo PTM and come to be recognized by autoreactive T cells. Therefore, the normal physiology of the β cell, which predisposes the cell to ER stress, may contribute to its own immune-mediated destruction in T1D.

2. Materials and methods

2.1. Mice

Mice were bred and housed under specific pathogen-free conditions at Rangos Research Center of Children's Hospital of Pittsburgh of University of Pittsburgh Medical Center. All experiments were approved by Institutional Animal Care and Use Committee of the University of Pittsburgh.

In 4 experiments, 13 NOD.*scid* mice were transplanted with 5×10^6 NIT-1 cells under the kidney capsule, and 10 nonrecipient mice were used as controls. Starting at day 2 post-transplant, blood glucose was monitored for hypoglycemia and serum was collected to measure insulin levels. Hypoglycemia was defined as 2 consecutive blood glucose readings ≤ 40 mg/dl. At onset, mice were sacrificed, the kidney was harvested, and the NIT-1 cells were explanted for further analysis.

2.2. Cell culture

Primary murine islets were harvested from NOD.*scid*, C57BL/6, or BALB/c pancreata as described [76–78]. Islet cells were 90% viable as determined by trypan blue (Gibco) exclusion assay. Islet cell-conditioned media was harvested from islet cultures after 1 hr at 37°C.

The NIT-1 insulinoma cell line was a gift from Clayton Mathews (University of Florida) and $CD4^+$, MHC class II-restricted BDC2.5 T cells were a gift from Kathryn Haskins (University of Colorado). NIT-1 cells were maintained at 37°C in a 5% CO_2 humid air incubator, in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Mediatech), 10 mM HEPES buffer (Gibco), 4 mM L-glutamine (Gibco), 200 μM nonessential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 61.5 μM β-mercaptoethanol (Sigma-Aldrich), and 100 μg/ml gentamicin (Gibco). NIT-1 cell-conditioned media was harvested from NIT-1 cultures after 1 hr at 37°C.

BDC2.5 T cell clones were maintained in supplemented DMEM as described [79].

2.3. BDC2.5 T cell assay

BDC2.5 T cells (2×10^4), NOD.*scid* splenocytes as APC (4×10^5), and antigen (1×10^3 dispersed islet cells/ml, 50 μl islet cell conditioned media, or 90 μM $CHgA_{351–370}$ peptide) were combined in 200 μl supplemented DMEM in triplicate in 96-well flat-bottom tissue culture plates (Greiner Bio-One) and incubated at 37°C for 72 hr. T_H1 effector function was determined by measuring IFNγ secretion by enzyme-linked immunosorbent assay (ELISA).

2.4. ELISA

IFNγ from T cell assays was measured with murine IFNγ ELISA antibody pairs (BD Biosciences) as described [80, 81]. Insulin in mouse serum was measured by Mouse Insulin Ultrasensitive ELISA (Alpco Immunoassays) following the manufacturer's instructions. Absorbances were measured at 450 nm with a SpectraMax M2 microplate reader (Molecular Devices). Data were analyzed with SoftMax Pro (Molecular Devices).

2.5. Induction of ER stress

Primary murine islets were incubated in low-binding tissue culture dishes (Corning) with 5 μM Thapsigargin (Thaps; Sigma-Aldrich) or control for 1 hr at 37°C. Prior to BDC2.5 T cell assay or other downstream analysis, the islets were washed extensively (50,000× original volume) to remove residual Thaps, and dispersed in Cell Dissociation Buffer (Gibco) at 37°C for 15 min with periodic vortexing.

NIT-1 cells were cultured in 25 cm^2 tissue culture flasks (Greiner Bio-one) with 5 μM Thaps or control for 1 hr at 37°C. Prior to BDC2.5 T cell assay or other downstream analysis, the cells were washed extensively (50,000× original volume) to remove residual Thaps, and removed from the flask with 0.05% Trypsin-EDTA (Gibco).

2.6. Preparation of Cell Lysates

Cells were lysed by sonication in 50 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM Na_3VO_4, and 1 mM PMSF. Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific).

2.7. Western blotting

Lysates were separated by SDS-PAGE with 4–20% polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked in 4% milk in TBST for 1 hr, and probed with antibodies to phosphorylated PERK (Cell Signaling Technology; 1:200), phosphorylated eIF2α (Cell Signaling Technology; 1:1,000), and β-actin (Sigma-Aldrich; 1:10,000) overnight at 4°C. Membranes were washed and incubated with HRP-conjugated goat anti-rabbit (Cell Signaling Technology; 1:2,000) or horse anti-mouse (Jackson ImmunoResearch 1:10,000) for 1 hr. Chemiluminescence was detected with Luminata Crescendo Western HRP Substrate (Millipore) and analyzed with Fujifilm LAS-4000 imager and Multi Gauge Software (Fujifilm Life Science).

2.8. Calcium tracing

Primary islets were dispersed and incubated in glass-bottom plates (MatTek) for 2 days until adherent. NIT-1 cells were seeded in glass-bottom plates overnight. Adherent cells were labeled with 1 μM Fluo-4 (Invitrogen) at 37°C for 1 hr. The cells were washed and the intensity of Fluo-4 at 488 nm was monitored by live imaging with the 40× objective lens of an Olympus Fluoview FV1000 microscope for 350 sec at room temperature. At 70 sec, the cells were exposed to 5 μM Thaps or control in supplemented DMEM. Data were analyzed with FV10-ASW imaging software.

2.9. Calcium chelation

Cells were incubated with 2.5 μM Bapta-AM (Invitrogen) or control for 1 hr at 37°C before the addition of 5 μM Thaps for 1 hr. Prior to BDC2.5 T cell assay or other downstream analysis, the cells were washed extensively (50,000× original volume) to remove residual Bapta-AM and Thaps. Conditioned media was obtained after 1 hr incubation at 37°C.

2.10 Antibody stimulation of BDC2.5 T cells

Stimulation of BDC2.5 T cells with plate-bound α-CD3 (BD Biosciences; 0.5 μg/ml) and α-CD28 (BD Biosciences; 1.0 μg/ml) was performed as described [82, 83], with the following modifications. BDC2.5 T cells (2×10^4) were combined with 50 μl conditioned media as indicated in 200 μl supplemented DMEM at 37°C for 72 hr. IFNγ secretion was measured by ELISA.

2.11. In Situ Tgase2 Activity Microtiter Plate Assay

The activity of Tgase2 was measured by the incorporation of biotinylated pentylamine into endogenous proteins as described [84, 85]. Briefly, primary murine islets or NIT-1 cells were incubated with 5 mM Pentylamine-Biotin (Thermo Fisher Scientific) or control for 2 hrs at 37°C prior to the addition of 5 μM Thaps or control for 1 hr. The cells were washed and lysed as described above. The concentration of the lysates was measured by BCA assay and adjusted to 40 μg/ml (islets) or 200 μg/ml (NIT-1) in coating buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EGTA, 5mM EDTA). The lysates were plated in triplicate in 96-well microtiter plates (Corning) and incubated overnight at 4°C. The wells were blocked with 200 μl blocking buffer (5% BSA, 0.01% SDS, 0.01% Tween-20 in borate buffered saline) for 2 hrs at 37°C. After washing (1% BSA, 0.01% Tween-20 in borate buffered saline), streptavidin HRP (Invitrogen) was diluted in wash buffer following the manufacturer's instructions and incubated at room temperature for 1 hr. After washing, TMB substrate (Dako) was added to the wells for 20 minutes at room temperature, and the reaction was stopped with 0.18 M H_2SO_4. Absorbance was measured at 450 nm. Values were normalized by subtracting the absorbance of cells not incubated with pentylamine-biotin (represented endogenous biotin). Data are reported as fold change in Tgase2 activity compared to cells incubated with pentylamine-biotin alone.

2.12. Synthesis of $CHgA_{351–370}$

$CHgA_{351–370}$ (REWEDKRWSRMDQLAKELTA) was synthesized at 95% purity and confirmed by HPLC analysis (Peptide2.0).

2.13. Expression of recombinant CHgA in E. coli

Full length murine CHgA was amplified from the pCMV6-Kan/Neo cDNA clone (Origene) with the following primers: F, 5'-TCTAGATCTTTCCGCACCGTCCG-3', 5'-GTCAGAATTCCCTACTCGAGCAGCAGTC-3'. The cycling parameters were as follows: 94°C for 6 mins, followed by 39 cycles of 94°C for 30 sec, 56°C for 60 sec, 72°C for 60 sec, and 72°C for 10 mins. The PCR product was ligated into the pTrcHis2 vector (Invitrogen). The pTrcHis2 vector was transformed into *E. coli* strain NiCo21 (DE3) (New England BioLabs) and the expression of HIS-tagged proteins was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 3.5 hr. The bacteria were lysed using B-PER (Thermo Scientific), following the manufacturer's instructions. CHgA was purified using the NI-NTA column (Qiagen), following the manufacturer's instructions.

2.14. In vitro modification with Tgase2

Tgase2 (Sigma-Aldrich;1.3 μM) was incubated with $CHgA_{351–370}$ (0.2 mM) in 50 mM Tris, pH 8.0, 10 mM $CaCl_2$, 1 mM DTT, 1 mM EDTA at 37°C for 3 hr. The reactions were repeated with 0 mM $CaCl_2$ or 10 mM EDTA. Reactions were separated with 16.5% Tris-Tricine gels (Bio-Rad), and stained with Coomassie G250 (Bio-Rad). Gels were imaged with Fujifilm LAS-4000 imager and Multi Gauge Software.

Tgase2 (1.3 μM) was incubated with full length recombinant CHgA (3.9 μM) in 50 mM Tris, pH 8.0, 10 mM $CaCl_2$, 1 mM DTT, 1 mM EDTA at 37°C for 3 hr.

2.15. Mass Spectrometry

Recombinant CHgA, incubated with Tgase2 or control, was separated by SDS-PAGE. The gel was stained with Gel Code Blue (Thermo Scientific). Gel samples were washed with 25 mM ammonium bicarbonate followed by acetonitrile. Samples were then reduced with 10 mM DTT at 60°C followed by alkylation with 50 mM iodoacetamide at room temperature. Samples were then digested with trypsin (Promega) at 37°C for 4 h. Following digestion, samples were quenched with formic acid, and the supernatant was analyzed directly by nano-LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Orbitrap Velos Pro mass spectrometer. Peptides were loaded on a trapping column and eluted over a 75 μm analytical column at 350 nL/min; both columns were packed with Jupiter Proteo resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed at 70,000 and 17,500 FWHM resolution, respectively. The 15 most abundant ions were selected for MS/MS. The MS/MS data were searched against the SwissProt database using Mascot search engine version 2.4 (Matrix Science, London, UK; version Mascot). Mascot was set up to search the SwissProt_Mouse_20150127 database assuming the digestion enzyme stricttrypsin. The precursor and product ion tolerances were 10 ppm and 0.5 Da, respectively. Carbamidomethylation of cysteines was set as a fixed modification. Gln->pyro-Glu of the N-terminus, deamidated of asparagine and glutamine, oxidation of methionine and acetylation of the N-terminus were specified as variable modification. Mascot DAT files were parsed into Scaffold (Proteome Software, Portland, OR) for visualization and validation using the Protein Prophet algorithm [86, 87]. Minimum protein probability was 90%, and peptide probability was 50%; a minimum of two unique peptides was necessary for protein identification. Putative modified peptides were confirmed by manual inspection of the raw MS/MS spectra.

2.16. Reduction of Tgase2 expression by shRNA

NIT-1 cells were incubated with lentiviral particles (multiplicity of infection 2.5) carrying vectors (pLKO.1) encoding control shRNA or Tgase2-targeting shRNA (Sigma-Aldrich) for 20 hr at 37°C. After 24 hrs in fresh supplemented DMEM, cells were selected with 7.5 μg/ml puromycin (Sigma-Aldrich). Five shRNA sequences were tested, and data shown were obtained with the sequence that most reduced Tgase2 expression: 5'–CCGGCCTGGAGAATCCCGAAATCAA–3'.

2.17. Quantitative Real Time PCR (qRT-PCR)

mRNA was isolated with RNeasy Kit (Qiagen) and cDNA was synthesized with RT^2 First Strand Kit (Qiagen). Quantification of cDNA was performed by qRT-PCR (iCycler, BioRad) with the iQ SYBR Green Supermix (BioRad). Cycling parameters were 95°C for 15 min and 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. Murine Tgase2 primers (*tgm2*), F 5'–GACAATGTGGAGGAGGGATCT–3', R 5'–CTCTAGGCTGAGACGGTACAG–3' were generated by Primer Bank, ID# 6678329a1. Murine RPLO (*rplo*) primers were previously published [88]. Gene expression was normalized using the $\Delta\Delta C_t$ method, where the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta Ct}$, where C_t is the cycle number of the detection threshold.

2.18. Glucose-stimulated insulin secretion assay

Glucose-stimulated insulin secretion assays were performed as described [89].

2.19. Ex Vivo Tgase2 Activity Assay

The activity of Tgase2 in explanted NIT-1 cell lysates was measured with Transglutaminase Colorimetric Assay Kit (Covalab) following the manufacturer's instructions, and normalized to total lysate concentration (mg/ml) determined by BCA assay.

2.20. Statistical analysis

For ELISA, data are mean IFNγ or insulin secretion ± S.E.M. For SDS-PAGE and western blotting, data are representative of 3 experiments. Densitometry data are phosphorylation levels normalized by β-actin and relative to that in control treated cells. For Ca^{2+} tracing, images are representative of 3 experiments and combined data are change in fluorescent intensity ± S.E.M. For *in situ* Tgase2 activity, data are mean relative Tgase2 activity ± S.E.M. For qRT-PCR, data are relative Tgase2 expression, normalized to *rplo* expression, ± S.E.M. For *ex vivo* Tgase2 activity, data are mean normalized mU/ml, ± S.E.M. Statistical significance was determined by Student *t* test and statistically significant differences are shown for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

For mass spectrometry data, data are representative of 3 independent experiments.

For NIT-1 transplant data, survival analysis was performed by Kaplan-Meier method with hypoglycemia as endpoint. Mice that did not become hypoglycemic were censored. Significance was determined by log-rank test. $p < 0.001$

3. Results

3.1. Primary islets from many mouse strains elicit effector responses from the BDC2.5 T cell clone

The diabetogenic murine $CD4^+$ T cell clone BDC2.5 proliferates in response to primary NOD.*scid* islets presented in the context of the NOD-derived MHC class II I-Ag^7 on antigen presentation cells (APC) [79]. To confirm that these T cells also exhibit effector function in response to primary NOD.*scid* islets, the immunogenicity of primary NOD.*scid* islets was

measured by BDC2.5 T cell assay. Briefly, 2×10^4 BDC2.5 T cells, 4×10^5 NOD.*scid* splenocytes acting as APC, and 5×10^3 NOD.*scid* islet cells were combined in 200μl and incubated for 72 hrs (Fig. 1A). T cell effector function was measured by IFNγ secretion. BDC2.5 T cells secreted significant levels of IFNγ in response to primary NOD.*scid* islets presented by NOD-derived APC ($p < 0.01$) (Fig. 1B), confirming the immunogenicity of these islet cells. However, supernatants harvested from cultures of NOD.*scid* islets did not elicit significant levels of IFNγ from BDC2.5 T cells (Supplemental Fig. 1). These data suggest that primary islet immunogenicity is contained in the β cell, not secreted into the assay milieu.

To determine whether BDC2.5 T cells respond to islets from other mouse strains, not only those from the autoimmune-prone NOD background, the immunogenicity of islets from non-autoimmune-prone C57BL/6 mice and BALB/c mice was measured by BDC2.5 T cell assay. BDC2.5 T cells also proliferated [79] and secreted IFNγ (Fig. 1C) in response to primary islets from the C57BlL6 and BALB/c murine backgrounds. These data demonstrate that islets from all backgrounds (both autoimmune prone and non-autoimmune prone) are equally immunogenic and capable of eliciting an immune response from autoreactive diabetogenic T cells. These data suggest that immunogenicity is, at least in part, the consequence of a factor that is common to islet cells from all individuals.

3.2. ER stress increases β cell recognition by BDC2.5 T cells

β cells from all individuals, regardless of genetic predisposition to autoimmunity, exhibit increased ER stress due to their normal secretory physiology [5–14]. Therefore, ER stress is one factor that all islets have in common. Since ER stress has been implicated in autoimmune pathology in a number of diseases such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus [15, 44–51], we hypothesized that ER stress may cause β cell immunogenicity, leading to autoreactive T cell activation. To test this hypothesis, ER stress was induced in primary islets with Thapsigargin (Thaps), which is a widely accepted chemical trigger of ER stress [50, 57]. To measure ER stress induction, the phosphorylation of UPR proteins PERK and eIF2α was assessed by Western blot. As discussed above, the UPR is activated during ER stress [29], and so the phosphorylation of these proteins is a reliable measure of ER stress levels. As expected, incubation with 5 μM Thaps for 1 hr increased phosphorylation of PERK (4.4-fold) and eIF2α (2.7-fold) compared to control-treated cells (Fig. 2A). Importantly, incubation with 5 μM Thaps for 1 hr did not induce apoptosis as determined by trypan blue exclusion (data not shown).

To determine whether Thaps-induced ER stress increased immunogenicity, these cells were analyzed by BDC2.5 T cell assay. Thaps-induced ER stress significantly increased the BDC2.5 T cell response to islets (27.6-fold, $p < 0.001$) (Fig. 2B), confirming a role for ER stress in β cell immunogenicity. Full IFNγ secretion required APC-mediated β cell antigen processing and presentation to BDC2.5 T cells (Fig. 2B). Without APC, any residual Thaps remaining in the β cells after washing did not elicit significant IFNγ.

These data suggest that BDC2.5 T cells recognize a β cell antigen that is altered due to ER stress-modified β cell physiology. Since incubation with 5 μM Thaps for 1 hr did not induce apoptosis, apoptosis is not likely the cause of this increased immunogenicity.

3.3. ER stress-induced immunogenicity is Ca^{2+} dependent

ER stress causes a release of Ca^{2+} stores from the ER [50, 57, 58, 90, 91], which increases the activity of cytosolic Ca^{2+}-dependent enzymes. To confirm the occurrence of this Ca^{2+} flux in Thaps-induced β cell ER stress, the cytosolic Ca^{2+} concentration was monitored with the Ca^{2+} indicator Fluo-4. As is evident from the images, Thaps significantly increased Fluo-4 fluorescence, indicating increased cytosolic Ca^{2+} ($p < 0.01$), while the fluorescence of control-treated cells remained constant (Fig. 3A).

To determine whether ER stress-induced immunogenicity depends on cytosolic Ca^{2+}, primary islets were incubated with 2.5 μM of the Ca^{2+} chelator Bapta-AM or control for 1 hr before incubation with 5 μM Thaps for 1 hr. Chelation of cytosolic Ca^{2+} significantly diminished ER stress-induced immunogenicity by 64% ($p < 0.001$) (Fig. 3B). Importantly, any residual Bapta-AM remaining in the β cells after extensive washing did not directly affect the BDC2.5 T cells, as conditioned media harvested from Bapta-AM-treated islets did not reduce IFNγ secretion from antibody-stimulated T cells (Supplemental Fig. 2). Together, these data demonstrate the crucial role of ER stress-induced Ca^{2+} release in β cell immunogenicity.

3.4. Tgase2 activity is increased during ER stress

Tgase2 is a Ca^{2+}-dependent cytosolic PTM enzyme that is associated with breaks in peripheral tolerance in several autoimmune disorders [44, 50, 51, 66, 67]. Also, recent findings suggest that Tgase2-mediated PTM increase the immunogenicity of synthetic peptides of known T1D autoantigens [69, 70, 72, 74]. But whether Tgase2 is relevant to whole β cell immunogenicity, or how Tgase2 becomes activated in β cells, remains unknown.

To determine whether Tgase2 is active in β cells undergoing ER stress, Tgase2 activity was measured in primary murine islets. Islets incubated with 5 μM Thaps for 1 hr contained 1.8-fold higher Tgase2 activity than control islet cells ($p < 0.01$) (Fig. 4). These data demonstrate that Tgase2 activity corresponds with high ER stress and immunogenicity in islets (Fig. 2) and suggest a possible role for Tgase2 in ER stress-induced immunogenicity.

3.5. Tgase2-mediated PTM increases immunogenicity of CHgA

Treatment of the CHgA-derived peptide WE14 with Tgase2 increased the BDC2.5 response to the peptide [69]. However, WE14 is a nontraditional T cell antigen since it contains only 14 amino acids and is predicted to bind MHC molecules outside the binding groove [68]. We studied a peptide, $CHgA_{351–370}$, that contains WE14 but is 20 amino acids long and includes traditional MHC binding motifs (Fig. 5A).

We first sought to determine whether Tgase2 would modify $CHgA_{351–370}$. Tgase2 modifies substrates through two mechanisms: the formation of ε(γ-glutamyl) isopeptide bonds between glutamine and lysine residues that crosslink and aggregate proteins, and through the deamidation of glutamine to glutamic acid [63]. The crosslinking of $CHgA_{351–370}$ was measured by SDS-PAGE analysis. Indeed, Tgase2 modified $CHgA_{351–370}$ by crosslinking and aggregation (Fig. 5B). Importantly, this modification was Ca^{2+}-dependent, since

aggregation did not occur when Ca^{2+} was omitted or chelated (Fig. 5B). In addition, recombinant full length CHgA was incubated with Tgase2 and analyzed by mass spectrometry. Mass spectrometry confirmed that Tgase2 modified CHgA by deamidation (Supplemental Fig. 3 and Supplemental Table 1). Tgase2-mediated deamidation was found throughout the protein, including within the $CHgA_{351–370}$ region. Together, these data demonstrate that Tgase2 is able to modify $CHgA_{351–370}$ by both crosslinking and deamidation.

To determine whether Tgase2 modification of $CHgA_{351–370}$ increases its immunogenicity, the native and modified peptides were analyzed by BDC2.5 T cell assay. Tgase2-modified $CHgA_{351–370}$ elicited significantly higher (27.9-fold) BDC2.5 T cell responses than untreated $CHgA_{351–370}$ ($p < 0.05$) (Fig. 5C). These data support the findings of Delong *et al.* [69] and demonstrate that Tgase2-mediated PTM increases immunogenicity of the more traditional $CHgA_{351–370}$ peptide.

3.6. ER stress in NIT-1 insulinomas increases immunogenicity through Ca^{2+}-dependent PTM

Thus far, the experiments described were performed with primary murine islets. These primary cells are the ideal cell type for elucidating the cellular mechanisms by which β cell immunogenicity occurs. However, primary murine islet cells are not ideal for experiments requiring longer *in vitro* culture, or for experiments requiring high cell numbers. For such experiments, we surmised that NIT-1 insulinomas, which are derived from NOD β cell tumors [92], would be better suited both for longer culture and for the collection of greater cell numbers. Although other studies have demonstrated that insulinomas are a useful model of β cell processes [93], we sought to confirm in NIT-1 cells the observations made in primary islet cells.

First, to determine whether ER stress increases NIT-1 immunogenicity, NIT-1 cells were incubated with 5 μM Thaps for 1 hr. Thaps increased phosphorylation of UPR proteins PERK (7.8-fold) and eIF2α (3.9-fold) compared to control-treated cells (Fig. 6A). As in primary islet cells, this Thaps-induced ER stress significantly increased the BDC2.5 T cell response to NIT-1 insulinomas (1451.1-fold, $p < 0.001$) (Fig. 6B). In addition, the response to Thaps-treated NIT-1 cells also required APC-mediated β cell antigen processing and presentation to BDC2.5 T cells (Fig. 6B).

Second, to assess whether Ca^{2+} stores are released from the ER into the cytosol in NIT-1 cells undergoing ER stress, the cytosolic Ca^{2+} concentration was monitored with Fluo-4. As in primary islet cells, Thaps significantly increased Fluo-4 fluorescence ($p < 0.01$), indicating a significant increase in cytosolic Ca^{2+} concentrations, while the fluorescence of control-treated cells remained constant (Fig. 6C). In addition, pre-incubation with Bapta-AM significantly reduced Thaps-induced immunogenicity in NIT-1 cells (27%, $p < 0.01$) (Fig. 6D) without directly affecting the BDC2.5 T cells (Supplemental Fig. 2), demonstrating that, as in primary islets, increased cytosolic Ca^{2+} is important for ER stress-induced NIT-1 immunogenicity.

Finally, Tgase2 activity was measured in NIT-1 insulinomas. NIT-1 cells incubated with 5 μM Thaps for 1 hr contained 1.7-fold higher Tgase2 activity than control NIT-1 cells ($p < 0.05$) (Fig. 6E). These data confirm that ER stress increases the activity of this cytosolic PTM enzyme in NIT-1 cells.

Taken together, these data demonstrate that ER stress contributes to NIT-1 immunogenicity through Ca^{2+}- and Tgase2-dependent mechanisms. These data confirm that, in these limited respects, NIT-1 cells are sufficiently similar to primary murine islets to be used for additional mechanistic studies.

3.7. Tgase2 contributes to ER stress-induced β cell immunogenicity

Tgase2 activity corresponds to heightened ER stress and β cell immunogenicity (Fig. 4 and 6E). To confirm the importance of Tgase2 in ER stress-induced immunogenicity, NIT-1 cells were stably transduced with either control shRNA or Tgase2-targeting shRNA. Tgase2-targeting shRNA reduced Tgase2 expression by 46% compared to control shRNA ($p < 0.01$) (Fig. 7A). Furthermore, Tgase2-deficient NIT-1 cells incubated with Thaps elicited significantly lower BDC2.5 T cell responses compared to control cells incubated with Thaps (59%, $p < 0.05$) (Fig. 7B). Therefore, even a modest decrease in Tgase2 expression significantly impacted ER stress-induced β cell immunogenicity. These data confirm the importance of Tgase2 during ER stress-induced immunogenicity, and suggest that Tgase2-mediated PTM of β cell proteins, like CHgA, contributes to the recognition of β cells by diabetogenic T cells.

3.8. Physiological cues in vivo increase β cell ER stress and immunogenicity

Islet β cells undergo high ER stress due to their normal physiology [5–14]. Indeed, primary islets exhibit high ER stress and elicit strong BDC2.5 T cell responses immediately after harvest (Fig. 1B and C) [79]. We therefore hypothesized that the normal physiology of β cells *in vivo* is sufficient to generate ER stress-dependent PTM and cause β cell recognition by autoreactive T cells. To test this hypothesis, NIT-1 cells, which exhibit low ER stress and immunogenicity (Fig. 6A and B), were transplanted under the kidney capsule of NOD.*scid* recipients and thus exposed to physiological triggers of ER stress (such as changes in glucose concentration). Similar to previous findings with mice injected with NIT-1 cells [94], NIT-1 recipient mice had 4.9-fold higher serum insulin than control mice ($p < 0.01$) (Fig. 8A) and became hypoglycemic (blood glucose levels ≤ 40 mg/dl) beginning at day 16 post-transplant ($p < 0.001$) (Fig. 8B). These data show that, unlike NIT-1 cells that do not secrete insulin in response to static glucose concentrations in culture (Supplemental Fig. 4), NIT-1 cells exposed to physiological cues (including, but limited to, dynamic changes in blood glucose levels) regain the ability to secrete insulin. Importantly, the grafted NIT-1 cells did not increase in number during the course of these experiments (Supplemental Fig. 5). This indicates that the increased insulin secretion was due to physiological changes within the NIT-1 cells, rather than proliferation of the transplanted cells.

At the onset of hypoglycemia, transplanted NIT-1 cells were explanted from the kidney and analyzed by Western blot. Since explanted NIT-1 cells exhibit increased insulin secretory capacity, we surmised that these cells, like primary islets, would experience heightened ER

stress. Indeed, compared to cultured NIT-1 cells, explanted NIT-1 cells showed greater phosphorylation of UPR proteins PERK (136.4-fold) and eIF2α (292.1-fold), confirming higher ER stress (Fig. 8C). This heightened ER stress further supports the hypothesis that physiological cues increase ER stress in β cells and β cell insulinomas.

Tgase2 activity correlates with ER stress (Fig. 4 and 6E) and is necessary for ER stress-induced β cell immunogenicity (Fig. 7B). We hypothesized that ER stress triggered by physiological cues would increase Tgase2 activity. To test this hypothesis, Tgase2 activity was measured in explanted and cultured NIT-1 cells. Explanted NIT-1 cells exhibited 42.0-fold higher Tgase2 activity than cultured NIT-1 cells ($p < 0.001$, Fig. 8D). These data confirm that physiological ER stress increases Tgase2 activity in β cells.

Finally, we hypothesized that increased ER stress and Tgase2 activity would increase immunogenicity in NIT-1 cells exposed to physiological cues. Explanted and cultured NIT-1 cells were analyzed by BDC2.5 T cell assay. As observed in Fig. 6B, cultured NIT-1 cells did not elicit IFNγ secretion from BDC2.5 T cells (Fig 8E). As a control, we confirmed that that these cultured NIT-1 cells did not directly inhibit BDC2.5 T cell activation, as conditioned media harvested from cultured NIT-1 cells did not reduce IFNγ secretion from antibody-stimulated T cells (Supplemental Fig. 6). However, explanted NIT-1 cells elicited 33.0-fold higher BDC2.5 T cell responses than cultured NIT-1 cells ($p < 0.05$) (Fig. 8E). These data demonstrate that physiological ER stress is sufficient to render β cells immunogenic.

Importantly, these observations were only made when NIT-1 cells were explanted at the onset of hypoglycemia. When NIT-1 cells were explanted after only 2 days *in vivo* (a time point at which NIT-1 recipient mice have similar serum insulin levels to control mice and are not hypoglycemic (Fig. 8A and B)), neither ER stress nor immunogenicity was different from that in cultured NIT-1 cells (Supplemental Fig. 7). These data suggest that the increased ER stress and immunogenicity observed in explanted NIT-1 cells (Fig. 8C and E) was not due to technical manipulation of transplantation and explantation. Rather, these data demonstrate that increased ER stress, Tgase2 activity, and immunogenicity, occur as the NIT-1 cells begin to secrete insulin in response to normal physiological cues.

Together, the data presented in Fig. 8 demonstrate that NIT-1 cells with low ER stress and low immunogenicity (Fig. 6A and B) can secrete insulin when exposed to similar physiological cues as those to which primary islets are exposed *in vivo.* Furthermore, insulin secretion is accompanied by high ER stress, high Tgase2, and high immunogenicity. These data implicate the normal physiology of the β cell in ER stress- and PTM-dependent β cell immunogenicity. Therefore, normal β cell physiology, and the ER stress inherent to this normal physiology, may predispose β cells to immunogenicity and immune-mediated destruction. In the context of genetic susceptibility to autoimmunity and the presence of autoreactive T cells in the periphery, these β cell processes lead to activation of the autoreactive immune response and T1D.

4. Discussion

While protein PTM has been implicated in pathology in other diseases [15, 44–51], this area of research has not been well explored in T1D [71]. Although recent literature shows that PTM of synthetic murine and human β cell peptides increases their recognition by autoreactive T cells [69–75], these studies did not explore the physiological relevance of cellular mechanisms that cause PTM. Here, for the first time, we demonstrate that β cell ER stress, such as that induced by physiological triggers, leads to PTM-dependent β cell recognition by diabetogenic T cells.

Primary murine islets analyzed immediately after harvest, but not islet-conditioned media, are highly immunogenic (Fig. 1B and C, Supplemental Fig. 1). In fact, primary islets from both autoimmune prone and non-autoimmune prone backgrounds are highly immunogenic (Fig. 1C). These data show that immunogenicity of some β cell antigens (such as CHgA) is not unique to islets from the autoimmune prone NOD mouse. Rather, these data suggest that islets from all mouse strains contain a common factor that may play an important role in generating β cell immunogenicity. β cells, as professional secretory cells, contain a more fully-developed ER than nonsecretory cells and are consequently more susceptible to ER stress due to their normal physiology [5–14]. Rises in blood glucose concentrations stimulate significant increases in insulin production by β cells [15] that, in turn, leads to heightened ER stress [16]. We hypothesized that normal β cell physiology, which includes heightened ER stress [5–16], contributes to β cell recognition by immune cells and breaks in peripheral tolerance.

ER stress has been linked to T1D through β cell malfunction and apoptosis [95–97]. However, β cell apoptosis may not be the only mechanism by which ER stress contributes to T1D. In other diseases, ER stress increases the generation of abnormally modified proteins that act as neo-antigens that, if presented by APC to autoreactive T cells, break peripheral tolerance [44, 50, 51]. Here, we show that β cell ER stress significantly increased BDC2.5 effector responses (Figs. 2B, 6B, and 8E). These data provide a novel link between β cell ER stress and β cell recognition by immune cells, namely that ER stress predisposes β cells to autoreactive T cell recognition.

ER stress releases Ca^{2+} from the ER lumen. In β cells, Ca^{2+} release occurs with insulin production and secretion [40–43]. As expected, Thaps-triggered ER stress in β cells significantly increased cytosolic Ca^{2+} (Fig. 3A and 6C). Higher Ca^{2+} concentrations increase the activity of Ca^{2+}-dependent PTM enzymes that play important roles in cells under stress. For instance, Tgase2 modifies caspase 3 to delay apoptosis during stress [64]. However, Tgase2 has also been implicated in modifying other proteins to produce neo-antigens in several autoimmune diseases [44, 50, 51, 66]. Recently, Tgase2 has been implicated in the modification of T1D peptide antigens [72, 74] including WE14 [69, 72] (Fig. 5B, Supplemental Fig. 3, Supplemental Table 1). Together, these findings support the hypothesis that BDC2.5 T cells optimally recognize CHgA after PTM. However, these data alone do not address the physiological mechanisms that lead to CHgA PTM in β cells. Here, we show that ER stress-induced Ca^{2+} flux into the cytosol is necessary for ER stress-induced β cell immunogenicity (Fig. 3B and 6D). Also, Ca^{2+}-dependent Tgase2 activity is

significantly higher in β cells under ER stress (Fig. 4 and 6E), and is crucial for ER stress-induced immunogenicity (Fig. 7B). These data demonstrate for the first time the importance of Tgase2-mediated PTM in eliciting heightened effector responses from BDC2.5 T cells in response to whole β cells, and provide a mechanism to explain the cellular events that lead to Tgase2 activation and the Tgase2-mediated PTM of β cell proteins described by other groups [69, 70, 72, 74].

Finally, primary islet cells exhibit high ER stress [5–16] and are immunogenic immediately after harvest (Fig. 1B and C). We therefore hypothesized that normal physiological cues *in vivo* sufficiently raise ER stress to facilitate Ca^{2+}-dependent PTM and immunogenicity. This hypothesis was supported by experiments in which non-immunogenic NIT-1 insulinoma cells were transplanted in NOD.*scid* mice and exposed to normal physiological cues. These NIT-1 cells regained the ability to secrete insulin (Fig. 8A, Supplemental Fig. 4) and the recipient mice suffered hypoglycemia (Fig. 8B). When examined *ex vivo*, NIT-1 cells exposed to normal physiological cues exhibited significantly higher ER stress and Tgase2 activity (Fig. 8C and D), both of which our studies showed to be necessary for β cell immunogenicity (Fig. 2, 4, 6A, 6B, 6E, and 7B). As with Thaps-induced ER stress, the physiologically heightened ER stress and Tgase2 activity led to increased responses from BDC2.5 T cells (Fig. 8E).

Interestingly, the BDC2.5 effector responses elicited by physiologically-stressed primary islets (Fig. 1B and C) or NIT-1 cells (Fig. 8E), were much lower than those elicited by Thaps-stressed β cells (Fig. 2B, 6B), demonstrating important differences between the two types of ER stress triggers. Thaps induces very strong and acute ER stress with strong Ca^{2+} fluxes, which cause apoptosis if exposure is prolonged beyond the 1 hr time point used in this study [98, 99]. In contrast, the physiological triggers of ER stress *in vivo* are less potent and induce less acute ER stress with smaller Ca^{2+} fluxes. This lower ER stress would be important for β cell survival and function *in vivo*, because lower stress would allow β cells *in vivo* to activate UPR, return to homeostasis, and avoid apoptosis. Although physiologically- and chemically-induced ER stress elicits different BDC2.5 T cell responses, these data still demonstrate that normal β cell physiology is sufficient to render β cells immunogenic through Ca^{2+}-dependent PTM.

While identifying β cell autoantigens is important, elucidating mechanisms by which β cell proteins become immunogenic is crucial to identify therapeutic targets to reduce β cell immunogenicity and prevent immune-mediated β cell destruction in T1D. Thus, the aim of these studies was not to identify novel immunogenic β cell proteins or peptides, but rather to understand how and why β cells become immunogenic and come to be recognized by autoreactive T cells. Our data show that normal islet physiology sufficiently increases β cell ER stress and leads to β cell immunogenicity through PTM. Because islets are particularly susceptible to ER stress [5–16], these processes are likely occurring in all β cells in every individual, regardless of whether autoimmune pathology is occurring. Indeed, heightened ER stress is detected in islets from nonautoimmune mice as early as 3 weeks of age [16]. In addition, islets from autoimmune- and nonautoimmune-prone mouse strains elicit equal BDC2.5 T cell responses (Fig. 1C). Together, these data support the hypothesis that inherent β cell ER stress may provide the necessary trigger for protein modification [100] and lead to

immunogenicity in all individuals, even in the absence of pancreatic inflammation. Therefore, T1D onset may not be determined by whether these ER stress-induced PTM are generated, but perhaps by genetic predisposition to autoimmunity. Individuals not genetically predisposed to autoimmunity would either not have autoreactive T cells in the periphery, or would have a higher frequency of circulating regulatory T cells. The presentation of abnormally-modified β cell proteins by APC would fail to elicit autoimmune β cell destruction. However, patients genetically predisposed to autoimmunity do have autoreactive T cells in the periphery due to failures in central and peripheral tolerance. In these individuals, the presentation of abnormally-modified β cell proteins may activate these autoreactive T cells, and lead to pathology.

In addition to inherent ER stress, exposure of β cells to additional triggers of ER stress (such as viral infection, exposure to chemicals or reactive oxygen species, dysglycemia, and inflammation) may increase ER stress above physiological levels, further increasing the activity of Ca^{2+}-dependent PTM enzymes and the subsequent modification of β cell proteins. This could explain how environmental triggers lead to the progression of T1D [100, 101]. Once autoreactive immune cells invade the islet, the inflammatory environment likely further increases β cell ER stress, leading to modification of additional β cell proteins [75]. Thus, once immune pathology in the islet begins, other β cell antigens may undergo PTM, which may contribute to epitope spreading.

Although these studies focused on recognition of one autoantigen (CHgA) by one T cell clone (BDC2.5), the mechanisms by which tolerance is broken by ER stress would be easily applicable to other autoantigens in T1D and other autoimmune diseases [15, 44–51, 100]. For instance, ER stress may cause PTM of other endogenous proteins, or may facilitate recognition of non-modified proteins by autoreactive T cells by changing β cell physiology.

5. Conclusion

In summary, we have demonstrated two important novel findings. It is known that modification of β cell peptides (such as WE14) by PTM enzymes (such as Tgase2) increases the response of autoreactive T cells [69– 75]. Here, we show that these PTM enzymes are activated and contribute to β cell immunogenicity when ER stress releases Ca^{2+} into the cytosol. Second, we show that β cell ER stress and the resulting PTM-dependent immunogenicity occur due to normal β cell physiology. Therefore, the normal physiology and function of β cell insulin secretion is sufficient to generate immunogenicity and activate diabetogenic T cells. We therefore propose a model (Fig. 9) in which ER stress constantly leads to abnormal PTM of β cell proteins as a result of normal physiology. This process may be exacerbated when β cells are exposed to additional triggers of ER stress, such as the environmental triggers thought to accelerate T1D and the inflammatory environment created by islet-infiltrating immune cells. In patients with genetic susceptibility to autoimmunity, presentation of modified β cell proteins by APC to β cell-specific T cells causes a break in peripheral tolerance and, ultimately, to autoimmune T1D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- β cells undergo high levels of ER stress due to normal physiology
- ER stress increases activity of cytosolic Ca^{2+}-dependent PTM enzymes
- PTM enzyme activity is necessary for full β cell immunogenicity
- Physiological ER stress predisposes β cells to immune-mediated destruction in T1D

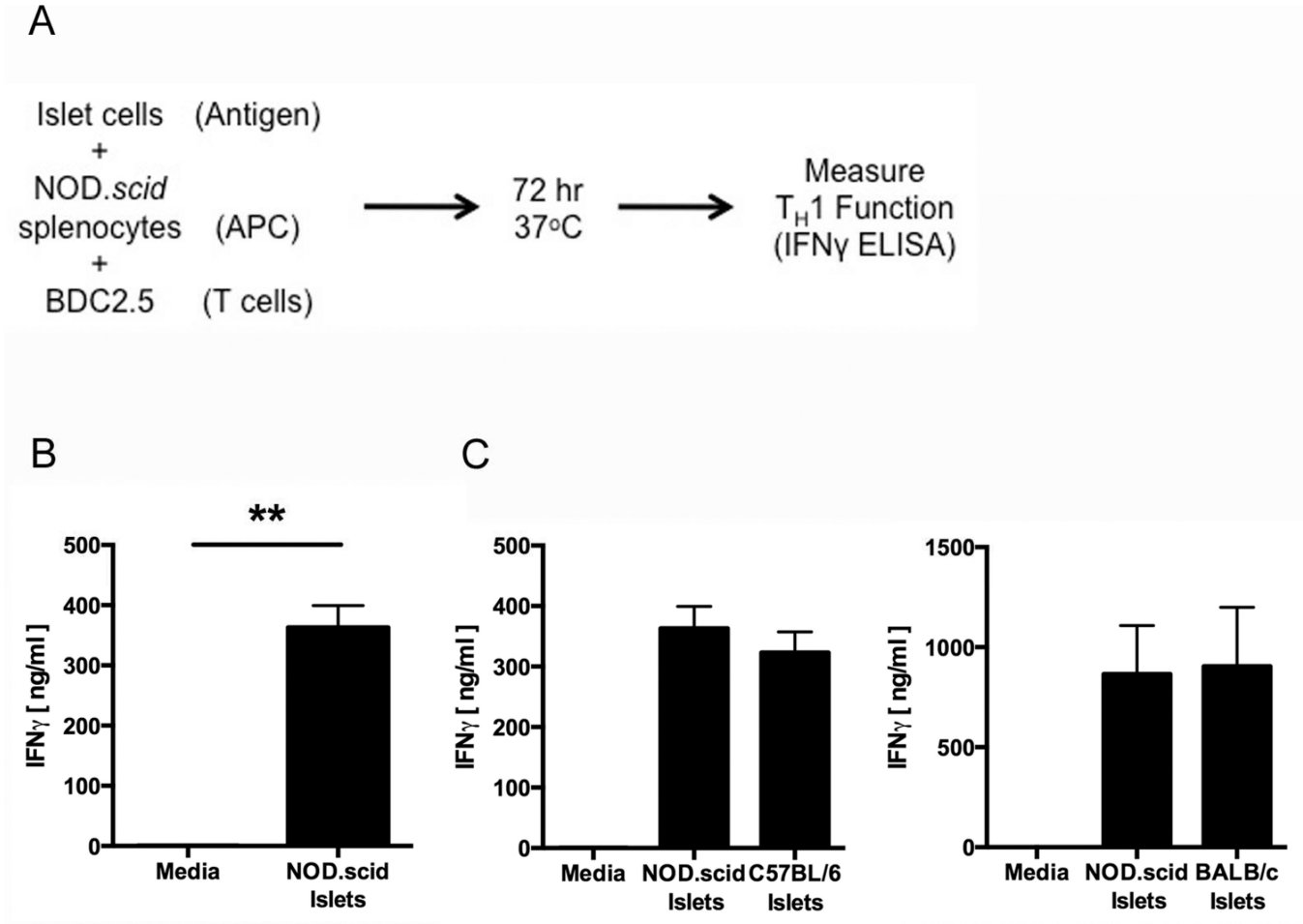

Fig. 1. Primary islets from many mouse strains elicit effector responses from BDC2.5 T cell clones

(A) Schematic of the BDC2.5 T cell assay protocol. Antigen presenting cell (APC). (B) The immunogenicity of NOD.*scid* islet cells was measured by BDC2.5 T cell assay. Data are mean IFNγ secretion ± SEM. ** $p < 0.01$. (C) The immunogenicity of NOD.*scid* and C57BL/6 (left) or BALB/c (right) islet cells was measured by BDC2.5 T cell assay. Data are mean IFNγ secretion ± SEM.

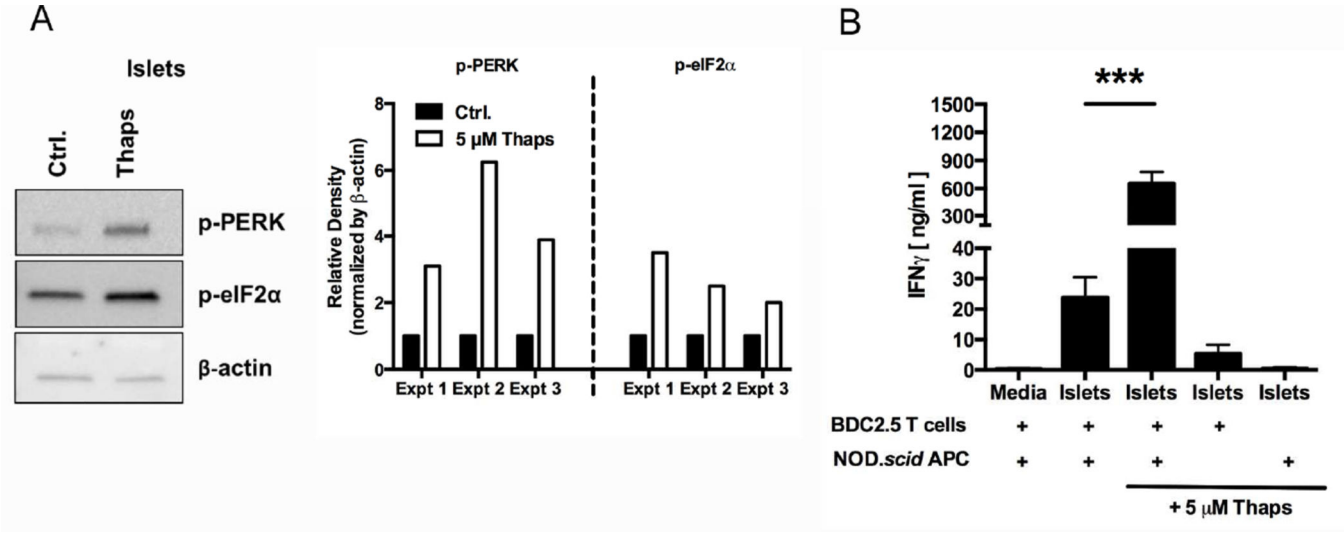

Fig. 2. β cell ER stress increases recognition by diabetogenic T cells

(A) Primary NOD.*scid* islets were incubated with 5 μM Thaps or control (Ctrl.) for 1 hr, washed extensively, and dispersed. Cell lysates were analyzed for the phosphorylation of UPR proteins PERK and eIF2α. Data are representative of 3 independent experiments. Densitometry data are phosphorylation levels normalized by β-actin and relative to that in control (Ctrl.) treated cells. (B) The immunogenicity of NOD.*scid* islet cells treated with 5 μM Thaps or control for 1 hr was measured by BDC2.5 T cell assay. Either APC or T cells were omitted from assay to demonstrate the necessity of both cell types for full IFNγ secretion. Data are mean IFNγ secretion ± SEM. *** $p < 0.001$.

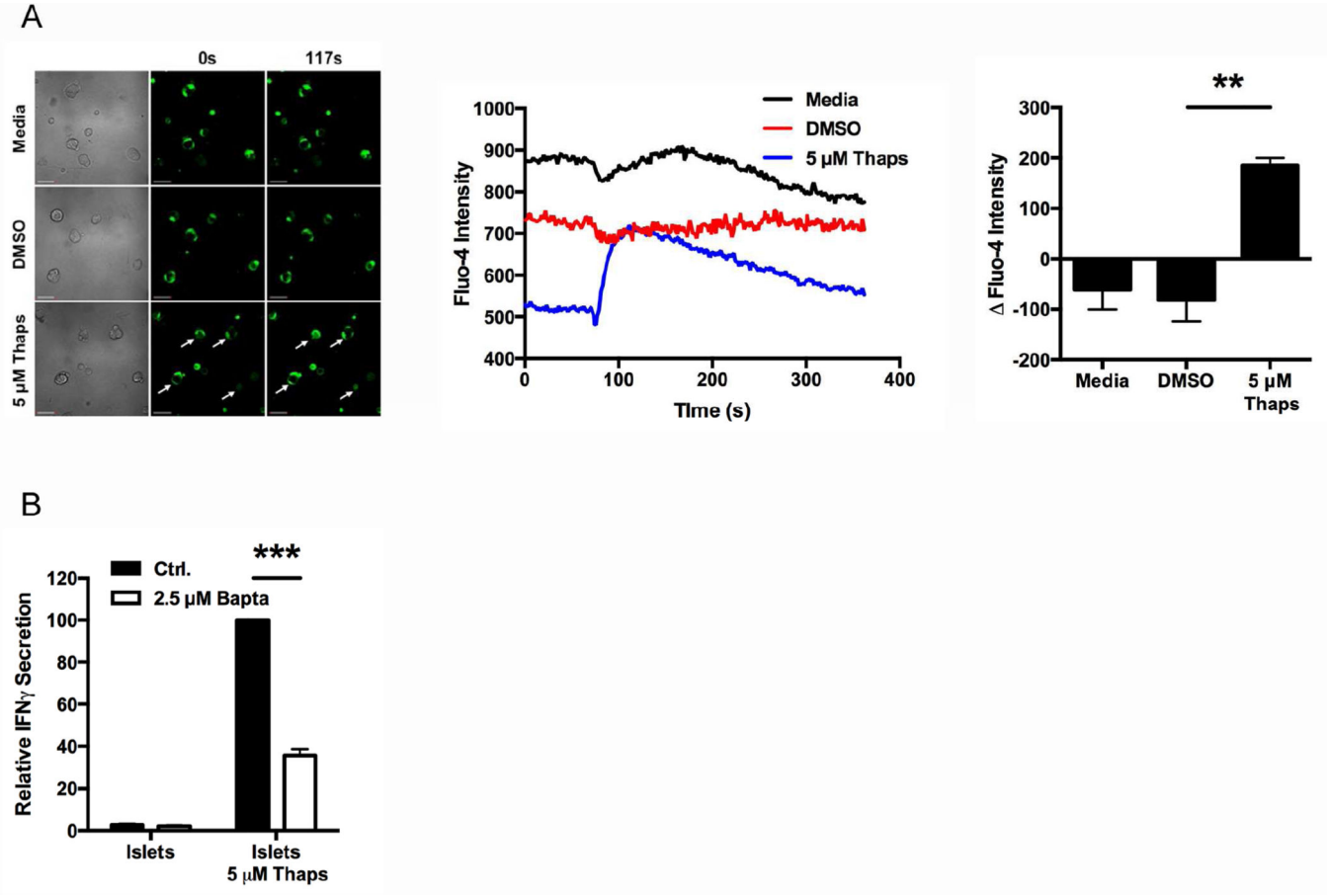

Fig. 3. ER stress-induced immunogenicity is Ca^{2+} dependent

(A) Dispersed NOD.*scid* islet cells were labeled with Fluo-4 and analyzed by live imaging with an Olympus Fluoview FV1000 microscope for 350 sec at room temperature. At 70 sec, the cells were exposed to 5 μM Thaps or controls. Scale bars, 50 μm. Arrows indicate cells with greater Fluo-4 intensity after exposure to Thaps. Line graphs represent the intensity of Fluo-4 in each sample over time. Data are representative of 3 independent experiments. Quantified data are change in Fluo-4 intensity from baseline to peak, with mean ± SEM. ** $p < 0.01$. (B) The immunogenicity of primary NOD.*scid* islet cells treated with 2.5 μM Bapta or control (Ctrl.) prior to incubation with 5 μM Thaps or control was measured by BDC2.5 T cell assay. Data are relative IFNγ secretion compared to 5 μM Thaps, with mean ± SEM. *** $p < 0.001$

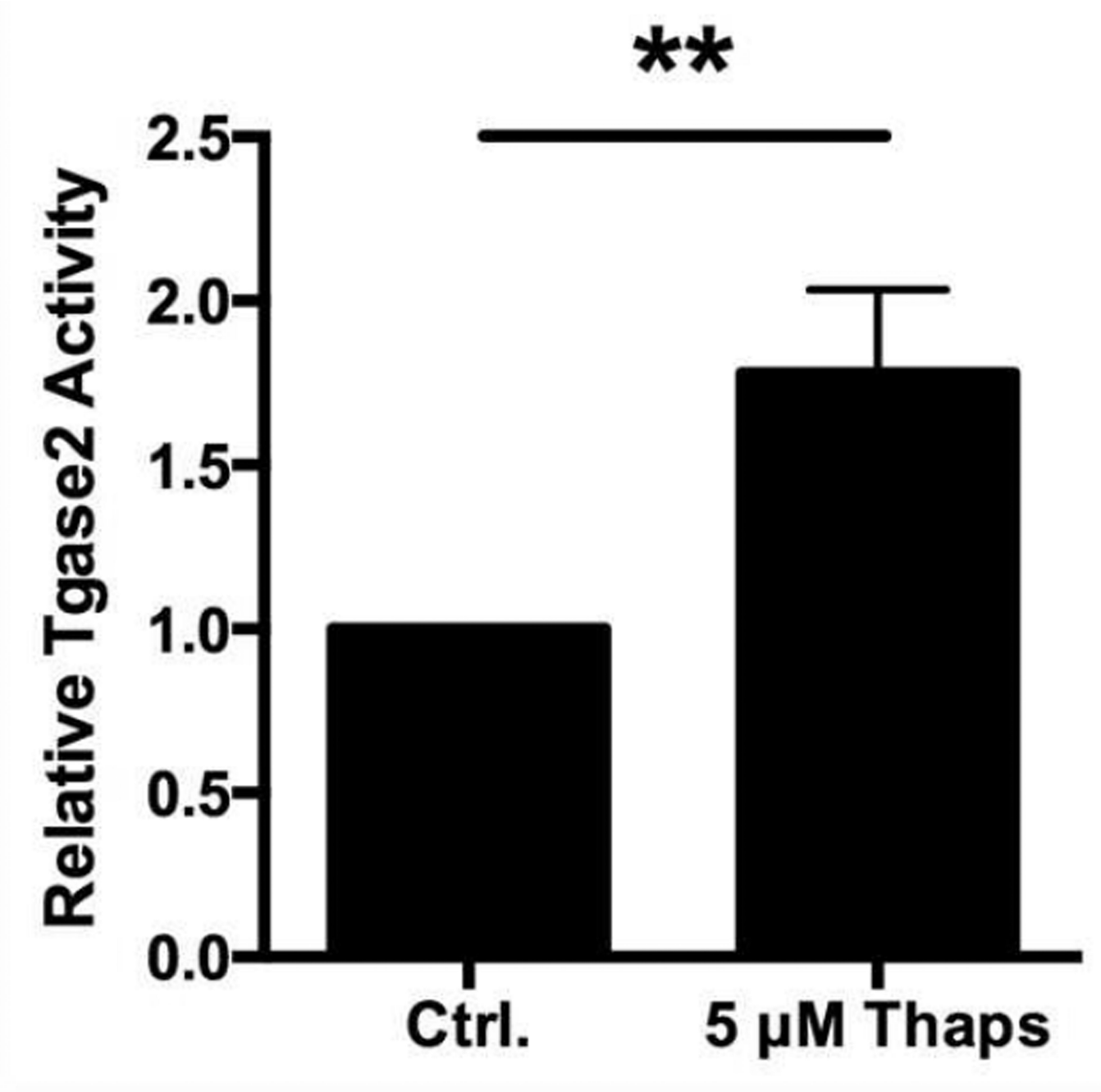

Fig. 4. β cell ER stress increases the activity of Tgase2

Primary NOD.*scid* islets were incubated with 5 μM Thaps or control (Ctrl.) for 1 hr. The cells were lysed and Tgase2 activity was measured by microtiter plate assay. Data are relative Tgase2 activity compared to control (Ctrl.) islet cells, with mean ± SEM. ** $p < 0.01$.

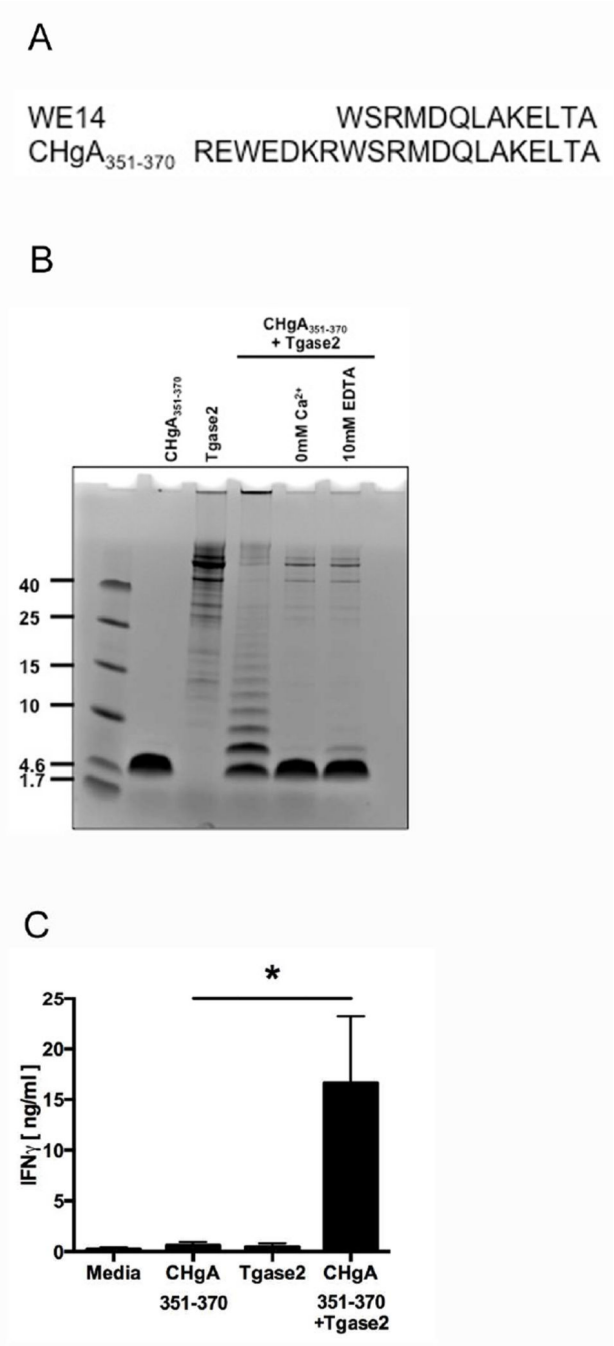

Fig. 5. Modification of CHgA by Tgase2 increases immunogenicity

(A) Alignment of the sequences of WE14 and $CHgA_{351–370}$. (B) Synthetic $CHgA_{351–370}$ was incubated with purified Tgase2 at 37°C for 3 hr. To confirm the dependence of these reactions on Ca^{2+}, the reactions were repeated without $CaCl_2$ (0 mM Ca^{2+}) or with increased EDTA (10 mM EDTA). The reaction products were analyzed by SDS-PAGE. Lane 1: $CHgA_{351–370}$ alone; Lane 2: Tgase2 alone; Lane 3: $CHgA_{351–370}$ incubated with Tgase2; Lane 4: $CHgA_{351–370}$ incubated with Tgase2 in 0 mM Ca^{2+}; Lane 5, $CHgA_{351–370}$ incubated with Tgase2 in 10 mM EDTA. (C) The immunogenicity of $CHgA_{351–370}$

incubated with Tgase2 was measured by BDC2.5 T cell assay. Data are mean IFNγ secretion ± SEM. * $p < 0.05$.

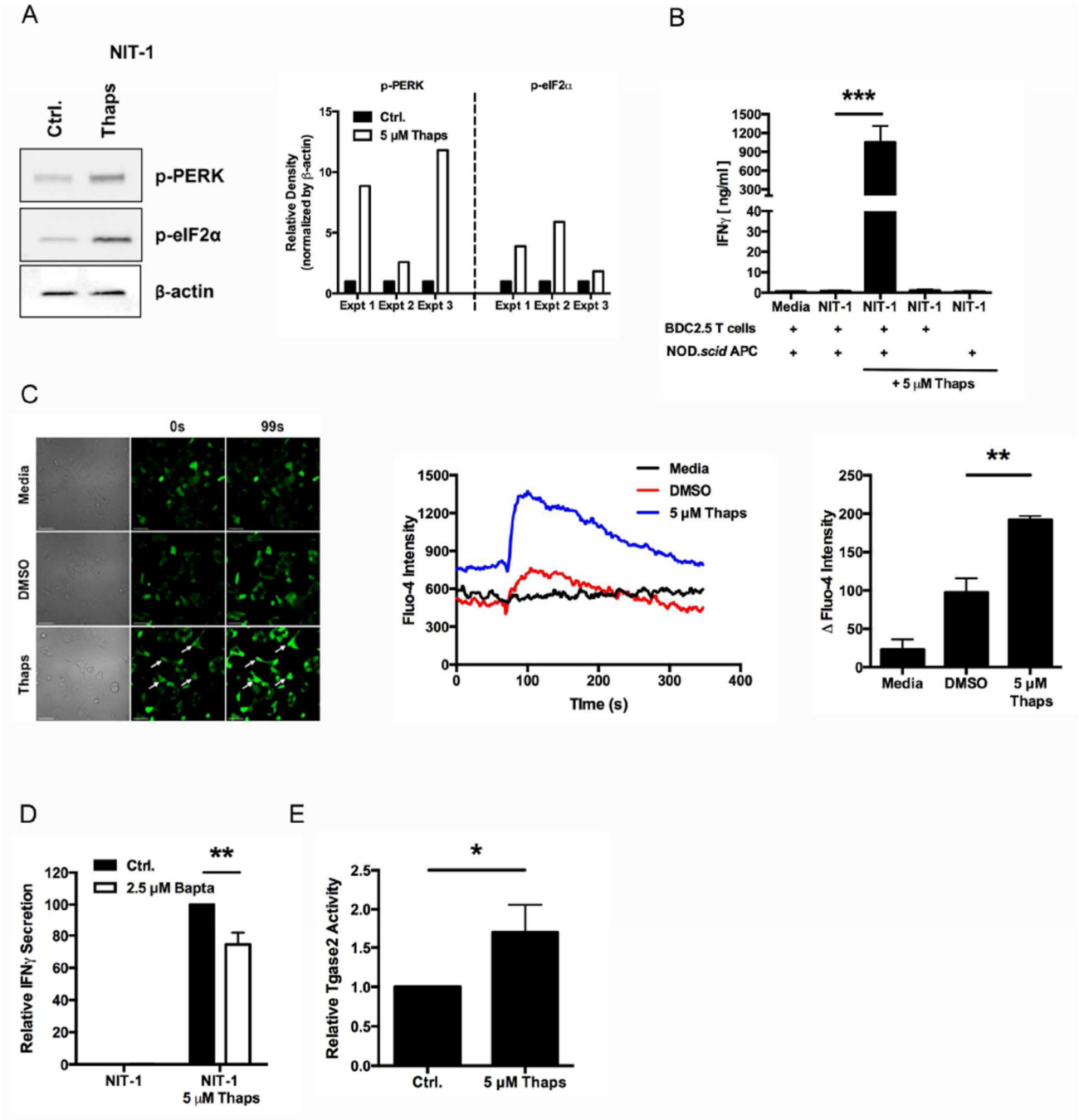

Fig. 6. ER stress in NIT-1 insulinomas increases immunogenicity in a Ca^{2+}-dependent manner

(A) NIT-1 insulinoma cells were incubated with 5 μM Thaps or control (Ctrl.) for 1 hr and washed extensively. Cell lysates were analyzed for the phosphorylation of UPR proteins PERK and eIF2α. Data are representative of 3 independent experiments. Densitometry data are phosphorylation levels normalized by β-actin and relative to that in control (Ctrl.) treated cells. (B) The immunogenicity of NIT-1 cells treated with 5 μM Thaps or control was measured by BDC2.5 T cell assay. Either APC or T cells were omitted from assay to demonstrate the necessity of both cell types for full IFNγ secretion. Data are mean IFNγ

secretion ± SEM. *** $p < 0.001$. (C) NIT-1 cells were labeled with Fluo-4 and analyzed by live imaging with an Olympus Fluoview FV1000 microscope for 350 sec at room temperature. At 70 sec, the cells were exposed to 5 μM Thaps or controls. Scale bars, 50 μm. Arrows indicate cells with greater Fluo-4 intensity after exposure to Thaps. Line graphs represent intensity of Fluo-4 in each sample over time. Data are representative of 3 independent experiments. Quantified data are change in Fluo-4 intensity from baseline to peak, with mean ± SEM. ** $p < 0.01$. (D) The immunogenicity of NIT-1 cells treated with 2.5 μM Bapta or control (Ctrl.) prior to incubation with 5 μM Thaps or control was measured by BDC2.5 T cell assay. Data are relative IFNγ secretion compared to 5 μM Thaps, with mean ± SEM. ** $p < 0.01$ (E) NIT-1 cells were incubated with 5 μM Thaps or control (Ctrl.) for 1 hr. The cells were lysed and Tgase2 activity was measured by microtiter plate assay. Data are relative Tgase2 activity compared to control (Ctrl.) NIT-1 cells, with mean ± SEM. * $p < 0.05$.

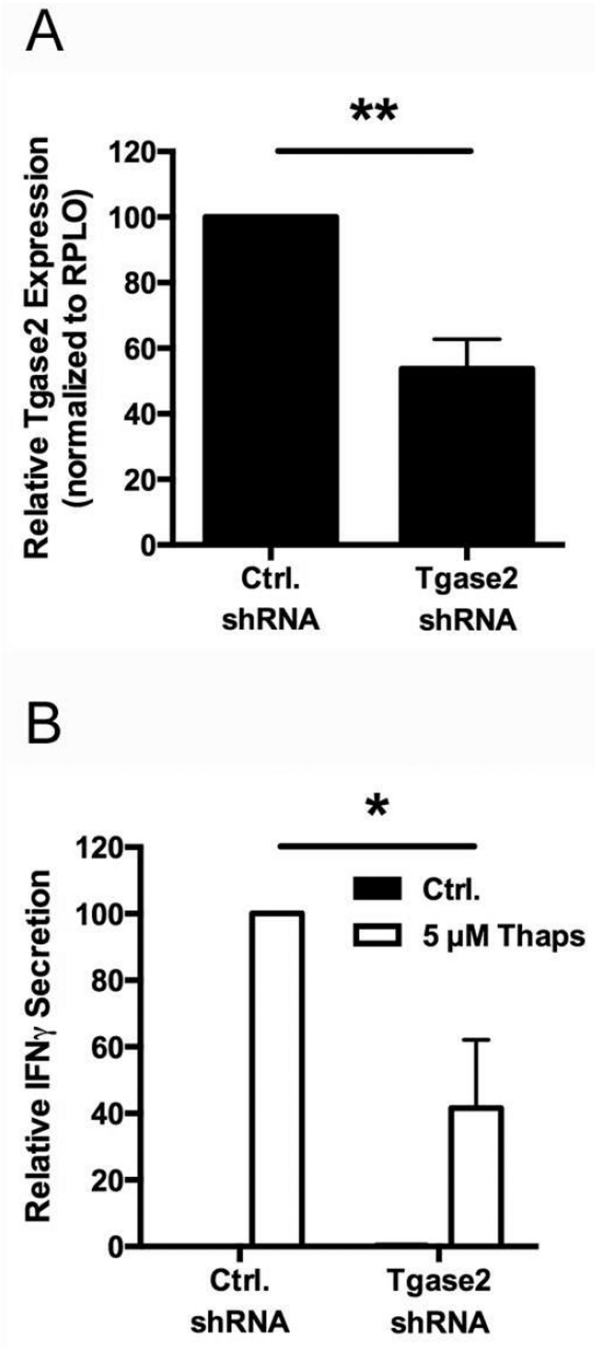

Fig. 7. Tgase2 activity contributes to β cell immunogenicity

(A) NIT-1 cells were transduced with control shRNA (Ctrl. shRNA) or Tgase2-targeting shRNA (Tgase2 shRNA). Tgase2 expression was measured by qRT-PCR. Data are relative Tgase2 expression compared to cells transduced with control shRNA, with mean ± SEM. ** $p < 0.01$. (B) The immunogenicity of NIT-1 cells transduced with control shRNA (Ctrl. shRNA) or Tgase2-targeting shRNA (Tgase2 shRNA) prior to incubation with 5 μM Thaps or control (Ctrl.) for 1 hr was measured by BDC2.5 T cell assay. Data are relative IFNγ

secretion compared to NIT-1 cells transduced with Ctrl. shRNA and incubated with 5 μM Thaps, with mean ± SEM. * $p < 0.05$.

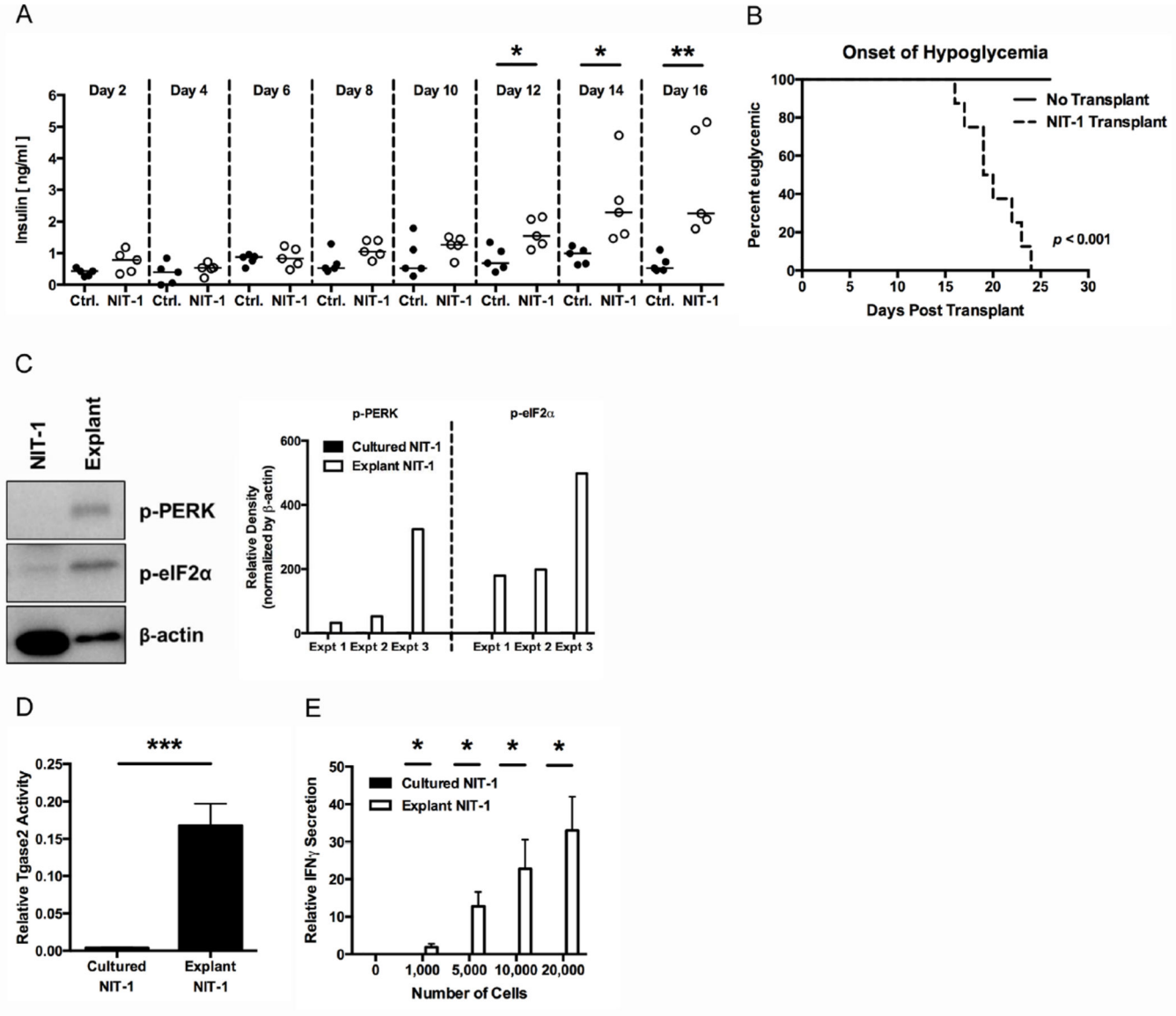

Fig. 8. Physiological cues increase β cell ER stress and immunogenicity

Non-immunogenic NIT-1 cells ($2–5\times10^6$) were transplanted under the kidney capsule of NOD.*scid* mice. (A) Serum insulin levels in control mice (Ctrl.) or mice transplanted with NIT-1 cells (NIT-1) were measured by ELISA. Medians are indicated with a line. Data are representative of 4 independent experiments. * $p < 0.05$, ** $p < 0.01$. (B) The onset of hypoglycemia in control mice (No Transplant) or mice transplanted with NIT-1 cells (NIT-1 Transplant) was monitored over time. $p < 0.001$ (C) At the onset of hypoglycemia, the mice were sacrificed and the NIT-1 cells were explanted. Cell lysates of cultured NIT-1 cells (NIT-1) or explanted NIT-1 cells (Explant) were analyzed for the phosphorylation of UPR proteins PERK and eIF2α. Data are representative of 3 independent experiments. Densitometry data are phosphorylation levels normalized by β-actin and relative to that in cultured NIT-1 cells. (D) Cultured NIT-1 cells or explanted NIT-1 cells were lysed, and Tgase2 activity was measured by *ex vivo* Tgase2 activity assay. Data are mean Tgase2

activity, with mean ± SEM. *** $p < 0.001$. (E) The immunogenicity of cultured NIT-1 cells or explanted NIT-1 cells was measured by BDC2.5 T cell assay. Data are mean IFNγ secretion ± SEM. * $p < 0.05$.

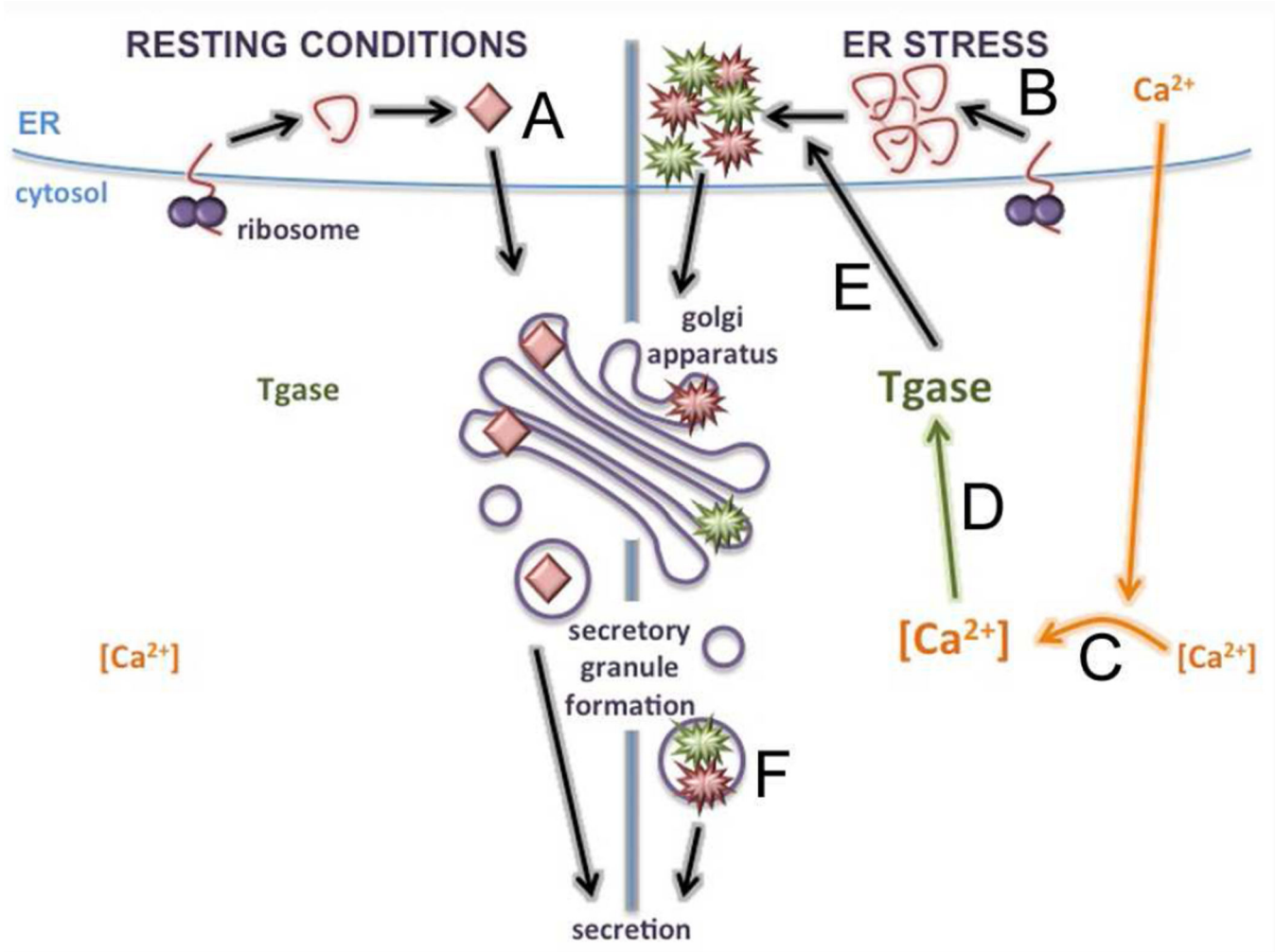

Fig. 9. Model

(A) Under resting conditions, CHgA is translated, folded in the ER, and packaged in secretory granules. Cytosolic Ca^{2+} remains low, and Tgase2 activity remains low. (B) β cell exposure to physiological or environmental triggers of ER stress leads to increased ER burden. (C) Ca^{2+} stores are released from the ER, increasing cytosolic Ca^{2+}, and (D) activating Ca^{2+}-dependent PTM enzymes such as Tgase2. (E) Activated Tgase2 translocates to the ER to modify CHgA, (F) which is packaged into secretory granules. If presented to auto-reactive T cells by APC, modified CHgA breaks peripheral tolerance and facilitates immune recognition of β cells.