

# GAD65-Specific Cytotoxic T Lymphocytes Mediate Beta-Cell Death and Loss of Function

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## ■ Abstract

Autoimmunity to islet cell antigens like glutamic acid decarboxylase 65kD (GAD65) is associated with the destruction of insulin-producing  $\beta$ -cells and progression to type 1 diabetes (T1D) in NOD mice and humans. T cell responses to GAD65 are detectable in the spleen of prediabetic NOD mice and in the peripheral blood of humans prior to the onset of overt hyperglycemia. Previous findings from our lab revealed that GAD65<sub>546-554</sub>-specific cytotoxic T lymphocytes (CTL) are present in naïve NOD mice and are able to induce islet inflammation upon adoptive transfer into NOD.scid recipients. Additionally, we found that professional antigen-presenting cells (APC) generate the p546-554 epitope from a soluble GAD65 fragment, p530-554, and

from GAD65 released by injured  $\beta$ -cells *in vivo*. Here, we report that the GAD65 fragment p546-554 is a dominant CTL-inducing epitope which is naturally processed and presented by a GAD65-expressing  $\beta$ -cell line. Further, coculture of GAD65<sub>546-554</sub>-specific CTL with the  $\beta$ -cells leads to a reduction in insulin production and the induction of perforin-mediated cell death. Collectively, these findings support a role for the cross-presentation of GAD65 antigen in the priming and enhancement of dominant GAD65-specific CTL responses, which can directly target  $\beta$ -cells that display GAD65 epitopes.

**Keywords:** type 1 diabetes · T cell · GAD65 · major histocompatibility complex class I · MHC · NOD.scid · antigen · FasL · beta-cell · pathogenesis

## Introduction

ype 1 diabetes (T1D) is an autoimmune disease characterized by the activation of autoreactive T cells specific for the insulin-producing pancreatic  $\beta$ -cells, resulting in  $\beta$ -cell destruction and ultimately, the loss of blood glucose homeostasis [1]. The non-obese diabetic (NOD) mouse is an animal model of human T1D. At 4-5 weeks of age, NOD mice spontaneously develop insulinitis after which 80-90% of females develop T1D [2-4]. While the NOD mouse has been extensively studied and characterized, the mechanisms that lead to the priming of spontaneous  $\beta$ -cell autoimmunity are still under investigation.

Normally, insulinitis and lymphocytic infiltration into the pancreatic islets precedes overt T1D, but such inflammation does not always lead to overt  $\beta$ -cell destruction and disease onset [3, 5]. The events that cause actual  $\beta$ -cell destruction and progression to clinical disease remain unknown, as is the determination of CD4<sup>+</sup> or CD8<sup>+</sup> T cells as the principal mediators of  $\beta$ -cell death. Previous studies have demonstrated T1D transfer into NOD.scid with CD8<sup>+</sup> CTL alone [6, 7], while others have shown disease transfer with CD4<sup>+</sup> T cell clones [8, 9]. These findings suggest that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are involved in the pathogenesis of T1D [10], and are thus capable of inducing  $\beta$ -cell death. However, pancreatic  $\beta$ -cells express major his-

to compatibility complex (MHC) class I but lack MHC class II proteins, suggesting that direct cytotoxicity can only be mediated by CD8<sup>+</sup> CTL that recognize peptide antigen:MHC class I complexes displayed on  $\beta$ -cells. Moreover, NOD mice deficient in MHC class I or MHC class I associated- $\beta_2$ M are protected from both insulinitis and T1D [11-14], demonstrating that MHC class I presentation to CTL is necessary for disease initiation and progression to T1D. In the inflamed islets, diabetogenic CTL populations can induce overt T1D [7], while other self-reactive CTL may contribute via exacerbation of insulinitis and/or the activation of T1D-causing T cells [15].

Several studies have shown that CTL are cytotoxic to islet cells [16-18]. CTL can kill target cells via perforin and granzyme B secretion and/or Fas-Fas ligand interactions upon epitope recognition [17, 19, 20]. The primary method of CTL-mediated islet cell death is likely via perforin/granzyme B [21], although CTL may also secrete cytokines such as IFN $\gamma$  and TNF $\alpha$  that can provide an indirect method for  $\beta$ -cell damage [22]. Islet cells treated with proinflammatory cytokines can upregulate MHC class I and increase their susceptibility to CTL-mediated cytotoxicity [22]. Additionally, treatment with cytokines can lead to increased expression of FasL (CD178), suggesting that Fas-FasL interaction may be a secondary form of CTL-mediated cytotoxicity [22].

Glutamic acid decarboxylase 65kD (GAD65), an enzyme expressed in the pancreatic  $\beta$ -cells, is a candidate autoantigen in the initiation and pathogenesis of T1D [9, 23, 24]. T cell responses to GAD65 are detectable in the spleens of prediabetic NOD mice and in the peripheral blood of humans with T1D prior to the onset of overt hyperglycemia [23, 24], suggesting that GAD65 autoimmunity is important in the initial and later stages of disease. While the CD4<sup>+</sup> responses to GAD65 determinants have been well characterized [23-25], less is known about the CTL response to GAD65 [26-29]. Previously, we have reported that peptide 546-554 is a pathogenic CTL-inducing epitope of GAD65 [26, 28]. GAD65<sub>546-554</sub>-specific CTL are detected in naïve NOD mice, produce inflammatory cytokines IFN $\gamma$  and TNF $\alpha$  upon culture with cognate antigen [26], and transfer islet inflammation [28]. Importantly, subsequent work has confirmed that GAD65-specific CTL, including those specific for p546-554, have diabetogenic potential [29]. Because of challenges to isolating pure  $\beta$ -cells from the pancreas, whole islet cell populations are often used to study CTL-mediated  $\beta$ -cell cytotoxicity. Pure  $\beta$ -cells offer the opportunity to investigate direct  $\beta$ -cell-CTL interac-

tions. In this report, we show direct communication between GAD65<sub>546-554</sub>-reactive CTL and a pancreatic  $\beta$ -cell line, which led to a loss of insulin production and  $\beta$ -cell death.

## Materials and methods

### *Mice and cells*

NOD and NOD.scid mice were purchased from Taconic Farms (Germantown, NY) and Jackson Laboratories (Bar Harbor, ME), respectively, and were housed in the University of Toledo Animal Care Facility. All experiments described in this report were reviewed and approved by the University of Toledo's Institutional Animal Care and Use Committee. NOD NIT-1 insulinoma cells were obtained from Dr. David Serreze (Jackson Laboratories; Bar Harbor, ME). GAD65-expressing NIT-1 cells were created by transfecting NIT-1 cells [30] with pcDNA3.GAD plasmid and subsequent selection with Genetecin (G418). Cloning of G418-resistant cells led to the recovery of several GAD-expressing NIT-1 cell lines, including NIT.GAD.A2. NIT.GAD.A2 cells were cultured in complete media (RPMI supplemented with fetal bovine serum, penicillin/streptomycin, 2-mercaptoethanol, sodium pyruvate, and nonessential amino acids). HIT-15 insulinoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in complete medium, while the B cell lymphoma line M12C3 (K<sup>d</sup>) and the 4G4 B cell hybridoma (H-2<sup>d/g7</sup>) were grown in RPMI supplemented with 10% horse serum.

GAD65<sub>546-554</sub>-specific CTL lines and clones were generated as previously described [28] following immunization of NOD mice with GAD65<sub>546-554</sub> (SYQPLGDKV) (Invitrogen, Carlsbad, CA). NP147-specific CTL were generated by immunization of NOD mice with the K<sup>d</sup>-binding peptide, 147-155 (IYQRTRALV), from the nucleoprotein (NP) of influenza. Briefly, mice were immunized subcutaneously with 20-50  $\mu$ g of peptide in CFA. Ten to 12 days later, single cell suspensions ( $8 \times 10^6$ /ml) from draining lymph nodes were cultured in complete medium with 10  $\mu$ g/ml of cognate peptide. Recombinant human IL-2 was added the next day (10 ng/ml). Peptide-responsive cells were restimulated every 2 wk with irradiated syngeneic spleen cells pulsed with cognate peptide, and maintained in IL-2-containing medium. Control cells include Th clones specific for GAD65 peptide 530-543 and Th clones specific for myelin oligodendrocyte glycoprotein (MOG) peptide 35-55.

*Immunohistochemistry*

Following adoptive transfer, pancreas organs from recipients were collected and fixed in 10% buffered formalin and embedded in paraffin. Serial sections, 5-7  $\mu\text{m}$  thick, and 20  $\mu\text{m}$  apart were placed on glass slides. Prior to immunostaining the pancreatic sections were deparaffinized with SafeClear (Fisher Scientific). NIT.GAD.A2 cells were grown overnight on glass cover slips and fixed with 4% paraformaldehyde. Both the pancreatic tissue and  $\beta$ -cell slides were treated with saponin and blocked with 2% horse serum prior to the addition of antibodies. GAD65-specific monoclonal antibodies, GAD-1 (ATCC) and GAD-6 (a gift from Dr. Dan Zekzer), were purified from the supernatants of the corresponding mouse B cell hybridomas by Protein-G column chromatography. The human/mouse/bovine insulin-specific antibody was purchased from R&D Systems (Minneapolis, MN), goat-anti-mouse IgG (H&L)-biotin was purchased from Southern Biotech (Birmingham, AL), while StreptAvidin-Alexa Fluor-488 and Avidin-HRP were purchased from Invitrogen and Vector Labs (Burlingame, CA), respectively. Primary antibodies were added and incubated overnight at 4°C, while secondary reagents were added for 1 hr each at room temperature. For sections treated with Avidin-HRP, a peroxidase staining kit (Vector Labs) was used to visualize enzyme deposition.

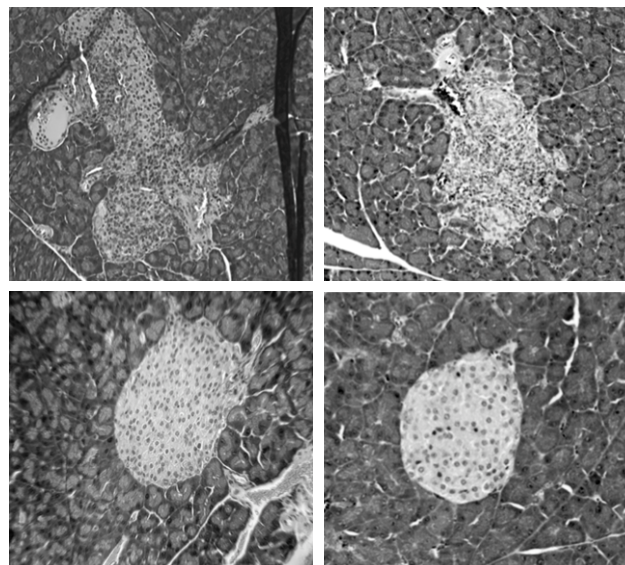
*Cytotoxicity assay*

An MTT assay was used to determine CTL-induced loss of viability among NIT.GAD.A2 cells ( $1 \times 10^5$  cells/well) cultured with GAD65<sub>546-554</sub>-specific or NP147-specific CTL ( $1 \times 10^5$  cells/well) in 96-well flat-bottom plates. Forty-eight hr after co-culture, triplicate wells were gently washed to remove nonadherent cells and debris before the addition of a MTT solution (0.5mg/ml) (Sigma Aldrich, St. Louis, MO) and incubated for 1 hr at 37°C. Finally, 10% SDS in 0.01N HCl was added to the wells overnight at 37°C, after which the absorbance of each well was determined at 595 nm using a Bio-Rad microplate reader. Viability was calculated as follows:

$$\frac{\text{Absorbance of experimental wells (CTL + target cells)}}{\text{Absorbance of control wells (target cells only)}}$$

To determine CD95 (Fas)-mediated loss of cell viability, NIT.GAD.A2 or M12C3 target cells were plated ( $2 \times 10^5$  cells/well) with soluble anti-Fas monoclonal Ab (0.5  $\mu\text{g/ml}$ ; Clone Jo2, BD Biosciences) and

crosslinked with protein G (1.0 g/ml) (Sigma) for 48 hr at 37°C. The MTT assay was performed as above to determine cell viability.

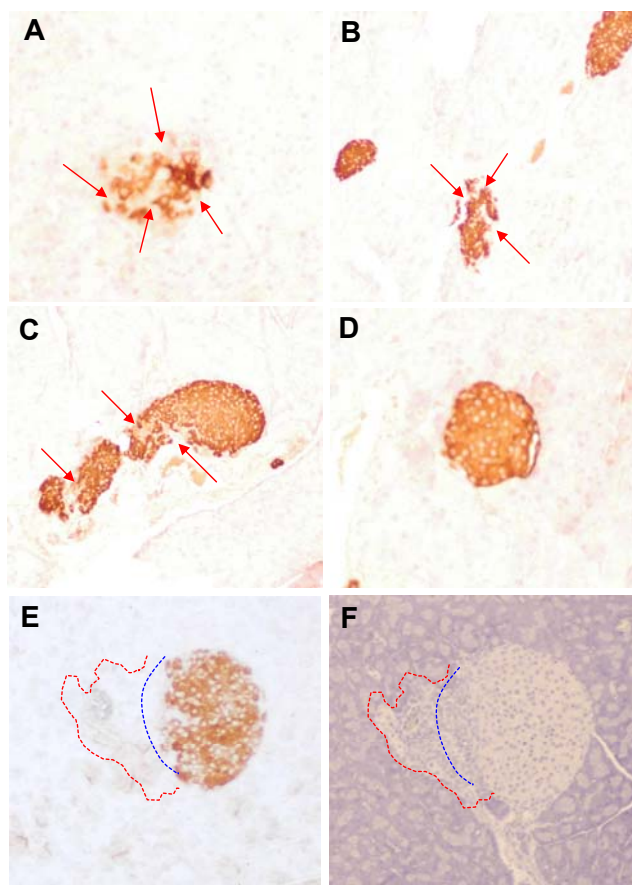


**Figure 1. Adoptive transfer of GAD65<sub>546-554</sub>-specific CTL induces insulinitis in NOD.scid recipients.** Recently activated GAD65<sub>546-554</sub>-specific CTL ( $1 \times 10^7$ ) were suspended in saline and injected into the peritoneal cavity of NOD.scid mice. Eleven days later the mice were sacrificed, and the pancreata were formalin-fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Representative sections were photographed and evaluated for insulinitis. **A:** islet from the recipient of CTL line GAD 546.14 (10X). **B:** islet from the recipient of CTL line GAD 546.16 (20X). **C:** islet from the recipient of CTL line GAD 268.11 (20X). **D:** islet from saline-treated NOD.scid (40X).

To evaluate the metabolic activity of NIT.GAD.A2 cells, an insulin-specific radioimmunoassay (Linco Research, St Charles, MO) was used to determine the hormone level in supernatants from co-culture experiments. Briefly, equal numbers of NIT.GAD.A2 cells ( $1 \times 10^5$  cells/well) and antigen-specific CTL were placed in complete medium, in 96-well flat bottom plates. Supernatants from replicate wells were collected after 48 hr and diluted with buffer as per the manufacturer's instructions, tested in triplicate, and subsequently compared to a standard curve of purified insulin.

*ELISPOT assay*

ELISPOTs were performed as previously described [28]. Briefly, Multiscreen HA filtration plates (Millipore, Billerica, MA) were coated with anti-mouse IFN $\gamma$



**Figure 2. Adoptive transfer of GAD65<sub>546-554</sub>-specific CTL leads to a loss of insulin production *in vivo*.** Recently activated GAD65<sub>546-554</sub>-specific CTL ( $1 \times 10^7$ ) were i.p. injected into NOD.scid mice. Eleven days later, the mice were sacrificed and the pancreata were fixed, embedded, sectioned, and immunostained to reveal sites of insulin production. Representative sections were photographed. **A:** islet from recipient of CTL clone GAD 546.14.46. **B:** islet from recipient of CTL line GAD 546.16. **C:** islet from recipient of CTL line GAD 546.14. **D:** healthy islet of a naïve NOD.scid. Red arrows indicate areas of localized insulin loss. The two bottom panels (**E** and **F**) are adjacent sections of a representative islet from a recipient of CTL line GAD 546.16. **E:** immunohistochemical staining with anti-insulin. **F:** hematoxylin alone. The dashed blue line outlines the theoretical extrapolation of the islets normal border, while the dashed red line encircles an area showing lymphocyte infiltration that disrupts islet architecture.

overnight. After blocking non-specific sites,  $1-10 \times 10^4$  antigen-specific CTL were co-cultured with  $3 \times 10^5$  NIT.GAD.A2, NIT, HIT-15, or M12C3 cells in complete medium for 24 hr. The plates were washed and biotinylated anti-mouse IFN $\gamma$  was added for at least 6

hr, followed by the addition of avidin-HRP for 1 hr. IFN $\gamma$ -secreting spots were detected by adding chromogenic substrate, and then enumerated using a stereo microscope.

#### Flow cytometry

To evaluate perforin expression, the CTL were fixed with 4% paraformaldehyde, resuspended in permeabilization buffer (1% saponin and 0.05% sodium azide in PBS), stained with PE-conjugated anti-Perforin or Rat Ig-PE (eBioscience, San Diego, CA), and analyzed by flow cytometry. CTL were identified by size and granularity (forward and side scatter). To detect IFN $\gamma$  production in CTL after culture with NIT.GAD.A2 cells, GAD65<sub>546-554</sub>-specific CTL were cultured with 4G4 cells, pulsed with p546-554 or buffer, or NIT.GAD.A2 cells in the presence of Brefeldin A (5  $\mu$ g/ml) (Sigma Adrich; St Louis, MO) for 6 hr at 37°C. The cells were washed, fixed with 4% paraformaldehyde, and resuspended in permeabilization buffer. Next, 0.4  $\mu$ g of PE-conjugated anti-IFN $\gamma$  (BD Biosciences, San Diego, CA) or isotope control (Ig-PE) was added for an additional 30 min at 4°C, after which the cells were washed and analyzed by flow cytometry. CTL were identified by size and granularity (forward and side scatter).

To evaluate CD95 expression on NIT.GAD.A2, the cells were trypsinized, washed, and stained stepwise with biotinylated anti-CD95 (eBiosciences, San Diego, CA) and StrepAvidin-AlexaFluor 488, and then analyzed by flow cytometry. Controls were stained with isotype control plus secondary or secondary reagent alone.

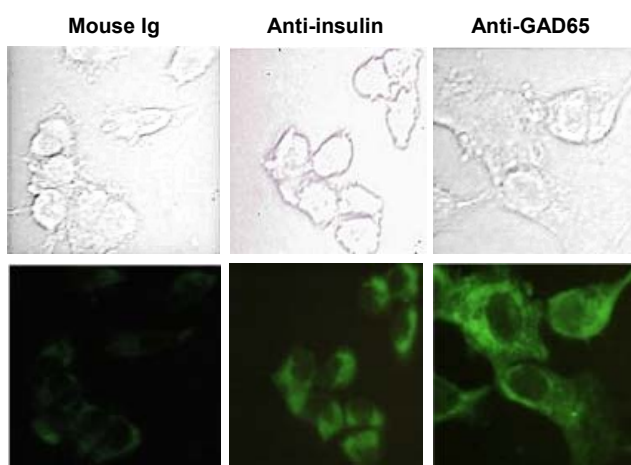
## Results

### *GAD65<sub>546-554</sub>-specific CTL lines and clones induce insulinitis and localized loss of $\beta$ -cell insulin production in NOD.scid recipients*

GAD65 p546-554-specific T cells are detectable in the spleens of prediabetic NOD mice. As we previously reported, adoptively transferred GAD<sub>546-554</sub>-specific CTL cause insulinitis in NOD.scid recipient mice [28]. The islets of recipient mice showed characteristic lymphocyte infiltration and disruption of islet architecture (Figures 1A-B); while the islets of mice that received an equal number of T cells from a CTL line specific for GAD268-78 (Figure 1C), or saline (Figure 1D), remained healthy and showed no signs of lymphocytic infiltration. Additionally, GAD65<sub>546-554</sub>-specific CTL induced localized islet-specific loss of in-



sulin production (Figures 2A-C and 2E), which was not obvious in healthy NOD.scid (Figure 2D) or the GAD268 controls (data not shown).



**Figure 3. NIT.GAD.A2 cells express insulin and GAD65.** NIT.GAD.A2 cells were grown overnight on glass cover slips, fixed, permeabilized, and incubated with mouse IgG, anti-insulin, or anti-GAD65. The slides were then treated stepwise with biotinylated goat-anti-mouse IgG and streptavidin-AlexaFluor 488. The top panels depict phase-contrast brightfield images, while the bottom panels are fluorescent images of the same cells captured under UV light.

Figures 2E and 2F reveal a relationship between lymphocyte infiltration and the loss of insulin staining (insulin or hematoxylin staining of adjacent sections). The H&E sections showed that the vast majority of the infiltrating cells in disrupted islets were lymphocytes. Given the purity of the CTL lines and clones that were transferred (>95% CD8<sup>+</sup> and TCR<sup>+</sup> at the time of transfer) and the lack of host lymphocytes in NOD.scid recipients, it is clear that the islet infiltrating cells contained the GAD546-specific CTL. The infiltrating cells were also associated with disruption of normal islet architecture (Figures 2E and 2F). However, despite the obvious damage to numerous islets, we did not observe overt diabetes in the NOD.scid recipients. Our GAD65 CTL may be important in the early phases of insulinitis and  $\beta$ -cell damage, which leads to the recruitment of Th cells and CTL of other specificities that may be needed to progress to diabetes in NOD.scid mice. Our findings indicate that infiltrating GAD-specific CTL can directly interact with insulin-producing  $\beta$ -cells, disrupt islet structure, and induce  $\beta$ -cell damage.

#### *GAD65-transfected NIT $\beta$ -cell insulinoma expresses GAD65 and insulin at detectable levels*

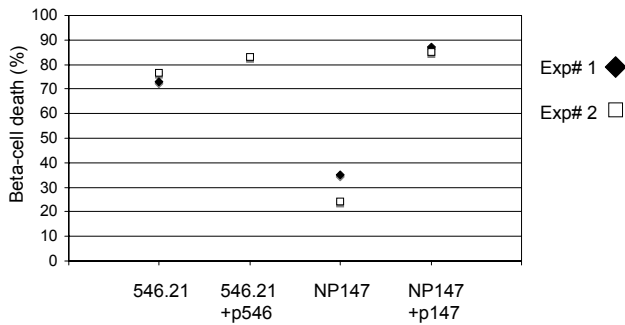
A key to unraveling autoimmune diabetes requires an understanding of the interaction between immune cells and insulin-producing  $\beta$ -cells. Investigations of immune-mediated  $\beta$ -cell death often involve the use of primary islet cells, which contain a mixture of  $\alpha$ -,  $\beta$ -, and  $\delta$ -cell populations. The isolation of purified  $\beta$ -cells from islet cell preparations requires tedious mechanical and enzymatic manipulations. Furthermore, typical cytotoxicity assays can be problematic when evaluating T cell activity because  $\beta$ -cells are sensitive to <sup>51</sup>Cr exposure and are poor loaders of <sup>3</sup>H thymidine (unpublished data). Because of the fastidious nature of purified islet  $\beta$ -cells, we chose NIT-1  $\beta$ -cell insulinoma cells to aid in our investigation of  $\beta$ -cell-CTL specific interactions. NIT-1 is a well-established NOD  $\beta$ -cell line [30] that produces insulin *in vitro*, but has lost the ability to express GAD65 at detectable levels, which can also be difficult to detect in preparations of mouse islets [31]. To ensure stable GAD65 expression, we transfected the NIT-1  $\beta$ -cell line with a GAD65-expressing plasmid and subcloned several stable cell lines. One of the resulting lines, NIT.GAD.A2, expresses both GAD65 and insulin at levels detectable by immunofluorescence (Figure 3).

#### *GAD65<sub>546-554</sub>-specific CTL kill NIT.GAD.A2 in an epitope-restricted manner*

We used an MTT assay to determine whether GAD65<sub>546-554</sub>-specific CTL could directly cause a loss of viability in NIT.GAD.A2  $\beta$ -cells. Viable NIT.GAD.A2 are adherent, so the gentle washing needed for analysis does not disturb a viable monolayer. The viability of NIT.GAD.A2  $\beta$ -cells was reduced by more than 70% when co-cultured with the GAD65<sub>546-554</sub>-specific CTL clone, 546.21 (Figure 4), but similar responses were not seen when the  $\beta$ -cells were cultured with the influenza-specific CTL-line, NP147 (Figure 4). In some experiments, we observed a slight loss of NIT.GAD.A2 viability when NP147 CTL were added (Figure 4). However, the level of killing never reached the magnitude observed with GAD-specific CTL, and was attributed to non-specific interactions since dramatic increases in cell death occurred when NIT.GAD.A2 cells were prepulsed with peptide NP147 (Figure 4). In contrast to this result, prepulsing with GAD<sub>546-554</sub> peptide did not increase the killing observed with the GAD-specific CTL (Figure 4).

In addition to cell death, we found that co-culture with GAD65<sub>546-554</sub>-specific CTL reduced the produc-

tion of insulin by NIT.GAD.A2 by greater than 75% (Figure 5). Similar to the cytotoxicity assays, only a marginal decrease in insulin production was observed in the presence of NP147-specific CTL, which increased almost 3-fold when NIT.GAD.A2 were first pulsed with cognate peptide, NP147-155 (Figure 5).

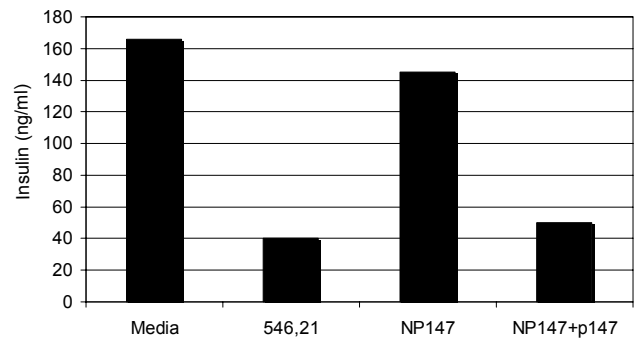


**Figure 4. GAD65<sub>546-554</sub>-specific CTL can kill GAD-expressing  $\beta$ -cells.** NIT.GAD.A2 cells ( $1 \times 10^5$  c/w) were incubated with an equal number of GAD-specific CTL line 546.21 or the control CTL line NP147 (specific for residues 147-155 of NP molecule of influenza), for 48 hr at 37°C in 96-well plates. Positive controls included NIT.GAD.A2 cells prepulsed with cognate peptide (10  $\mu$ g/ml of p546-554 or p147-155). The plates were washed to remove CTL and debris before cell viability was measured in each well. Cell viability (% cell death) was calculated as described in the materials and methods section. The filled circles and open squares reflect results from two independent experiments, but they are representative of multiple experiments with a number of different GAD65<sub>546-554</sub>-specific CTL lines and clones. The mean values were calculated from triplicate wells.

#### *GAD65<sub>546-554</sub>-specific CTL kill NIT.GAD.A2 via a FasL-independent mechanism*

To determine the mechanisms of cytolytic activity of GAD65<sub>546-554</sub>-reactive CTL against NIT.GAD.A2, we evaluated the expression of the death-related molecules CD178 and perforin on our CTL. We found that CTL clone 546.14.46 and CTL line 546.21 each expressed perforin (Figures 6A and 6C). However, CD178 was undetectable on both lines (Figures 6A and 6B). Additionally, in spite of their susceptibility to the GAD-specific CTL, we could not detect CD95 expression on NIT.GAD.A2  $\beta$ -cells (Figure 7A), suggesting that Fas-FasL interactions were not centrally involved in GAD65<sub>546-554</sub>-specific CTL mediated  $\beta$ -cell cytotoxicity. We readily detected CD95 on M12C3 cells (Figure 7A), which were also killed by the GAD-

specific CTL when pulsed with cognate peptide. To confirm that NIT.GAD.A2 were not killed in a Fas-FasL-dependent manner, we performed a cytotoxicity assay with soluble anti-CD95 monoclonal antibody, which induced apoptosis in CD95-expressing cells. While NIT.GAD.A2 were completely refractive to apoptosis when cultured with anti-CD95, similar treatment of the M12C3 cells induced a greater than 80% loss of viability (Figure 7B). Collectively, these data demonstrated that the GAD65<sub>546-554</sub>-specific CTL could kill  $\beta$ -cells in CD178-independent manner.



**Figure 5. GAD65<sub>546-554</sub>-specific CTL diminish insulin production *in vitro*.** NIT.GAD.A2 ( $1 \times 10^5$  c/w) were incubated with an equal number of GAD-specific, 546.21, or control CTL NP147 for 48 hr at 37°C in 96-well plates. NP147 CTL were also incubated with cognate peptide-pulsed NIT.GAD.A2 cells (NP147+peptide). Culture supernatants were collected and analyzed in an insulin-specific RIA. Mean values were calculated from triplicate wells.

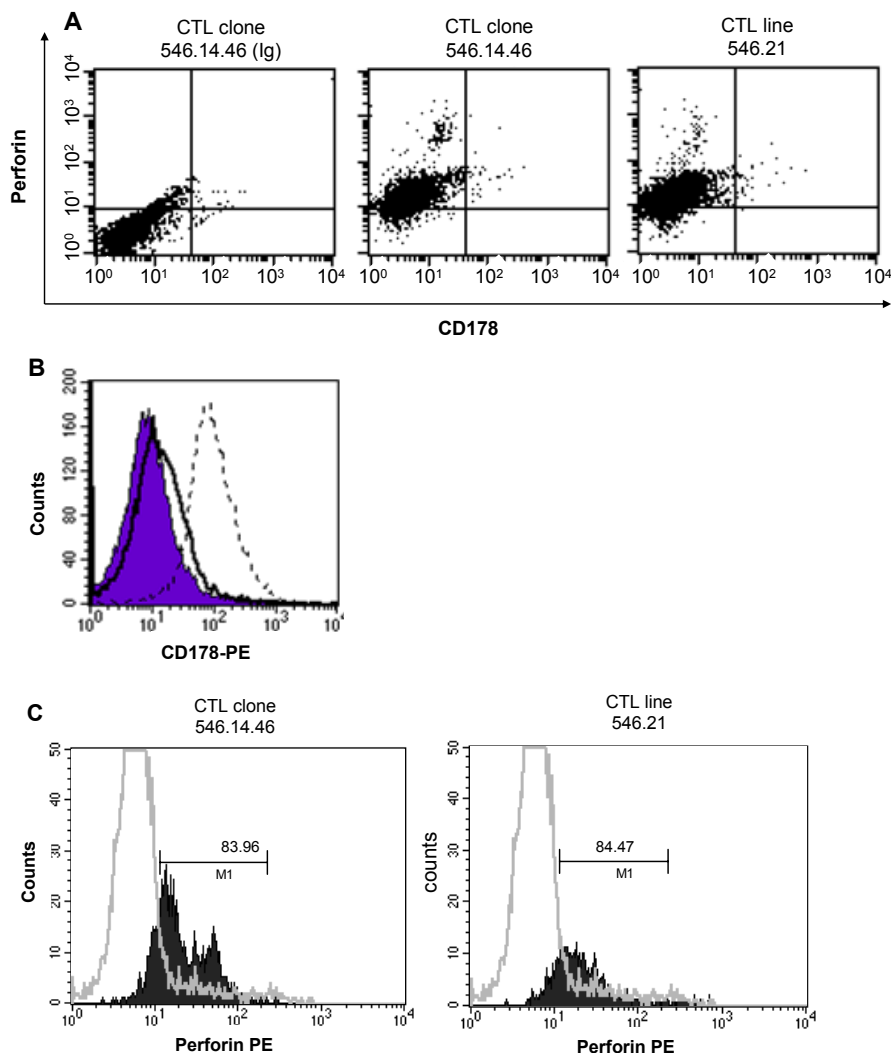
Cytokine production is an additional indicator of CTL activation and recognition of cognate epitope. GAD65<sub>546-554</sub>-specific CTL produced IFN $\gamma$  when cultured with NIT.GAD.A2, as shown by intracellular cytokine staining (Figures 8A and 8B) and ELISPOT assay (Figure 8C). Interestingly, compared to the M12C3 cells, the  $\beta$ -cell line was relatively poor at inducing cytokine release (Figure 8C), which we could not compensate for by adding more  $\beta$ -cells to the culture. Two-thirds fewer peptide-pulsed M12C3 cells yielded 5-fold more spots than the  $\beta$ -cells (Figure 8C). Nevertheless, the results demonstrated  $\beta$ -cell display of the CTL epitope and subsequent T cell activation. Therefore, NIT.GAD.A2 cells were capable of processing 546-554 from the GAD65 molecule. However, despite their ability to produce GAD65 (detected by immunofluorescence, data not shown), hamster-derived HIT-15 insulinomas were incapable of inducing IFN $\gamma$

release from the GAD-reactive CTL (Figure 8), which confirmed that the CTL are both GAD65-specific and K<sup>d</sup>-restricted [28], and this crosspresentation was not contributing to our assay results.

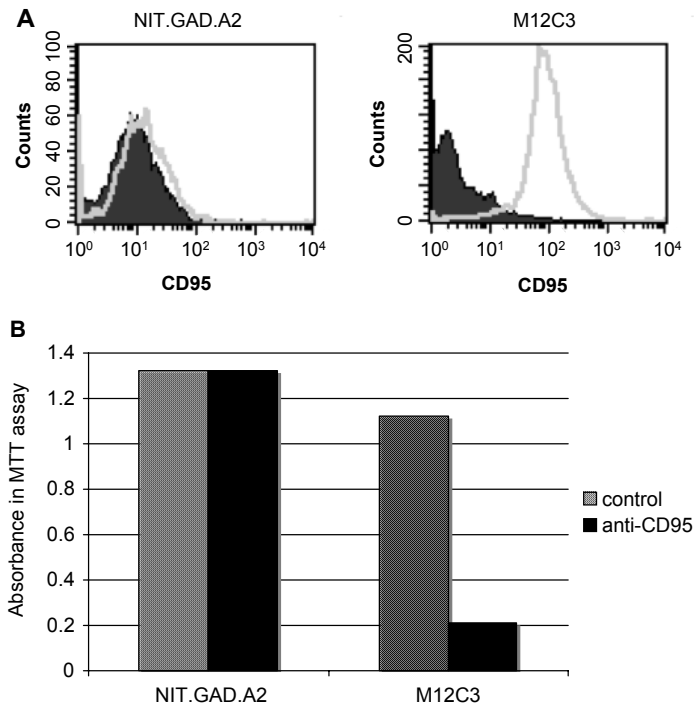
## Discussion

Much of our knowledge about T cell to  $\beta$ -cell interactions emanates from previous investigations of self-antigen-specific CTL-mediated  $\beta$ -cell death performed with islet cell populations or investigations utilizing monoclonal T cells specific for a non- $\beta$ -cell antigen expressed as a transgene in islet cells [19]. Previously, we have shown that the peptide p546-554 contains a dominant K<sup>d</sup>-restricted determinant of GAD65 that induces CTL capable of driving insulinitis in NOD mice [28]. We now report evidence of direct interaction between GAD65<sub>546-554</sub>-specific CTL and a  $\beta$ -cell line. Importantly, we found that GAD65-specific CTL, and not NP147-specific CTL, mediated intense  $\beta$ -cell cytotoxicity in the absence of professional APC or phagocytes. The lack of comparable NIT.GAD.A2  $\beta$ -cell death with the irrelevant CTL demonstrates that, in spite of their sensitivity to the deleterious effects inflammation, the direct presentation of MHC:peptide ligands to CTL accounts for the majority of the  $\beta$ -cell death we observed in co-culture experiments.

Previous studies suggest that perforin/granzyme B secretion is the primary method of CTL-induced  $\beta$ -cell cytotoxicity [32] and that Fas-FasL-



**Figure 6. GAD65<sub>546-554</sub>-specific CTL express perforin but not CD178.** GAD-specific CTL clone 546.14.46 and CTL line 546.21, and the MOG-specific (myelin oligodendrocyte glycoprotein) Th cell clone SC5 were stained with isotype control antibodies (IgG-PE), or PE-conjugated anti-perforin or biotinylated anti-CD178 (FasL) plus avidin-Alexa Fluor 488, as described in the materials and methods section. The cells were analyzed by flow cytometry. **A:** cells within the lymphocyte gate are displayed as dot plots. **B:** CTL 546.14.46 was stained with anti-CD178 (solid line) or isotype control (filled histogram). The Th cell clone GAD530.1 was also stained with isotype control (data not shown) or anti-CD178 (broken line) as a positive control for CD178 expression. **C:** Data from a second experiment are displayed as histograms. The MOG-specific Th cell clone is shown as the unfilled histogram. The values in the histogram reveal the percentage of cells that were positive compared to isotype control. These results are representative of two experiments.



**Figure 7. GAD CTL kill NIT.GAD.A2 cells via a CD95-independent mechanism.** **A:** NIT.GAD.A2 or M12 C3.PIA cells ( $1.5 \times 10^5$ ) were stained with isotype control (filled histogram) or biotinylated-anti-CD95 (Fas) (unfilled histogram) followed by StrepAvidin-AlexaFluor 488. **B:** NIT.GAD.A2 cells or M12C3.PIA were plated ( $2 \times 10^5$  c/w) with soluble anti-Fas monoclonal Ab (0.5  $\mu\text{g/ml}$ ) and protein G (1.0  $\mu\text{g/ml}$ ). After 48 hr at 37°C, an MTT assay was used to evaluate cell viability. The mean values and SD were calculated from triplicate wells and the results are representative of two experiments.

dependent killing is likely to occur in the absence of perforin [17, 19, 20]. Collectively, our data supports this conclusion as GAD65<sub>546-554</sub>-specific CTL kill NIT.GAD.A2  $\beta$ -cells in a FasL-independent, and likely perforin-dependent manner. While combinations of proinflammatory cytokines can induce the expression of CD95 on NIT cells [33], we did not detect increased CTL-mediated cytotoxicity of NIT.GAD.A2 in the presence of IFN $\gamma$  and TNF $\alpha$  (unpublished data).

In addition to direct target cell cytolysis, we report a loss of insulin production in  $\beta$ -cells co-cultured with GAD65<sub>546-554</sub>-specific CTL, but not with NP147, suggesting that insulin loss is related to antigen-specific presentation by the  $\beta$ -cells and is due to direct  $\beta$ -cell damage and not CTL absorption of secreted insulin. *In vivo*, we observed localized insulin loss in the islets of NOD.scid mice that received GAD65<sub>546-554</sub>-specific

CTL, and the loss coincided with islet infiltration by lymphocytes. Further investigation is required to define the kinetics of insulin loss and clarify whether substantial loss of the metabolic function occurs prior to cell death.

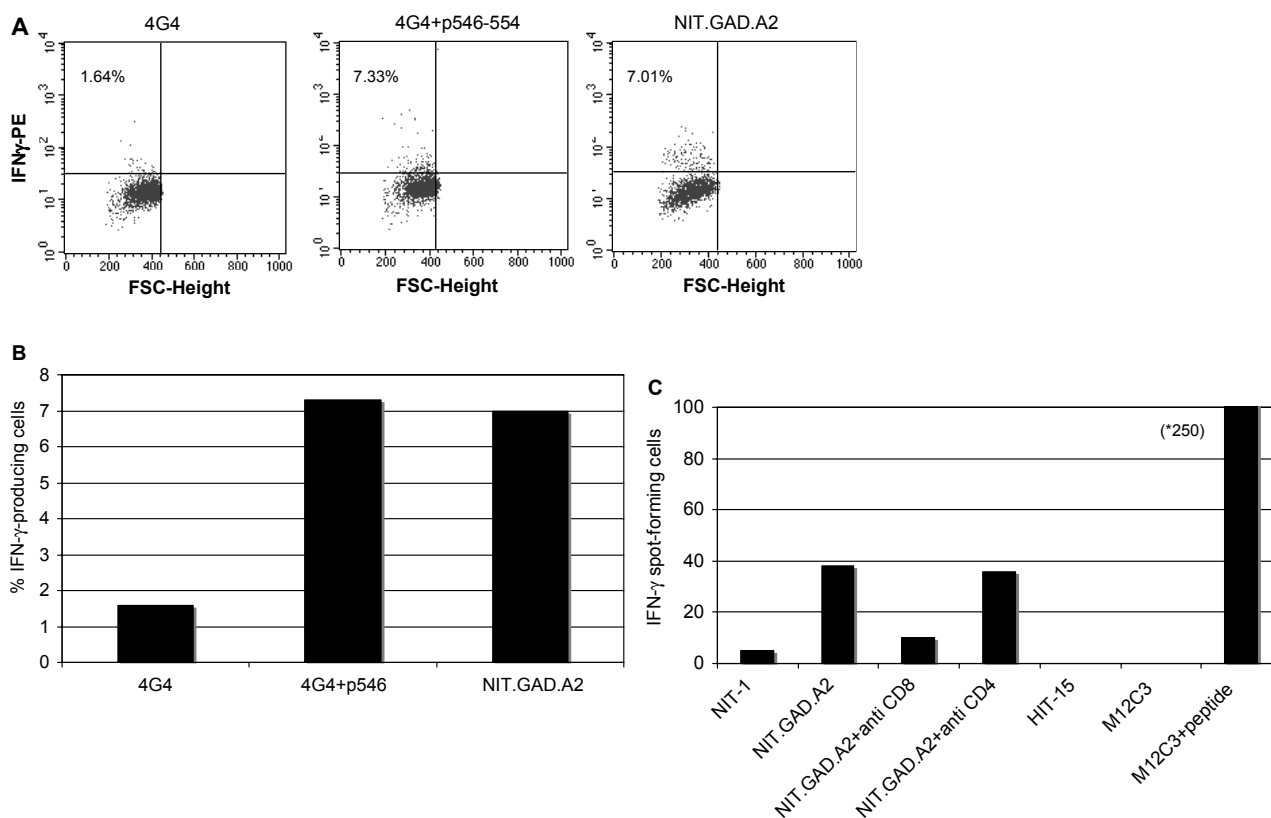
Additionally, NIT.GAD.A2 cells induced low level CTL secretion of IFN $\gamma$ . IFN $\gamma$  signaling through the Jak-STAT pathway is prolonged in  $\beta$ -cells [34], and may upregulate MHC class I, rendering them more susceptible to CTL-mediated cytolysis [35]. Therefore, in the inflamed islet, IFN $\gamma$  is implicated in both direct and indirect  $\beta$ -cell damage, as localized tissue damage cytokine release by CTL may promote pancreatic inflammation and recruit other immune mediators including APC and Th cells.

Our demonstration of direct  $\beta$ -cell cytotoxicity and CTL activation highlights the ability of  $\beta$ -cells to naturally and preferentially process and present CTL-inducing GAD65 epitopes that have diabetogenic potential [29]. Importantly, p546-554 is processed and presented in GAD65-transfected B cells, as well as macrophages/dendritic cells and  $\beta$ -cells that can capture or express GAD65 *in vivo*, respectively [28].

This finding shows that the determinant is intrinsically accessible and readily excised from GAD65 by both  $\beta$ -cells and professional APC. Therefore, cross-presentation of  $\beta$ -cell antigens released following tissue damage by infectious agents [36], age-related tissue remodeling [37], and/or normal cellular turnover may be a significant mechanism of priming autoreactive CD8<sup>+</sup> T cells.

In the pancreas, activated GAD65-specific CTL can exclusively initiate an immune response via direct TCR-MHC I interactions with  $\beta$ -cells, a feature not possessed by Th cells. The mechanisms by which Th cells mediate T1D remains unclear; however, such cells may secrete inflammatory cytokines or other soluble mediators that preferentially damage surrounding  $\beta$ -cells, but leave other islet cells unscathed. Speculations on the precise contribution of GAD65 to T1D pathogenesis have been controversial, and its role in islet in-





**Figure 8. GAD<sub>546-554</sub>-specific CTL produce IFN $\gamma$  in the presence of GAD-expressing  $\beta$ -cells.** CTL clone 546.14.74 CTL ( $5 \times 10^4$  c/w) and 4G4 B cell hybridomas ( $1 \times 10^5$  c/w), prepulsed with cognate peptide p546-554 or saline, or NIT.GAD.A2 were co-cultured in the presence of BFA for 6 hr at 37°C. The cells were fixed, permeabilized, and stained with PE-conjugated (A) anti-IFN $\gamma$  or isotype control (data not shown). The figure shown is representative of two independent experiments. Figure B is a graphical representation of the FACS dot plots in A. C: ELISPOT analysis for IFN $\gamma$  production. CTL ( $5 \times 10^4$  c/w) were co-cultured with  $3 \times 10^5$  NIT.GAD.A2, NIT-1, or HIT-15 insulinomas ( $3 \times 10^5$  c/w) in 96 well ELISPOT plates and then processed as described in the materials and methods section. As positive control,  $1 \times 10^3$  CTL were cultured with  $1 \times 10^5$  M12C3 cells, pulsed with or without cognate peptide. The data represent mean spot-forming cells of triplicate wells. The standard deviations were less than 5%.

flammation also remains elusive. We and others have found GAD65<sub>546-554</sub>-specific CTL in naïve NOD mice [26, 29]. Treatment of NOD mice with GAD65 protein or peptides can induce tolerance and protection from T1D, or enhance the progression of the disease [9, 29]. GAD65 autoimmunity may play an even greater role in human T1D pathogenesis [38], as human  $\beta$ -cells synthesizes far more GAD65 than murine  $\beta$ -cells.

Clearly, not all GAD65 epitopes are immunogenic and it is likely that only a limited number are able to recruit T cells that influence the course of T1D. Moreover, some epitopes with diabetogenic potential may

not be readily processed *in vivo* by the appropriate cells. The processing and presentation of relevant islet cell antigens by  $\beta$ -cells may contribute significantly to the susceptibility and progression of T1D. The ability to promptly identify these critical epitopes will be important in our efforts to design therapeutic interventions that can arrest  $\beta$ -cell-specific inflammatory mechanisms.

**Conflict of interest statement:** The authors declare that they have no competing conflict of interests with respect to financial or other issues.

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